STUDIES ON THE FUSION OF LIGNINOLYTIC ENZYME cDNAS AND THEIR EXPRESSION

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Manganese peroxidase (MnP) and lignin peroxidase (LiP) are two major peroxidases involved in lignin biodegradation. The cDNA *mnp1* encoding a kind of MnPs, and cDNA *clg5* encoding a kind of LiPs were fused to one cDNA *mnp1- clg5* (rmc15) by over-lap PCR technology in this research. Then the recombinant cDNA rmc15 was cloned into a vector pTrcHisB to construct its efficient expression plasmid pTHmc15 in *Escherichia coli*. The *E. coli* transformed by pTHmc15 was induced by isopropyl-β-D-thiogalactoside. The expressed protein was analyzed by SDS-PAGE, and a new one was observed with a molecular weight of about 77KD. Enzyme activities of MnP and LiP could not be observed in the unfolded fused protein. However, the enzyme activity of MnP was detected in the recombinant protein after it was refolded and activated by Ca^{2+} and heme, while the activity of LiP was not detected. These results show that the enzyme activity of the protein at N-terminal was not affected, but at C-terminal it was affected in the fusion protein of ligninolytic enzymes. Therefore, it is unfeasible to construct the gene of bifunctional ligninolytic enzyme with the fusion of the cDNA *mnp1* encoding MnP and cDNA *clg5* encoding LiP.

Keywords: *Ligninolytic Enzymes; Over-lap PCR technology; Gene Expression; Folding and Activating of proteins*

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INTRODUCTION

Phanerochaete chrysosporium is a model microorganism to investigate the degradation of lignin, which can secrete peroxidases in nitrogen-limited growth medium, i.e., manganese peroxidase (MnP) and lignin peroxidase (LiP). The protein sequences (Mayfield et al. 1994; Tien and Kirk 1984; Timofeevski et al. 1999; Zhang 1987) of MnP and LiP indicate that the third amino acid of their mature protein is Cys, and it can interact with other Cys moieties to form a double-sulfur bond, essential in enzyme construction. So it can be deduced that if MnP or LiP is fused with another peroxidase, the activity of one protein may be influenced, while another would not be.

In order to verify the above-mentioned hypotheses, over-lap PCR technology was used to fuse the encoding area of two kinds of peroxidases, i.e., cDNA *mnp1* and cDNA *clg5*, then the fused gene *mnp1-clg5* was expressed in *Escherichia coli* (*E. coli*). After folding and activating the protein, the enzyme activities of these two kinds of enzymes were monitored to determine their different activities.

MATERIALS AND METHODS

Bacteria, Vector and Plasmid

E.coli DH5α, clone vector pUC18 and expression vector pTrcHisB were provided by the molecular biology laboratory of Sichuan University. Plasmid pATTMnP1 and pBSCLG5 was constructed by Xie (Xie 2001)

Reagents

The enzymes and PCR purification kits were bought from Japan TaKaRa Bioengineering Company (Dalian), Germany Boehringer Mannheim Company and U.S.A. BRL Company, separately. Isopropyl -β-D-thiogalactoside (IPTG) and bovine chlorohemin, oxidized glutathione (GSSG), dithiothreitol (DTT), and Triton X-100 were bought from Sigma. All other reagents were of analytical grade.

Primers and DNA fragments were synthesized and purified by Japan TaKaRa Bioengineering Company (Dalian).

Gene Operation

Plasmid extraction, the enzyme cut reaction, the recovery of DNA fragments, joint reaction, the transform and culture of bacterium, SDS-polyacrylamide gel electrophoresis, Bradford protein detection, and the PCR overlap extension technique were all performed with little modification (Sambroo et al. 1989).

The expression of fusion protein was carried out according to the expression of H2 gene of LiP in *E. coli* by Nie et al. (1998) and H8 gene by Doyle et al. (1996).

The folding and activating of mutated protein were previously reported (Nie et al. 1998; Timofeevski et al. 1999; Doyle et al.1996).

The enzyme activity of MnP was similar to what has been previously reported (Gold and Glenn 1988), while the detection of the enzyme activity of LiP was detected by veratryl alcohol (Tien and Kirk 1984).

RESULTS

The Fusion of rmc15 cDNA and the Construction of Its Plasmid

According to the total sequences of cDNA *mnp1* of MnP and cDNA *clg5* of LiP, primers were designed by DNASIS software. There are no *Nco*I, *BamH*I sites in *mnp1* and *clg5* (Mayfield 1994; Tien and Kirk 1984; Timofeevski 1999; Zhang 1987). To clone the amplified fragments into the vector pTrcHisB, *Nco*I and *BamH*I sites were designed at the 5'-end of P1 and P4, respectively.

- *mnp1* forward primer (P1): 5'ATT CCA TGG CAG TCT GTC CAG ACG G3'
- *mnp1* backward primer (P2) backward: 5'CTT GCC GTT CGA GCA GGT CGC AGC AGG GCC ATC GAA CTG3'
- *clg5* forward primer (P3): 5'GTT CAG TTC GAT GGC CCT GCC GCG ACC TGC TCG AAC GG3'
- *clg5* backward primer (P4): 5'ATT GGA TCC TTA AGC ACC AGG AGG AGG 3'

A 1.07 Kb DNA fragment, *mnp1*, was obtained by using the plasmid pATTMnP1 DNA as the template and PCR amplification of P1 and P2 primers, while a 1.03 Kb DNA fragment, *clg5*, was obtained by using the plasmid pBSCLG5 DNA as the template and PCR amplification of P3 and P4 primers.

Then, *mnp1* and *clg5* were purified by PCR purification kits and used as the template; thus a 2.1 Kb DNA fragment could be obtained by the second PCR amplification of P1 and P4 primers. This DNA fragment was *mnp1-clg5* cDNA (1.07+1.03 Kb) and named as *rmc15* (Lane 3 and 4 in Fig.1). If *rmc15* was directionally inserted into the corresponding sites of expression vector pTrcHisB in *E. coli*, the expression plasmid of fusion cDNA was constructed and named as pTHmc15.

By using *Nco*I and *Bam H*I to restrict pTHmc15, two DNA fragments were detected by gel electrophoresis. Among them, the DNA lane with 4.3 Kb was the same with pTrcHisB restricted by *Nco*I/*Bam H*I. Another DNA lane with 2.1Kb (Lane 2 in Fig. 2) was similar to the fusion cDNA fragment as expected a priori.

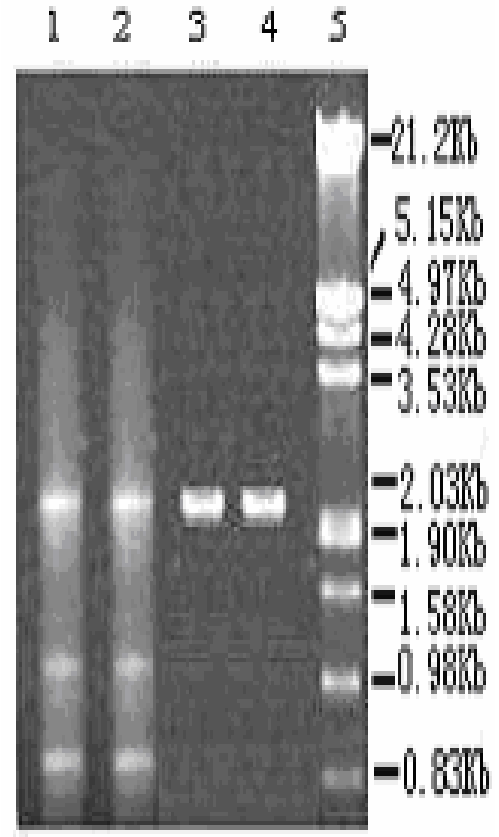

Fig. 1. Overlap-PCR amplification of fused cDNA *rmc15.* Lanes 1-2: the amplification product of fused cDNA *rmc15*; Lanes 3-4: the purified product of fused cDNA *rmc15*; Lane 5: λ/ *Hind* III-*Eco*R I DNA standard.

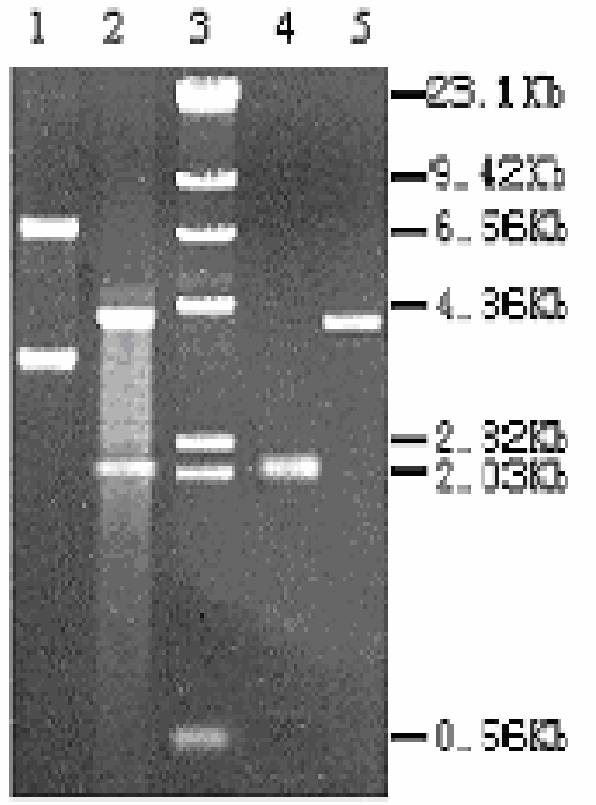

Fig. 2. Restriction analysis of recombinant plasmid pTHmc15. Lane 1: unrestricted pTHmc15; Lane 2:The restriction product of pTHmc15 by *Nco* I/*Bam* HI; Lane 3: λ/ *Hind* III-*Eco*R I DNA standard; Lane 4: The PCR product of pTHmc15; Lane 5: The restriction product of pTrcHisB by *Nco* I/*Bam* HI.

The Expression of Fused cDNA *rmc15* in *E. coli*

There are 358 amino acid residues (108-1181) in the *mnp1* encoded area of natural MnP, while there are 344 amino acid residues (115-1146) in the *clg5* encoded area of the natural LiP; therefore, a fusion protein will contain 702 amino acid residues, and its molecular weight will be approximately 77.2 kD. In Fig. 3, when *E. coli* with recombinant plasmid was induced by IPTG with a different time, and analyzed with SDS-PAGE, a protein of approximately 77 kD, termed rMnP1-LiP5 (abbreviated as rML15), could be observed after a one-hour inducement, while this protein could not be observed if it was not induced by IPTG.

E.coli DH5 α, which contained expression plasmid pTHMC15, was cultured and induced for 4 h by 0.5 mmol/L IPTG. The cultured bacteria were then suspended in lysate. After being broken by ultrasonic waves, a small fraction of the protein solution was removed, and the enzyme activities of MnP and LiP were detected, respectively. The remaining protein solution was centrifuged, and the precipitate was stored at -20°C. The experimental results indicate that the protein expressed by *E. coli* that was transformed by either pTrcHisB and pTHmc15 had no enzymatic activity for MnP and LiP.

After thawing, the protein was folded and activated according to the literature (Nie et al. 1998; Timofeevski et al. 1999; Doyle et al. 1996), and then its enzyme activity was determined. In Fig. 4 the curve (MnP) is the absorbance at 240 nm of the solution with 0.06 mg/mL of total protein, 0.1 mmol/L Mn^{2+}, 0.1 mmol/L H_2O_2, and 0.1 mol/L lactic acid buffer (pH 4.5). The solution without recombinant protein was used as a reference. The curve (LiP) is the absorbance at 310 nm of the solution with 0.22 mg/mL of total protein, 2.0 mmol/L veratryl alcohol, 0.4 mmol/L H_2O_2, 0.1% Tween 80, and 0.1 mol/L tartaric acid buffer (pH3.0).

The results showed that the protein concentration of *E.coli* transformed by pTrcHisB was 1.50 mg/mL, with no enzyme activity of LiP and MnP. The protein concentration of *E.coli* transformed by PTHmc15 is 1.53 mg/mL, and the concentration of the recombinant protein (rML15) was about 0.1 mg/mL by SDS-PAGE. It can be seen from Fig. 4, after folding and activating, if the recombinant protein was treated with veratryl alcohol, the value at A_{310} value did not change with time, while the value at A_{240} increased with increase in reaction time if it was treated with Mn^{2+}. Therefore, after folding and activating, this fusion protein showed the enzyme activity of MnP, but no activity of LiP. The enzyme activity of MnP was calculated as 26.96 U/L, with specific activity as 0.3 U/mg, while the specific activity from natural MnP can be up to 100 U/mg (Gold and Glenn 1988).

DISCUSSION

The Folding Conditions of the Expression Result of Fusion Gene and Its Enzyme Activity

The above results show that after folding and activating of rML15, the specific activity of MnP was approximately 0.3 U/mg, just 0.3% of natural MnP. This indicates only approximately 0.3% of rML15 was correctly folded, so it is necessary to optimize the folding conditions.

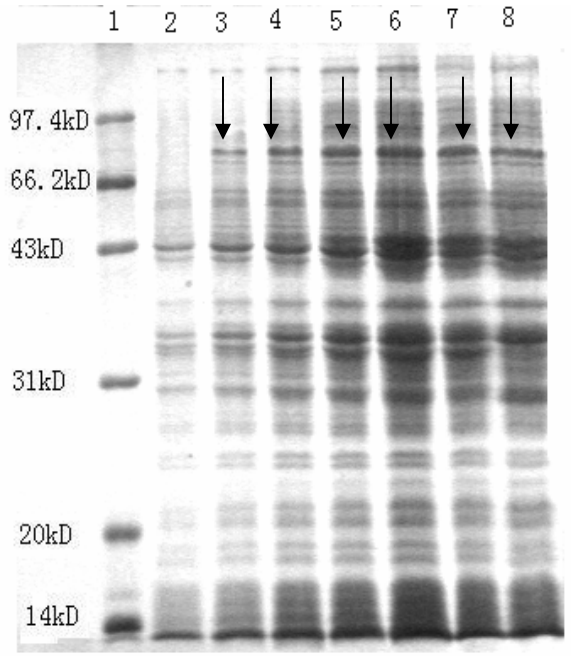

Fig. 3. SDS-PAGE analysis of the total proteins of *E.coli* containing expression plasmid pTHmc15.

Lane 1: molecular weight of standard protein;Lane 2: the recombinant *E.coli* without IPTG inducement;Lanes 3-8: the recombinant *E.coli* with IPTG inducement for different times: 1, 2, 3, 4, 5, 6h.

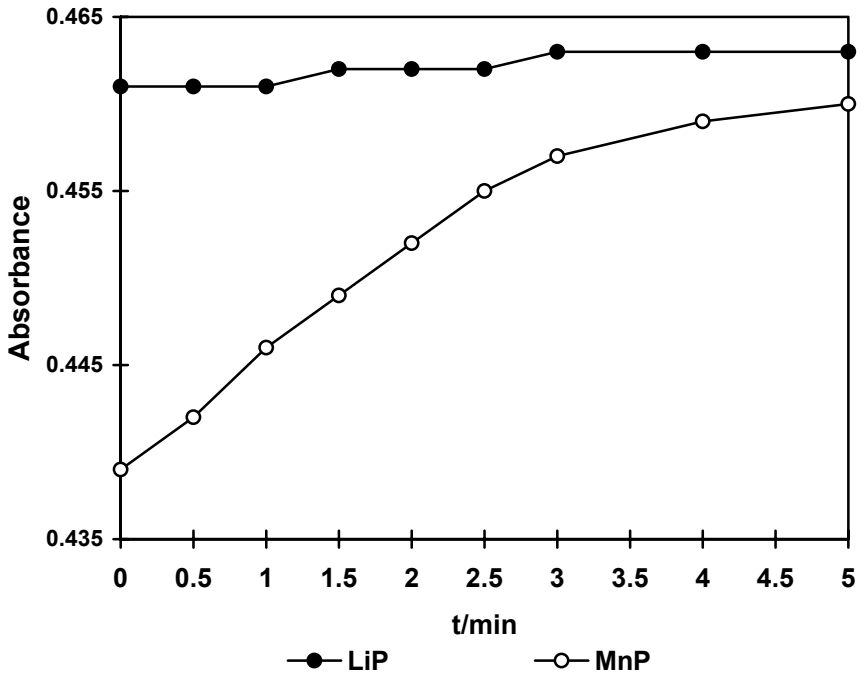

Fig. 4. Absorbance changes of the oxidation of veratryl alcohol and Mn^{2+} by rML15.

The folding conditions of MnP and LiP in *E.coli* were performed according to earlier literature reports and on the basis of their protein structure. Nie et al. (1998), Timofeevski et al. (1999) and Doyle et al. (1996) have also discovered that the folding conditions used were all suitable for MnP and LiP. Therefore, it can be concluded that enzyme inactivity of the last kind of protein of rML15 was not due to the folding conditions. Since rML15 had been correctly translated, the main influence factor for enzyme activity of LiP is the structure of fusion protein.

The Influence of Gene Fusion of MnP and LiP on Enzyme Activity

If the 3'-end of cDNA *mnp1* encoded MnP was directly fused with the 5'-end of cDNA *clg5* encoded LiP, the protein encoded by this fusion cDNA should be the fusion protein by direct association of the carboxyl end of MnP with the amino end of LiP. So, the third amino acid of the expressed protein should still be Cys of MnP, but the Cys of the corresponding LiP is the 361st. If this fusion protein is folded and activated by ferroheme and Ca^{2+}, the enzyme activity of MnP can be detected, while LiP cannot.

This result has proven that if another peroxidase gene was fused into MnP or LiP gene, the enzyme activity of the first protein will not been influenced, while that of the second protein will be affected. The essential reason may be that the existence of the first protein has influenced the correct folding of the second protein, so it is difficult for the second protein to form a three-dimensional conformation, electron shift channel and substrate binding sites, so there is no enzyme activity for the second protein.

Therefore, it is unfeasible to construct a bifunctional enzyme gene by fusing the cDNA in the encoding area of MnP and LiP.

CONCLUSION

The mature protein coding regions of MnP cDNA mnp1 and LiP cDNA CLG5 were fused to form one cDNA called rMC15 in which the mnp1 is upstream of CLG5 by employing extend-overlap PCR.

The *E.coli* transformants containing recombinant plasmid pTHMC15 were induced with IPTG, and the total proteins were analyzed by SDS-PAGE. A new protein was observed, in which the molecular weight was approximately 77 kD, as expected.

After folding and activation by Ca^{2+} and heme, the fused protein rMC15 showed only the activity of MnP but not LiP, indicating that the MnP activity was not affected, but the LiP activity was lost.

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