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Research article

Cloning and heterologous expression of a hydrophobin gene *Ltr.hyd* from the tiger milk mushroom *Lentinus tuber-regium* in yeast-like cells of *Tremella fuciformis*

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ABSTRACT

Background: Hydrophobins are small proteins secreted by filamentous fungi, which show a highly surface activity. Because of the signally self-assembling abilities and surface activities, hydrophobins were considered as candidates in many aspects, for example, stabilizing foams and emulsions in food products. *Lentinus tuber-regium*, known as tiger milk mushroom, is both an edible and medicinal sclerotium-producing mushroom. Up to now, the hydrophobins of *L. tuber-regium* have not been identified.
Results: In this paper, a Class I hydrophobin gene, *Ltr.hyd*, was cloned from *L. tuber-regium* and expressed in the yeast-like cells of *Tremella fuciformis* mediated by *Agrobacterium tumefaciens*. The expression vector pGEH-GH was under the control of *T. fuciformis* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter. The integration of *Ltr.hyd* into the genome of *T. fuciformis* was confirmed by PCR, Southern blot, fluorescence observation and quantitative real-time PCR (qRT-PCR). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that recombinant hydrophobin rLtr.HYD with an expected molecular mass of 13 kDa was extracted. The yield of rLtr.HYD was 0.66 mg/g dry weight. The emulsifying activity of rLtr.HYD was better than the typical food emulsifiers sodium caseinate and Tween 20.
Conclusions: We evaluated the emulsifying property of hydrophobin Ltr.HYD, which can be potentially used as a food emulsifier.

1. Introduction

Hydrophobins are small proteins of around 100 amino acids uniquely secreted by filamentous fungi, which are characterized by eight cysteine residues in conserved position [1,2]. Based on the differences in hydropathy profiles, physical characteristics and amino acid sequence similarity, hydrophobins are divided into class I and II [3,4]. All hydrophobins can naturally form amphipathic membranes by self-assembling and convert hydrophobic surfaces to hydrophilic ones or vice versa [5]. Due to the self-assembling abilities and surface activities, hydrophobins are useful for many aspects, including separation technologies [6], surfactants [7], biomaterials applications [8,9], cosmetic and pharmaceutical purposes [8,10] and antifouling [11]. Particularly, hydrophobins have the ability to stabilize foams and emulsions, which will be novel stabilizer and emulsifier in commercial food [12]. However, producing sufficient amounts of hydrophobins is the prerequisite of commercial applications, so heterologous expression of hydrophobins in different hosts have been developed [4,12].

Lentinus tuber-regium (Fr.) Fr., synonym *Pleurotus tuber-regium* (Rumph. ex. Fr.) Singer, known as tiger milk mushroom, is both an edible and medicinal sclerotium-producing mushroom. Nowadays, *L. tuber-regium* is of popularity and economic importance due to the fact that it contains a variety of bioactive compounds, including glycoproteins, polysaccharides, and phytochemicals [13,14]. However, the hydrophobins of *L. tuber-regium* have not been identified.

In the present study, a hydrophobin gene was cloned from *L. tuber-regium* and heterologously expressed in the yeast-like cells of *Tremella fuciformis*. Moreover, the emulsifying activity of recombinant *L. tuber-regium* hydrophobin was also evaluated.

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2. Materials and methods

2.1. Strains and culture media

The *L. tuber-regium* strain ACCC 50657 and the *T. fuciformis* strain Y32 (monokaryotic yeast-like cell), provided by Laboratory of Food Microbiology, Huazhong Agricultural University, were maintained and sub-cultured on potato dextrose agar (PDA, Difco, USA) at 32°C and 25°C, respectively. *A. tumefaciens* strain EHA105 (Invitrogen, China), grown at 28°C in YEB medium (tryptone 5 g/L, nutrient broth 5 g/L, sucrose 5 g/L, yeast extract 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.49 g/L) containing 50 μg/mL rifampicin, was used to transform the yeast-like cells. The pGEH-GH vector used in this study was constructed by Zhu et al. [15].

2.2. cDNA and DNA cloning of hydrophobin

The total RNA of *L. tuber-regium* mycelia was extracted using RNAiso Plus reagent (TaKaRa, Japan) following the manufacturer's instructions. Then, the RNA was transcripted into cDNA using the anchored primer (AnchorP, Table 1) according to the M-MLV RTase cDNA synthesis kit (TaKaRa, Japan) manufacturer's instructions. The hydrophobin gene was amplified using the primers of arbitrary primer (ArbitraryP) and AUAP (Table 1), with the diluted 10-fold cDNA as the templates. The PCR reaction condition was: 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and then 72°C for 10 min.

Genomic DNA was extracted using CTAB method and used to amplify the hydrophobin gene with the specific primers (hyd-F/R, Table 1) designed according to the cDNA sequences of hydrophobin. The PCR reaction condition was: 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, and then a final extension at 72°C for 10 min. The PCR products were purified with E.Z.N.A Gel Extraction Kit (Omega, Shanghai, China), then cloned into pMD18-T (Takara, Dalian, China) for sequencing (Invitrogen, Shanghai, China) using primer M13-F or M13-R (Table 1).

2.3. Sequence analysis

Sequence similarity analysis was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The characteristics of proteins, including molecular weight, amino acid composition, and theoretical isoelectric point were predicted by Compute PI/MW. Protein secondary structure prediction was carried out by the SOPMA secondary structure prediction software. The protein hydrophobicity pattern was predicted by ProScale. The difference was conducted between hydrophobin DNA sequence and CDS sequence using DNAMAN software.

2.4. Construction of hydrophobin expression vector

The plasmid DNA of pGEH-GH was extracted using the E.Z.N.A Plasmid Mini Kit (Omega, Shanghai, China), digested with *Mlu*I and *Asu*II, then the vector pGEH-GH backbone was purified with E.Z.N.A Gel Extraction Kit (Omega, Shanghai, China). The hydrophbin PCR product (hyd1-F/R, Table 1) was purified with E.Z.N.A Cycle Pure Kit (Omega, Shanghai, China), and digested with the same enzymes then cloned into pGEH-GH backbone. The resulting recombinant plasmid pGEH-LH contained the hydrophobin CDS sequence (Fig. 1). The pGEH-LH was transformated into *A. tumefaciens* EHA105 strain through electroporation.

2.5. Agrobacterium-mediated transformation of T. fuciformis

The preparation of bacteria and yeast-like cells of *T. fuciformis* were based on previous description [15,16]. The pre-induced *A. tumefaciens* cells (500 μL) were mixed with *T. fuciformis* cells (at a concentration of 10^7 cells/mL, 500 μL). The mixed cells (200 μL) were plated onto a sterile microporous membrane (0.45 μm, 50 mm) on solid IM (with 200 μM acetosyringone) and incubated at 25°C for 3 d. After co-cultivation, the membranes with fungal and bacterial colonies were transferred onto PDSA plates containing 200 μg/mL of cefotaxime sodium and 50 μg/mL of hygromycin to select putative transformants.

2.6. Stability test and molecular analysis of transformants

The putative transformants were subcultured on PDSA containing 50 μg/mL of hygromycin and 200 μg/mL of cefotaxime for four rounds and transferred to PDSA without antibiotics. These putative transformants were then cultured on PDSA containing 50 μg/mL of hygromycin to check the stability. The *hph-egfp* and *hydrophobin* were amplified to detect the introduced sequence using primers hph-egfp-F/R and hyd1-F/R, respectively (Table 1). The amplification condition was: 94°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s, and then a final extension at 72°C for 10 min.

For Southern blot analysis, approximately 10 μg of genomic DNA, isolated from transformants and controls, digested with restriction enzymes *Xho*I, were separated on a 1% agarose gel and transferred to Hybond-N^+ nylon membranes (Amersham, Little Chalfont, UK) using 20 × SSC. The hydrophobin DNA fragment amplified by PCR with primers hyd1-F and hyd1-R was digoxigenin (DIG) labeled as the specific probe (Table 1). The labelling, hybridization and signal detection were performed following the manufacturer's protocol (Roche, Germany).

Table 1
Primers for PCR amplification in this work. The recognition sequences for restriction enzyme are underlined.

Primers	Sequence(5′ → 3′)	Description
AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT	Primers for transcription
arbitrarily P	CCGGAATTCTCGCTGCACGCTTCTCGAC	Primers for hyd cDNA
AUAP	GGCCACGCGTCGACTAGTAC	
Hyd-F	TCGCTCTTTACCAGCCAC	Primers for hyd DNA
Hyd-R	ATGCCCAGACCCTTCAGC	
Hyd1-F	ACGCGTATGTTCTTCCAAACTACCATC	Primers for hyd CDS
Hyd1-R	TTCGAATTACAAGGCAACGTTGATGG	
Hph-egfp-F	GCAGAAGAACGGCATCAAGGTG	Primers for *hph-egfp*
Hph-egfp-R	CAGGCTCTCGCTAAACTCCCC	
tubulin-F	GATGACCATTTCTTGCTTC	Tubulin primers for qRT-PCR
tubulin-R	GTTCTGACATTTGCTACCG	
qhyd-F	CCTCTCGCACTTCGCACTGA	hyd primers for qRT-PCR
qhyd-R	CCGACAATGGGAGAACACTGC	
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	Primers for sequencing
M13-R	AGCGGATAACAATTTCACACAGGA	

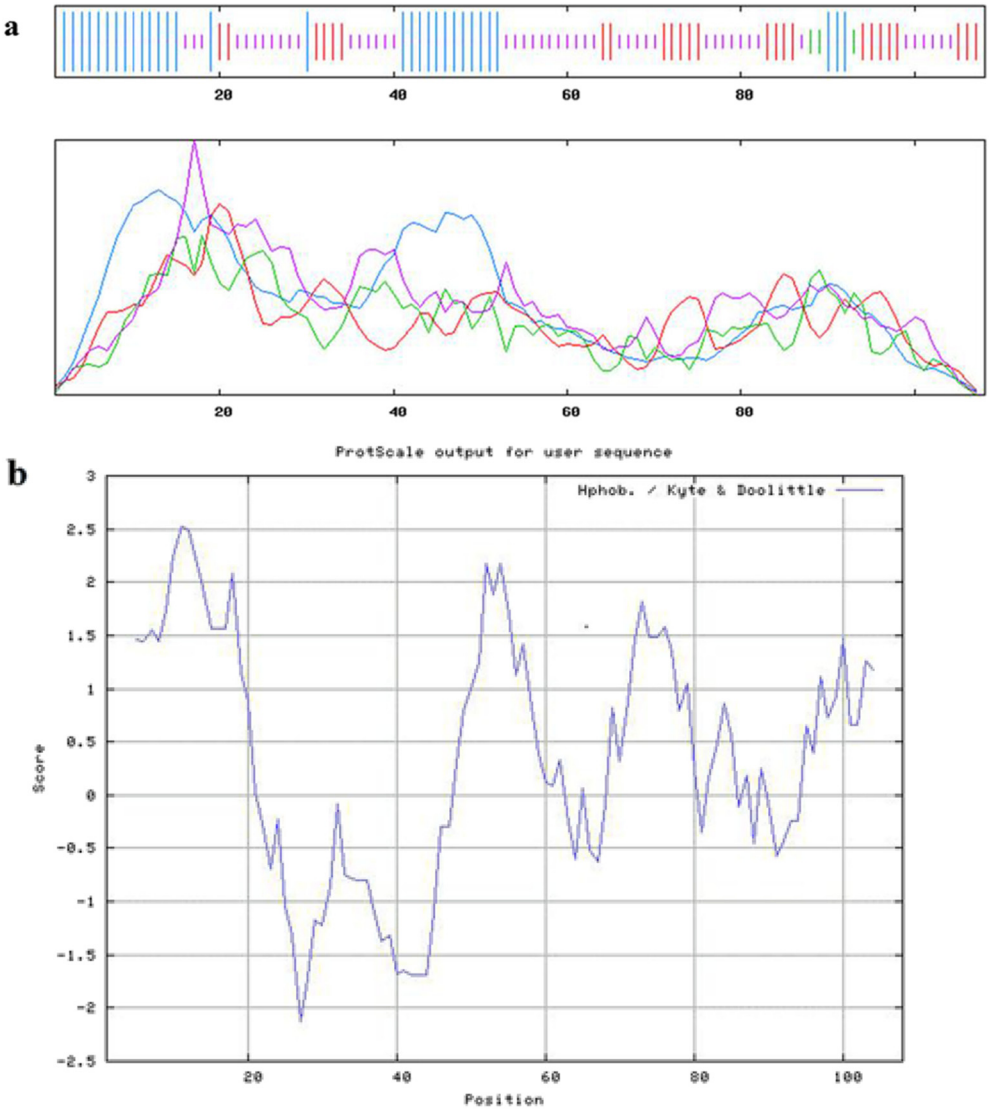

Fig. 1. The secondary structure and the hydropathy profile of Ltr.HYD. (a) The result of the secondary structure prediction (the blue line represents the alpha helix, the red line represents the extended strand, the green line represents the beta turn, and the purple line represents the random coil). (b) The hydropathy profile was calculated according to the Kyte and Doolittle algorithm using a window size of nine amino acid residues.

2.7. Fluorescence microscopy

The enhanced green fluorescent protein (*egfp*) expression was assessed by immobilizing the yeast-like cells of transformants and wild strain Y32 on glass slides. The samples were examined with a fluorescence microscope (DM 6000 B, Leica Microsystems, Germany) using a green fluorescence filter (546 nm). Images were taken under 40 × objective for randomly selected transformant and processed with imaging software OCULAR.

2.8. Quantitative real-time PCR analysis

To identify the expression of the hydrophobin, mRNA was extracted by means of RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instructions. The reverse transcription reaction was performed according to the M-MLV RTase cDNA synthesis kit (TaKaRa, Japan) manufacturer's instructions. The reverse transcription product as template was transferred to qRT-PCR. The qRT-PCR condition was: 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, followed by 40 cycles of 60°C for 30 s. The primers for hydrophobin gene (qhyd-F/R) and β-tubulin reference gene (tubulin-F/R) were listed in Table 1. Each reaction was replicated three times to estimate error and the gene expression was normalized by the $2^{-\triangle\triangle Ct}$ analysis.

2.9. Extraction, purification, and emulsifying activity of hydrophobin

The separation and purification of hydrophobin from the yeast-like cells of transformants and wild strain 32Y of *T. fuciformis* (negative control) referred to the method of Ma et al. [17]. The monomerized protein samples were detected by SDS-PAGE (15% acrylamide), and stained by coomassie blue. The yield of hydrophobin was determined by Bradford assay (BioRad).

The emulsifying capacities of hydrophobin was examined and compared with the typical emulsifying agents, sodium caseinate and Tween 20 (Sigma, St. Louis, MO, USA). Before emulsion, the water solutions of 100 μg/mL hydrophobin, 200 μg/mL sodium caseinate and 200 μg/mL Tween 20 were prepared. Each 2-mL liquid containing the above-mentioned water solutions and 8% (v/v) soybean oil was completely mixed for 2 min using a vortex mixer. The stabilities of the emulsions were observed visually with an optical microscope.

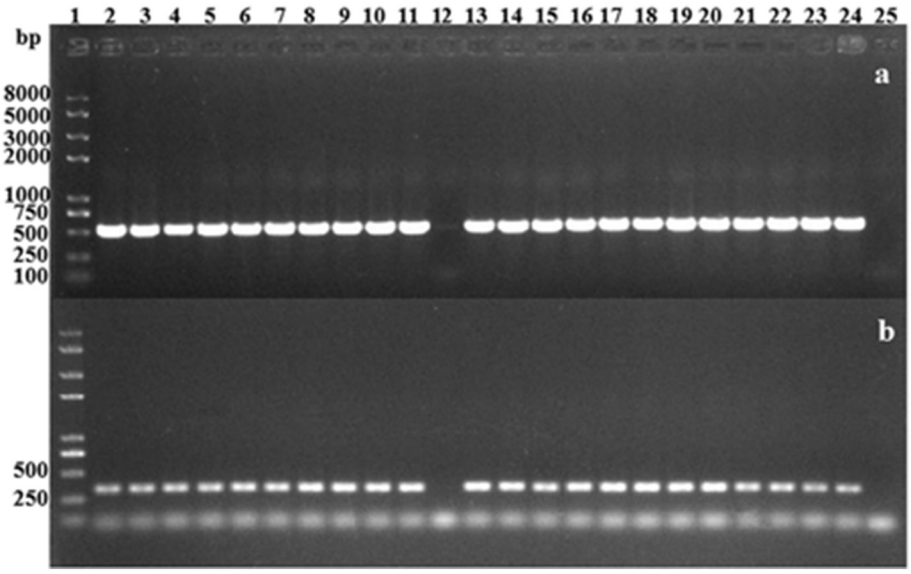

Fig. 2. PCR assays of the *hph-egfp* (a), and *Ltr.hyd* (b) genes in the transformants, recombinant plasmid, and the *T. fuciformis* wild-type strain Y32. Lane 1, DNA marker; Lane 2–23, products obtained from the genomic DNA extracted from 23 random selected transformants; Lane 24, PCR products obtained from recombinant plasmid pGEH-LH; Lane 25, PCR products obtained from genomic DNA extracted from the wild-type strain Y32. The molecular weight of DNA marker (bp) is shown on the left.

3. Results

3.1. Sequence analysis of hydrophobin gene

A hydrophobin gene, *Ltr.hyd*, was cloned from the tiger milk mushroom *L. tuber-regium* in this study. The full CDS sequence and DNA sequence of *Ltr.hyd* was obtained and submitted to NCBI GenBank, and the accession number is KY646147 and KY983590, respectively. The CDS sequence of *Ltr.hyd* was 327 bp, which encoded 108 amino acids with the molecular weight of about 13 kDa and theoretical pI of 6.11. The DNA sequence of Ltr.HYD consists of 452 bp. It contains three exons (1–241, 241 bp; 295–333, 39 bp; 406–452, 47 bp) and two introns (242–294, 53 bp; 334–405, 72 bp). Ltr.HYD has eight conserved cysteine residues: C-X_6-C-C-X_{32}-C-X_{11}-C-X_5-C-C-X_{12}-C-X_7, it is coincided with the array of the cysteine residues for the hydrophobins of class I: C-X_{5-7}-C-C-X_{19-39}-C-X_{8-23}-C-X_5-C-C-X_{6-18}-C-X_{2-13} [18]. So, it verifies that Ltr.HYD is a class I hydrophobin.

Secondary structure prediction suggested that the Ltr.HYD was a highly random coil and alpha-helix protein (Fig. 1a). The hydropathy profile showed that most of the peaks were up zero (score > 0), which means the regions of these peaks represented highly hydrophobic areas (Fig. 1b).

3.2. Agrobacterium-mediated transformation and verification of transformants

After subcultured on PDSA with and without hygromycin, all the putative transformants showed stable resistance to hygromycin. To confirm the presence of introduced *Ltr.hyd* gene, the genomic DNA isolated from 22 randomly selected putative hygromycin-resistant

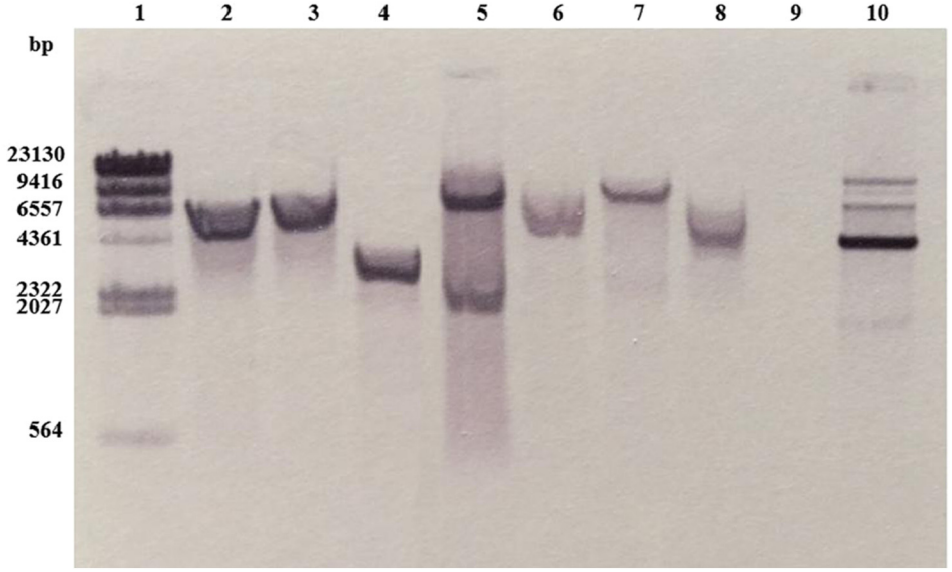

Fig. 3. Southern blot analysis of *T. fuciformis* transformants. Genomic DNA digested with *Xho*I was probed using DIG-labeled *Ltr.hyd*. Lane 1, DNA marker; Lane 2–8, *T. fuciformis* transformants; Lane 9, *T. fuciformis* wild-type strain Y32 (negative control); Lane 10, recombinant plasmid pGEH-LH (positive control). The molecular weight of DNA marker (bp) is shown on the left.

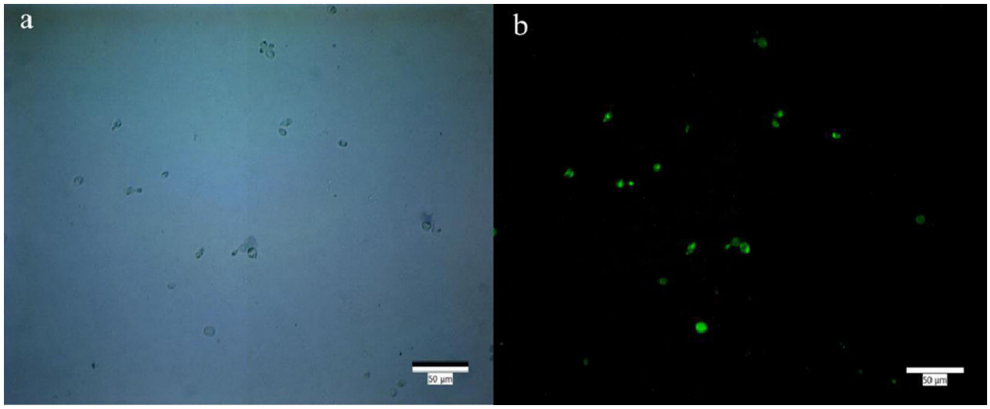

Fig. 4. Detection for green fluorescence under bright light (a) and UV light (b). Images were taken with 40 × fields of view, bar = 50 μm.

transformants and 32Y strain was analyzed by PCR. The 500-bp *hph-egfp* DNA product and the approximately 350-bp *Ltr.hyd* DNA product verified the presence of the introduced gene (Fig. 2).

To investigate the fate of transforming DNA, 7 positive transformants were randomly selected to carry out Southern blotting (Fig. 3). The hybridization result yielded single bands or double bands of different sizes, indicating random insertion of genes into the fungal genome. The positive control plasmid showed bands and the wild strain Y32 showed no hybridization.

The enhanced green fluorescent protein (eGFP) reporter gene was used as another evidence to verify the success of transforming vector pGEH-LH into *T. fuciformis*. The transformants were inspected by fluorescence microscope. The pattern revealed a stable eGFP expression in one transformant (Fig. 4).

3.3. Quantitative real-time PCR of transformants

qRT-PCR results revealed that all of the four randomly selected transformants had expressed hydrophobin with different expression levels. The transformant No. 4 has the highest relative mRNA level and was sub-cultured for further extraction of recombinant hydrophobin rLtr.HYD (Fig. 5).

3.4. rLtr.HYD expression analysis

The hydrophobin was extracted from transformant No. 4 and wild Y32 strain. The SDS-PAGE analysis showed that a band of about 13 kDa in transformant was obtained, indicating the *Ltr.hyd* was successfully expressed in *T. fuciformis*, while the same size band was absent in wild Y32 (Fig. 6). The yield of rLtr.HYD was 0.66 mg/g dry weight.

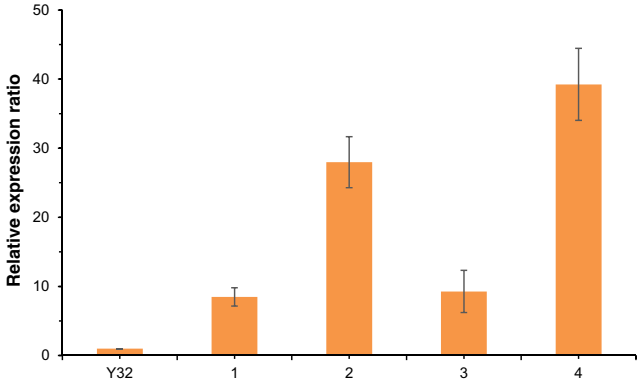

Fig. 5. Relative expression ratio of the hydrophobin in *T. fuciformis* transformants and wild-type strain Y32. Data were presented as mean values of three replicates with the corresponding standard deviations.

3.5. Emulsifying properties of rLtr.HYD

The emulsifying capacity of rLtr.HYD was compared with sodium caseinate and Tween 20, which were usually used as typical food emulsifiers. Emulsification was not observed in pure water, but white emulsions were formed when soy oil (8% v/v) was emulsified in rLtr.HYD, sodium caseinate and Tween 20 solutions. Fig. 7a showed that the emulsifying properties of rLtr.HYD was better than the other emulsifiers after 3 h, and optical microscopy demonstrated that the rLtr.HYD emulsion had much more small oil droplets compared to others (Fig. 7b). After 3 d, the rLtr.HYD emulsion remained turbid, and its oil droplet size and quantity showed little changes, while other emulsions became almost clear, and their oil droplets became evidently larger (Fig. 7a, b). It showed that rLtr.HYD had the ability to

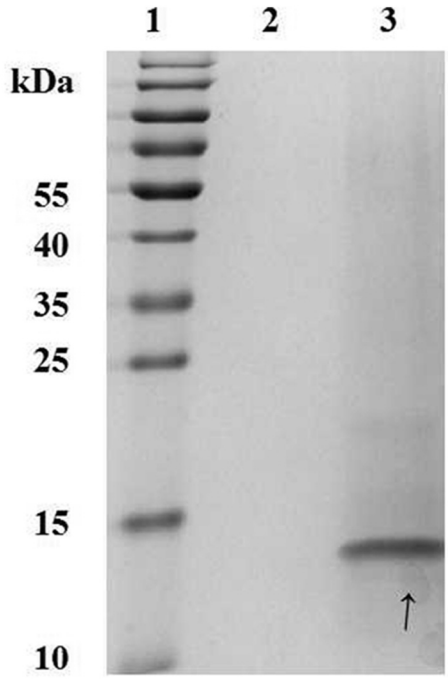

Fig. 6. SDS-PAGE analysis of rLtr.HYD expression by coomassie blue staining. Lane 1, protein marker; Lane 2, hydrophobin extracted from *T. fuciformis* wild-type strain Y32; Lane 3, rLtr.HYD extracted from *T. fuciformis* transformant No. 4. The arrow on the right indicates the size of the recombinant hydrophobin. The molecular weight of protein marker (kDa) is shown on the left.

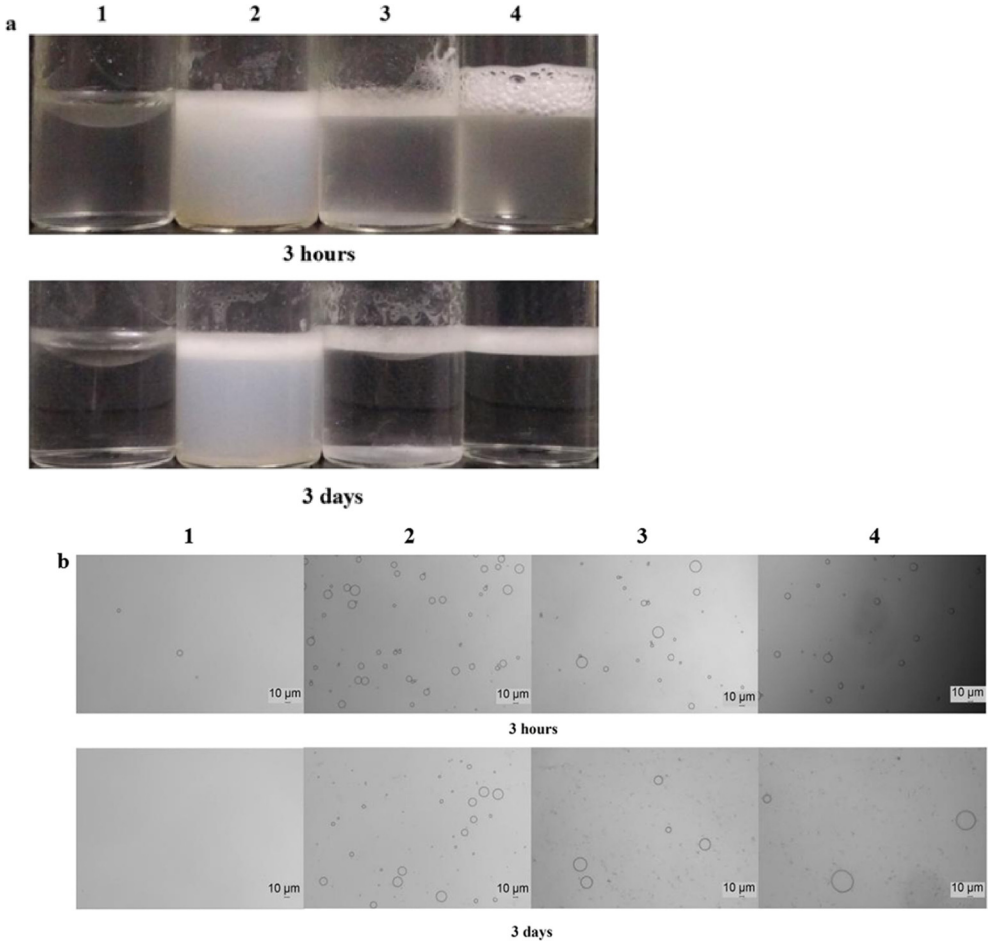

Fig. 7. Emulsifying activity of rLtr.HYD. Each solution was thoroughly mixed with soy oil and observed after 3 h and 3 d. (a) Picture of emulsified solutions: 1, water; 2, rLtr.HYD; 3, sodium caseinate; 4, Tween 20. (b) optical microscopy images of water phase corresponding to each emulsified solution. Scale bar = 10 μm.

stabilize oil droplets for longer time and stronger emulsifying properties compared to sodium caseinate and Tween 20.

4. Discussion

A comparison of the DNA and cDNA sequence indicated that *Ltr.hyd* gene contained two introns, and the size of two introns were 53 bp and 72 bp, respectively. The second intron splice motif is typical class-II introns based on the splice site consensus (5′ GT-AG 3′) and internal sequence required for lariat formation [19]. It has been reported that the presence of an intron is necessary for the expression of hydrophobin genes in *Schizophyllum commune,* so intron splicing may affect genetic expression levels [20].

To evaluate the relationships between Ltr.HYD and hydrophobins from other basidiomycetes, sequence similarity was analyzed using the NCBI BLAST program and a phylogenetic tree constructed based on the amino acids sequence. Ltr.HYD showed the highest similarity to *Pleurotus* hydrophobins. In the phylogenetic tree, Ltr.HYD was clustered with *Pleurotus* hydrophobins (data not shown).

Because of the self-assembling abilities and surface activities, hydrophobins have many applications, such as the foams stabilizing and emulsifying properties in the food industry. The foams generated by hydrophobins can retain bubble stability for several weeks or even longer, which lead to improve physical characteristics of food for example cakes, ice-cream and chocolate milk shakes [21]. The results showed that rLtr.HYD have the ability to stabilize oil droplets for longer time and stronger emulsifying properties compared to sodium caseinate and Tween 20. rLtr.HYD offers a potential to be a emulsifying agent in commercial food.

In this study, the yeast-like cell of *T. fuciformis* was chosen as host for the production of rLtr.HYD. *T. fuciformis*, a jelly fungus, belonging to the Tremellaceae family of the class Heterobasidiomycete, is an edible mushroom without toxicity [22,23]. Because the yeast-like cell of *T. fuciformis* is monokaryotic and easy to culture by fermentation as yeast, it has great potential to be a microbial cell factory system [15,22]. The yeast-like cells of *T. fuciformis* can be transformed into potential biofactory systems using the protoplast method, electroporation or *Agrobacterium* [22]. To develop *T. fuciformis* yeast-like cells-based hydrophobin, we delivered the hydrophobin gene under the control of the *T. fuciformis gpd* promoter using the *Agrobacterium* mediated method.

5. Conclusion

In the present work, a class I hydrophobin gene *Ltr.hyd* from *L. tuber-regium* was cloned and was successfully expressed in *T. fuciformis* yeast-like cells. The recombinant hydrophobin rLtr.HYD has better emulsifying properties than the typical food emulsifiers, sodium caseinate and Tween 20, which provides the potential to be a novel emulsifying agent in commercial food.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary material

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