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Heterologous Expression of Lignin Peroxidase H2 in *Escherichia coli*: *In Vitro* Refolding and Activation

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An engineered cDNA from *Phanerochaete chrysosporium* encoding both the mature and propeptide-sequence regions of lignin peroxidase H2 (Lip H2) was overexpressed in *Escherichia coli* BL21 (DE3) to evaluate its catalytic characteristics and potential application as a pollution scavenger. All expressed proteins were aggregated in an inactive inclusion body, which might be due to inherent disulfide bonds. Active enzyme was obtained by refolding with glutathione-mediated oxidation in refolding solution containing Ca^{2+}, heme, and urea. Propeptide-sequence region was not processed as evidenced by N-terminal sequence analysis. Recombinant Lip H2 (rLip H2) had the same physical properties of the native protein but differed in the k_{cat}. Catalytic efficiency (k_{cat}/K_m) of rLip H2 was slightly higher than that of the native enzyme. In order to express an active protein, fusion systems with thioredoxin or Dsb A, which have disulfide isomerase activity, were used. The fused proteins expressed by the Dsb A fusion vector were aggregated, whereas half of the thioredoxin fusion proteins were recovered as a soluble form but still catalytically inactive. These results suggest that Lip H2 may not be expressed as an active enzyme in *Escherichia coli* although the activity can be recovered by *in vitro* refolding.

Keywords: Expression, *In vitro* refolding, Lignin peroxidase.

Introduction

The white rot basidomycetes fungi organisms are capable of degrading lignin extensively to CO_2 and H_2O in pure culture (Crawford *et al.*, 1981) and degrade lignin more rapidly and extensively than other studied microbial groups (Blanchette, 1984). Lignin peroxidases (Lip) of *Phanerochaete chrysosporium* have been the focus of intensive study ever since their discovery in 1983 because of their potential for applications in biopulping and biobleaching, in the conversion of lignocellulosic materials to chemicals and fuels, and in the detoxification of a broad range of toxic xenobiotics (Kirk and Farrell, 1987). The Lip family consists of multiple isozymes with a molecular weight over 38,000 to 43,000 and isoelectric points ranging from 3.3 to 4.7 (Dass and Reddy, 1990). Lips are oligomannose-type glycoproteins with one or more *N*-glycosylation sites and a number of possible *O*-glycosylation sites (Farrell *et al.*, 1989). The lignin and manganese peroxidases are synthesized and secreted by *Phanerochaete chrysosporium* in response to nutrient (nitrogen, carbon, and sulfur) starvation (Keyser *et al.*, 1978, Jeffries *et al.*, 1981). Lignin is degraded by this organism only during secondary idiophasic metabolism, whose onset is triggered by depletion of nutrient nitrogen, carbon, or sulfur. Nitrogen limitation is most commonly used in experiments with *Phanerochaete chrysosporium.*

Until now, the only method of producing the active enzyme has involved purification from cultures of the native fungi (Tien and Kirk, 1984). However, since the expression of peroxidases in fungi is regulated by nitrogen content, white rot fungus should be cultured in low nutrient and low volume media for six days. Consequently, the productivity of Lips is extremely low. Several investigators have attempted to express Lips in *Escherichia coli (E. coli)* (Wendy and Andrew, 1996) but all the proteins were expressed in an inactive form as an inclusion body. Heterologous expressions of Lip H2 and H8 in baculovirus expression systems were tried and very low levels of active peroxidase were secreted into the growth medium (Johnson and Li, 1991).

The aim of the present investigation was to express Lip H2 in *E. coli* as an active enzyme. Despite several

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approaches to express Lip H2 in *E. coli* systems, all expressed proteins were aggregated in an inclusion body. The catalytic activity of recombinant lignin peroxidase (rLip) H2 was recovered by *in vitro* refolding and its spectral and catalytic characteristics were described.

Materials and Methods

Strains and plasmids *Phanerochaete chrysosporium* BKM-F-1767 (DSM 6909) was obtained from Deutsch Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *E. coli* strains, DH5α and BL21 (DE3) (Novagen, Madison, USA) were used as hosts for the propagation of plasmids and expression of recombinant proteins. AD494 (DE3) (Novagen, Madison, USA) strain was used for expression of thioredoxin fusion proteins. Plasmids pBluescript II KS (Keyser *et al.*, 1978) (Stratagene, La Jolla, USA), pET3a (Novagen, Madison, USA), pETRX, pTrxFus (Invitrogen Co., San Diego, USA), and pET39b (Novagen, Madison, USA) were used for subcloning and expression of the recombinant proteins. Mouse anti-thioredoxin antibody was obtained from Invitrogen.

Construction of the expression vector for Lip H2, pET3aLipH2 Cultivation of *Phanerochaete chrysosporium* was performed according to the methods by Fenn and Kirk (1979) and Kirk *et al.* (1978) with a slight modification. The total RNAs of *Phanerochaete chrysosporium* were purified as described by Chomczynski and Sacchi (1987). Reverse transcription (RT) from fungal mRNAs was performed by AMV reverse transcriptase according to Sambrook *et al.* (1989). Amplification of the mature plus propeptide sequence regions of the Lip H2 cDNA was achieved using the PCR technique (Salki *et al.*, 1988), utilizing the proofreading enzyme *Pfu* polymerase (Stratagene, La Jolla, USA). The 3 prime of oligonucleotide H2-R (AGAATT-CCATATGGCTGCGGTGATCGAGAAG) was complementary to the last codons of the Lip H2 cDNA, while, at the 5 prime, an additional sequence containing a *Bam*HI restriction site was present. Oligonucleotide H2-F (AGAATTCCATATGGCCCC-GAACCTCGACAAG) was designated to span the six-residue (23–28) propeptide sequence of the Lip H2 cDNA. Furthermore, this oligonucleotide was designed so that an *Nde*I restriction site was created at an engineered initiation codon (Kim *et al.*, 1996). A denaturation of 3 min at 94°C was followed by 30 cycles of denaturation (40 s at 94°C), annealing (45 s at 58°C), and extension (2 min at 72°C) using a programmable heating block (Gene Amp System 2400, Perkin-Elmer). PCR products were subcloned by restriction digestion with *Bam*HI, *Nde*I, and ligation with pretreated pET3a. pETLipH2 was transformed to BL21 (DE3). The cloned Lip H2 sequence was verified by an automatic sequencer.

Expression and refolding conditions IPTG-induced, cultured host cells harboring pET3aLipH2 were harvested, sonicated, and then inclusion bodies were collected by centrifugation at 10,000 rpm for 15 min. The pellet was solubilized in resolubilizing buffer [50 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol (DTT), 6 M Urea, 1 mM EDTA] and recentrifuged to remove insoluble material. Small-scale test folds were carried out in 1 ml for 24 h. Constant conditions were 20 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mg/ml protein. Conditions which were systemically varied included urea (0.6–3.8 M), oxidized glutathione (GSSG) (0.1–1.3 mM), $CaCl_2$ (0–100 mM), heme (0–20 μM), pH (6.4–9.5), and temperature (4–37°C). Refolding of denatured proteins took several hours. After refolding, each sample was dialyzed against 5 mM succinate buffer (pH 5.5) and centrifuged to remove reaggregates. Measurement of the relative folding activities was performed using a modified 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay. ABTS is used for the general peroxidase substrate or electron radical trap (Wendy and Andrew, 1996). The reaction mixture for the ABTS assay included 500 μM veratryl alcohol, 400 μM H_2O_2, 50 mM sodium acetate (pH 4.0), and 500 μM ABTS which was incubated at room temperature for 30 min and then read at 405 nm. After large-scale refolding from the inclusion body fraction under optimal conditions, they were purified through a DEAE-sephacel column (Park and Song, 1996).

Kinetic analysis of native and rLip H2 The kinetic assay was performed in a reaction mixture containing 2 mM veratryl alcohol, 25 mM tartaric acid (pH 2.5), 0.4 mM hydrogen peroxide, and 42 nM enzyme. The reaction was started by the addition of hydrogen peroxide and initial velocity was monitored by measuring the absorbance change at 310 nm. From nonlinear regressions performed using the software Enzifitter (Leatherbarrow, 1987), K_m and V_{max} values for veratryl alcohol and hydrogen peroxide were calculated.

Expression of thioredoxin or Dsb A fusion protein pET39b was used for fusion of Dsb A with Lip H2 and pETRX was used for thioredoxin fusion. To make pETRX in this study, the thioredoxin gene from pTrxFus was obtained by digestion with *NdeI*/*Bam*HI and subcloned to pET3a. For subcloning to pET39b and pETRX, the Lip H2 gene was reamplified using PCR with H2-R and H2-MF (AAACCCGGGGATCCTGTCGCTTGCCC-CGACGGC). The amplified gene was inserted into each vector through a *Bam*HI site. The expression test and determination of solubility of its product was performed by the previous same manner. Trx-Lip fusion protein was expressed in the *E. coli* AD494 strain. Western blot analysis was done with anti-thioredoxin antibody (Chang *et al.*, 1996).

Results

Expression of the Lip H2 gene in *E. coli* BL21 (DE3) Lip H2 cDNA containing the propeptide sequence was subcloned to the pET3a expression vector and transformed to *E. coli* BL21 (DE3). Expression of Lip H2 was induced by addition of IPTG to the medium. After the induction, the cells were harvested and lysed by freeze-thawing. The cell supernatant and pellet were subjected to SDS/PAGE. The size of the protein estimated from SDS/PAGE was found to be 38 kDa, well consistent with the size predicted from the engineered gene sequence (Fig. 1). Translation is therefore assumed to be initiated at the engineered AUG codon. Like other peroxidase expressions, all protein products were aggregated in an inclusion body even if cells were cultured at low temperature.

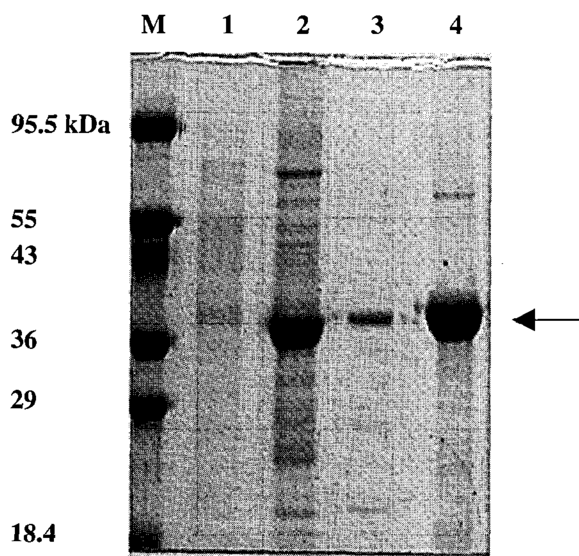

Fig. 1. Expression and purification of the cloned lignin peroxidase H2 gene in *E. coli* BL21 (DE3). Twelve percent polyacrylamide gel electrograms were stained with Coomassie Brilliant Blue R-250. Lane M, 10 μg of protein standard mid-range marker (MBI fermentas, Vilnius, Lithuania); Lane 1, inclusion body fraction; Lane 2, IPTG-induced inclusion body fraction; Lane 3, purified rLip H2 fraction; Lane 4, native Lip H2 fraction.

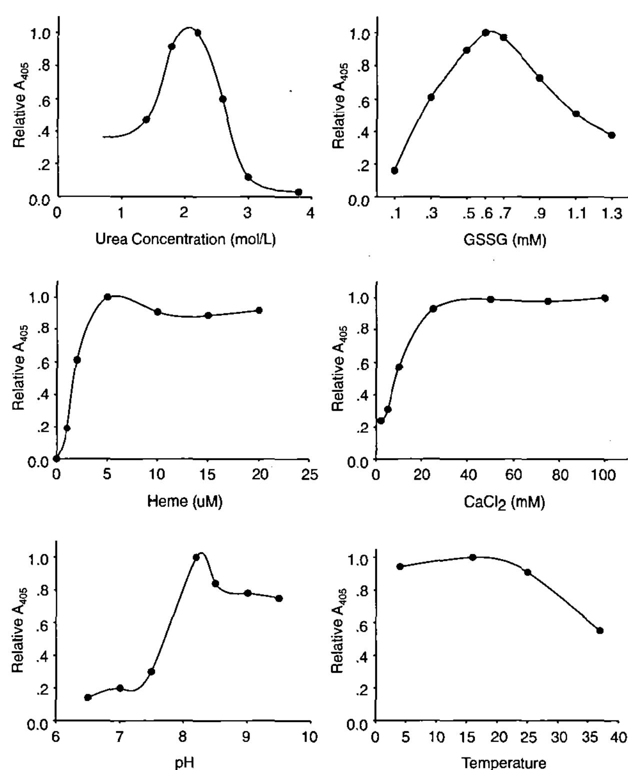

Fig. 2. Effects of various parameters on *in vitro* refolding of rLip H2 polypeptides. Shown are the results of ABTS assays of small-scale folding reactions demonstrating the effect of urea, GSSG, heme, $CaCl_2$, pH, and temperature. The ABTS assay is described in Materials and Methods.

Refolding of inactive polypeptides The inclusion body obtained from bacterial expression was first solubilized and denatured in resolubilizing buffer containing 6 M urea. Lips have been known to possess five intramolecular disulfide bonds, and appropriate amounts of DTT and GSSG were included in the refolding solution to prevent improper formation of disulfide bonds. Post-translational modification of this native enzyme entails the incorporation of heme and two calcium ions, thus bovine hemin and $CaCl_2$ are essential for proper refolding into an active form. Small-scale folding experiments were performed with inclusion body preparations by systemically varying the parameters (0.6–3.8 M urea, 0.1–1.3 mM GSSG, pH 6.5–9.5, 0–20 μM bovine hemin, 0–100 mM $CaCl_2$, and 4–36°C) while concentrations of protein and DTT were fixed. Complete refolding took more than 20 h. Each refolding sample was dialyzed against succinate buffer and then the activity was determined by the ABTS assay. The effects of each factor on the refolding are shown in Fig. 2. Maximal activity was obtained with a concentration of 5 μM heme, 50 mM $CaCl_2$, 2.1 M urea, and 0.65 mM GSSG. The reconstitution of Lip H2 exhibited an absolute dependence on $CaCl_2$ and heme. Because heme corresponds to approximately a 1 : 1 molar ratio of heme to peptide, the yield of refolding efficacy was more sensitive to heme content than to the other parameters. For proper formation of disulfide bonds, the optimal refolding condition required a 6.5 : 1 molar ratio of GSSG to DTT. The optimal refolding pH was about 8.2–8.3. No refolding was achieved at pH 6 where Lips showed its activity. Effect of temperature on refolding was trivial although refolding at 37°C showed a half of maximal activity. The small amounts of active protein could be recovered by reconstitution of inactive inclusion bodies. Approximately 2.4% of inclusion body was converted into active Lip H2. Refolded protein was purified as spectroscopically pure enzyme. The rLip H2 was obtained by DEAE-sephacel column chromatography with 95% purity. Its molecular weight and isoelectric point was similar to that of native enzyme. Specific activity towards veratryl alcohol was estimated to be 55.6 unit/mg protein. This result was also similar to that of native Lip H2. The protein concentration determined by the heme content assay was identical to that estimated by the Lowry method, suggesting that the purified heme proteins were pure.

***N*-terminal analysis of rLip H2** *N*-terminal analysis demonstrated that its *N*-terminus was MAPNLDKRVAC. Native Lip H2 mRNA consists of signal peptide-, propeptide- and mature peptide-segments. Signal peptide facilitates the secretion of peptides and propeptide plays a role in its proper folding and heme binding (Ritch *et al.*,

1991). In this study, expressed rLip H2 was designed to possess both mature peptide and propeptide which was cleaved before secretion in native system. *N*-terminal analysis revealed that the propeptide region was not cleaved in the *E. coli* system.

Kinetic analysis of Lips rLip H2 and native H2 were purified with a sufficient purity to allow measurement of the steady-state kinetic parameter. A comparison of the steady-state kinetic properties of each enzyme is described in Table 1. The K_m of rLip H2 for veratryl alcohol was identical to that of native Lip H2 and the activity was inhibited in the presence of a high concentration of hydrogen peroxide. However, there was a slight difference in the k_{cat} between native and rLip H2. The catalytic efficiency of rLip H2 was slightly higher than that of native Lip H2.

Table 1. Kinetic properties of native enzyme and rLips.

Enzyme[b]	Substrate	K_m (μM)	k_{cat} (1/s)	k_{cat}/K_m (s/μM)
rH2	VA[a]	122.2 ± 6.8[c]	55.5 ± 1.12	0.454
	H_2O_2	64.3 ± 7.93	68.1 ± 2.62	1.059
Native H2	VA	121.2 ± 11.1	40.6 ± 1.35	0.335
	H_2O_2	50.6 ± 8.74	49.3 ± 3.09	0.974

[a] Veratryl alcohol was labeled by VA.
[b] Recombinant Lip H2 and native Lip H2 were labeled by rH2 and native H2, respectively.
[c] Each data represents the mean ± SD of three determinations.

Expression of fused proteins Based on the assumption that aggregation of expressed polypeptide might be due to improper disulfide bonding, a fusion vector encoding the Dsb A or thioredoxin genes was used for the expression of an active Lip H2. Dsb A and thioredoxin have disulfide isomerase activity and can catalyze intramolecular disulfide bond formation depending on the redox potential in the hosts. pET39b vector (Novagen, Madison, USA) and pETRX were used for the Dsb A and thioredoxin fusion proteins, respectively. For the preparation of pETRX, the thioredoxin gene from pTrxFus was inserted pET3a through *Nde*I and *Bam*HI sites and this plasmid was named as pETRX. The Lip H2 gene was amplified with specific primers (H2-MF and H2-R) containing an engineered *Bam*HI site and then inserted into pET39b and pETRX. The expressed Dsb A fusion protein resulted in aggregates, whereas half of the thioredoxin fusion proteins were expressed as a soluble form, but they were still catalytically inactive (Fig. 3). The expressed Trx-Lip was purified through a DEAE-sephacel column and its heme content was measured. However, the expressed proteins had no heme that was critical in catalytic activity. These results suggest that the fused thioredoxin might facilitate proper protein folding but interfere with the incorporation of heme into the catalytic site. For precise disulfide bond formation, thioredoxin fusion proteins were expressed in an AD494 strain which has high oxidizing potential due to a mutation in Trx B reductase (Derman *et al.*, 1994), but no active protein was recovered even in this system.

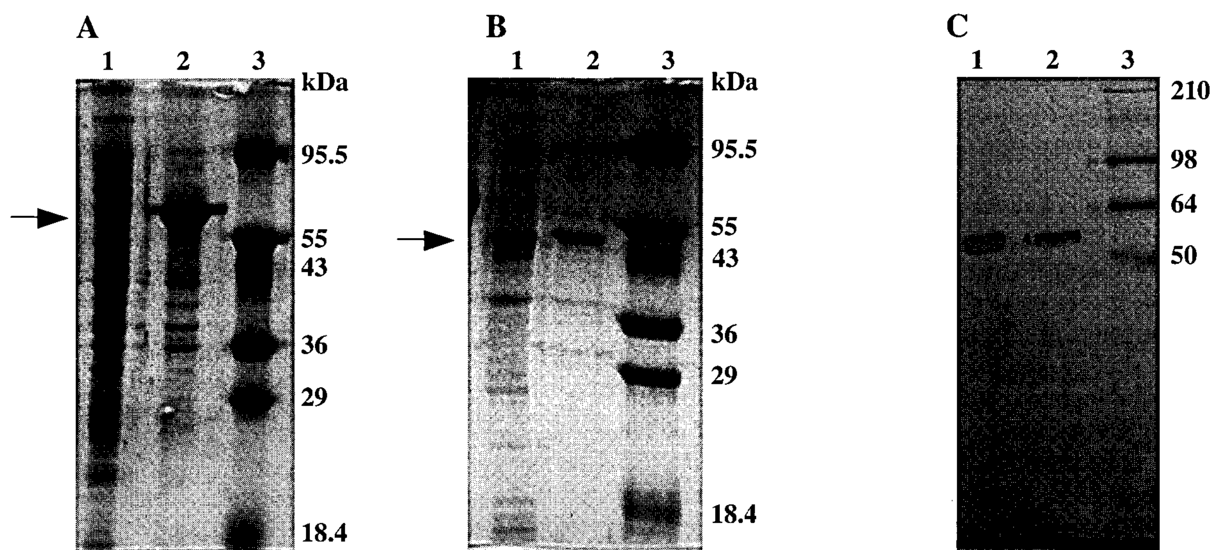

Fig. 3. SDS-PAGEs of Dsb A-fused Lip H2 (**A**), thioredoxin-fused Lip H2 (**B**), and Western blot of thioredoxin-fused Lip H2 (**C**). Samples were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Western blot analysis was done with mouse anti-thioredoxin antibody. Samples are: Lane 1, cytosolic fraction; lane 2, inclusion body fraction; lane 3, molecular weight marker.

Discussion

White rot fungi, responsible for the biodegradation of lignin in wood, have been of concern in their ability to degrade a variety of environmental pollutants (Bumpus and Aust, 1987). In general, it is thought that this biodegradative ability is related to the Lips of these fungi to degrade lignin (Tien and Kirk, 1983). This ability, which is unique to white rot fungi, is mostly attributed from a family of peroxidases secreted by the fungi. Among the peroxidase isozymes, Lip H2 has the highest oxidizing potential and can metabolize a wide variety of chemicals. Therefore, development of a genetically engineered microorganism harboring Lip genes can be applied to environmental remediation and pulp industries. For this purpose, Lips should be expressed in bacterial strains which can survive in harsh sludge.

In this study, the expression of Lip H2 in *E. coli* resulted in an inclusion body, and previous attempts at other prokaryotes have yielded only inactive inclusion bodies (Whitwam and Tien, 1996; Wendy and Andrew, 1996). This leads to the question of why rLips expressed in prokaryotes should be present in an insoluble fraction. One possibility is that Lips have many disulfide bonds. Most secretory proteins contain many disulfide bonds and Lips also include 5 disulfide bonds (Sinclair *et al.*, 1992). In the *E. coli* cytosol, these expressed proteins might be cross-linked through the formation of intermolecular disulfide bonds and consequently intramolecular disulfide bonds for the exact three-dimensional structure may not be formed. A second possibility is that rLip H2 in *E. coli* was not modified by glycosylation or phosphorylation. In a natural fungal system, all Lips are subject to modification with oligomannose-type glycosylation and phosphorylation on their *N*-linked carbohydrate moiety in the form of mannose 6-phosphate, which may facilitate Lips folding and heme binding (Wendy and Andrew, 1996). Therefore, post-translational modification may be critical in the active expression of rLips in *E. coli* and also in keeping away from aggregates.

The physical properties of rLip H2 were similar to those of native Lip H2 in isoelectric point, molecular weight, etc. The kinetic properties of rLip H2 was similar to the native form in the K_m for veratryl alcohol and hydrogen peroxide, but the k_{cat} and catalytic efficiency were slightly higher than that of native enzyme. rLip H2 was found to contain a propeptide region as judged by *N*-terminal sequencing, suggesting that the propeptide and modifying motif do not interfere with catalytical activity. In fungal systems, the propeptide of Lips plays a significant role in its folding and heme binding, which is cleaved prior to secretion due to its dibasic amino acid cleavage site (Ritch *et al.*, 1991). In our experiment, the expression vector was designed to include a propeptide sequence for facilitating the protein folding, but the propeptide was intact and not cleaved in the expressed polypeptide. This result might be due to the lack of specific peptidases related to protein sorting for secretion in *E. coli*.

The soluble Trx-Lip fusion protein could be obtained by using a thioredoxin fusion vector. The fraction of soluble protein was increased as the temperature was lowered. However, these soluble proteins still did not possess catalytic activity. Based on the assumption that proper disulfide bonds may be formed in the AD494 (DE3) strain, whose redox potential is relatively high (Derman *et al.*, 1994), this strain was used as host. However, no active proteins were recovered even in this strain.

Despite of several approaches to express Lip H2 in *E. coli* systems, all expressed proteins were aggregated in an inclusion body, although the catalytic activity of rLip H2 can be recovered by *in vitro* refolding under appropriate conditions. These results suggest that the expression of Lip enzymes in a prokaryotic system is extremely difficult. The expression of Lip in yeast is being studied.

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