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# Improved Heterologous Expression of the White-Rot Fungal Ligninase H8 by Crossover Linker Mutagenesis

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## ABSTRACT

Using the crossover-linker mutagenesis method, the 5′ noncoding region of the λML-1 cDNA, which encodes the ligninase H8 isozyme of the white-rot fungus, *Phanerochaete chrysosporium*, was deleted with the simultaneous insertion of the putative *Spodoptera frugiperda* ribosome-binding sequence (RBS) (TATAAAT) directly in front of the translation-initiation codon of this gene. A recombinant baculovirus, pVL-Mu-H8, carrying the ligninase-H8 gene was successfully constructed, as determined by both sequence analysis and dot blot hybridization. A more than 18-fold increase in the expression of ligninase H8, compared to the previous pEV11-1A.3 recombinant baculovirus, was detected in the Sf-21 insect cells. This enzyme was detected within 3 d postinfection and was biologically active, capable of oxidizing the model lignin compound, veratryl alcohol. The molecular weight of the overexpressed 42 kD protein was similar to that of the native fungal ligninase-H8 isozyme and it also reacted specifically with the anti-H8 monoclonal antibody (MAb 2D4.9) in Western blot analysis.

**Index Entries:** Recombinant baculovirus; crossover-linker mutagenesis; ligninase isozyme; protein expression.

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## INTRODUCTION

The white-rot fungus *Phanerochaete chrysosporiuma*, when subjected to deprivation of nutrient nitrogen, carbon, or sulfur, can secrete a family of glycosylated-peroxidase isozymes into the extracellular medium *(1,2)*. From their substrate specificities, modes of oxidation, and dependence on or independence of manganese II, most peroxidases can be placed into two distinct groups. The first group is named MnP because it is manganese II dependent. The second group requires no manganese II for its enzyme activity and is referred to as LiP. In the presence of hydrogen peroxide, LiPs can oxidize 3,4-dimethoxybenzyl (veratryl) alcohol and many other nonphenolic aromatic-lignin compounds directly, and thus are the major fungal enzymes responsible for the breakdown of wood lignins *(1–8)*.

Various isozymes of the ligninase family from the white-rot fungus have been isolated. Those LiP isozymes capable of oxidizing veratryl alcohol include H1 (pI 4.7), H2 (pI 4.4), H6 (pI 3.7), H7 (pI 3.6), H8 (pI 3.5), and H10 (pI 3.3). Each of these isozymes is post-translationally glycosylated *(8)*. However, the identities and composition of the N-linked and/or O-linked carbohydrate moieties and the sites of carbohydrate attachment of each isozyme are still unknown.

The genes that encode the H8 and H2 proteins have been cloned into baculovirus expression vector pEVmod, and these two lignin peroxidases, having active and biological functions, were heterologously expressed in Sf-9 insect cells *(7,8)*. However, the small yield of the extracellular and biologically active enzymes hinders the subsequent analyses of the structure and functions which will provide a better understanding of the enzyme mechanisms of this family of ligninolytic enzymes.

In this study, we designed and utilized a crossover linker (P24) which permitted us to delete the entire 5′ non-coding flanking sequence of the original λML-1 cDNA and to insert the putative *Spodoptera frugiperda* ribosome-binding sequence (RBS) (TATAAAT) directly in front of the translation-initiation codon of the ligninase-H8 gene by the crossover-linker mutagenesis method *(9,10)*, resulting in a more than 18-fold increase in H8 over that of the previous pEV11-1A.3 recombinant baculovirus *(7)*. An overexpressed 42 kD protein with an apparent molecular weight similar to that of the fungal ligninase H8 and which reacted specifically with the anti-H8 monoclonal antibody (MAb) was detected in the culture medium of the Sf-21 insect cells within only 2–3 d post-infection (p.i.).

## MATERIALS AND METHODS

### Expression Vectors, Baculovirus, and Insect Cells

Plasmid pAT153-λML-1 containing the ligninase H8 gene, the recombinant virus pEV11-1A.3-AcNPV, and the anti-H8 MAb (2D4.9) were

described previously by Johnson and Li *(7)*. Wild-type AcNPV (*Autographa california* Nuclear Polyhedrosis Virus) and transfer-vector pVL1393 were purchased from Invitrogen. The bBluescript-IIKS+ plasmid was obtained from Stratagene, Sf-21 cells were obtained from Clontech Laboratories.

## Extraction of Plasmid DNA and DNA Sequencing

Plasmid DNA was purified according to the methods of He et al. *(11)*. Large amounts of plasmid DNA were obtained by alkaline extraction followed by CsCl ultracentrifugation. DNA restriction-enzyme digestion, calf intestine-phosphatase (CIP) treatment, agarose-gel electrophoresis, DNA ligation and transformation were carried out according to the protocols of Sambrook et al. *(12)*. The DNA bands excised from the agarose gel were extracted using the GeneClean Kit, Bio-101. DNA sequence analysis was performed with the T7 Sequence Kit, Pharmacia.

## Production and the Isolation of AcNPV

The purification of AcNPV DNA was carried out according to the methods of Summers and Smith *(13)* and Maeda *(14)* with some minor modifications. Sf-21 cells grown in suspension in Corning tubes were infected with the virus at a multiplicity of infection (MOI) of 5 for 4 d. The virus particles in the growth medium were collected by ultracentrifugation at 74,000 *g* for 1 h using a linear 10–50% sucrose-density gradient (w/v). The viral DNA was purified by resuspending the virus in 4% sarkosyl-TE buffer, and incubating it at 60°C for 2 h, followed by CsCl density gradient centrifugation for 18 h at 210,000*g*. Both the supercoiled and open circular viral DNAs purified by this protocol retained high transfection activity.

## Cotransfection of Sf-21 Cells and Plaque Purification of Recombinant Baculoviruses

Five micrograms of pVL-Mu-H8 and 1.8 μg of AcNPV viral DNA were used to cotransfect $2.0 \times 10^6$ Sf-21 cells grown in 60 $mm^2$ culture flasks using the Lipofectin (BRL) procedure *(15)*. Plaques containing potential recombinant baculoviruses were picked and screened by the method of Summers and Smith *(13)* except 0.8% low-melting agarose was used as overlay.

## Isolation of Total Cellular DNA from AcNPV-infected Sf-21 Cells

The extraction of total DNA from infected cells was performed according to the methods of Summers and Smith *(13)* except that the cells were lysed by incubation at 60°C for 2 h in 4% sarkosyl (*N*-lauroyl sarcosine). The presence of the recombinant DNA was confirmed and detected by DNA dot-blot hybridization analysis using the nonisotopically labeled λML-1 DNA probe and the reagents included in the ECL system (Amersham).

### SDS-PAGE and Western Blot Analysis

The recombinant baculovirus-infected Sf-21 cells were washed with cold phosphate-buffered saline (PBS), resuspended in a small amount of PBS and lysed in a lysis buffer (50 mM Tris-HCl pH 6.9, 10% SDS, 25% glycerol, 25% 2-mercaptoethanol, and 0.25% bromophenol blue in a 5X solution). Samples were separated and analyzed by 12.5% SDS-PAGE. Proteins from the gel were electrically transferred to a nylon membrane in a transfer buffer (25 m*M* Tris base, 0.192*M* glycine, 20% methanol) at 4°C for 1 h at 100V using a Bio-Rad Transblot apparatus. After blocking, the membrane was first incubated with anti-H8 MAb (2D4.9) and was then washed with TBS (50 m*M* Tris-HCl, 150m*M* NaCl, pH 7.5) extensively before alkaline-phosphatase-conjugated rabbit anti-mouse IgG (Sigma) was added. The substrates used were NBT/BCIP dissolved in a reaction buffer containing 100m*M* Tris-HCl, 100 m*M* NaCl, and 5m*M* $MgCl_2$ (pH 9.5).

### Enzyme Assay of the Expressed Extracellular H8

Two 500 mL Sf-21 cell suspensions ($1 \times 10^6$ cells/mL) were infected with the recombinant baculovirus at an MOI of 10. After incubation at 27°C for 52 h, the cells were pelleted, resuspended in serum-free TC100 medium (Sigma) containing hemoglobin (1 μg/mL), and incubated for an additional 24 h. Phenylmethylsulfonyl fluoride (PMSF) was then added to a final concentration of 1 μg/mL. The media was collected and concentrated at 4°C to 50 mL using an Amicon concentrator with a YM10 membrane. After dialysis, the crude-enzyme sample was chromatographed on a DEAE-Sephadex A50 column (30 × 1.5 cm) at 4°C. The proteins were eluted by 200 m*M* sodium tartaric acid (pH 5.0) with an linear gradient of NaCl from 0 to 0.5*M*. The enzyme activity of each eluted fraction was determined by mixing 50 μL of the eluate with 450 μL of the substrate buffer (0.22*M* sodium tartaric acid, 3.33 m*M* veratryl alcohol, pH 2.5) followed by the addition of 50 μL 10 m*M* $H_2O_2$. The absorbance at 310 nm (25°C) was read and recorded immediately. The enzyme activity was calculated according to the formation of the reaction product, veratryl aldehyde (VAH). One enzyme unit was defined as 1 μmol VAH produced per min. Protein concentration was determined using the Bio-Rad Protein Assay Kit.

## RESULT AND DISCUSSION

### Construction of pBL-λML-1 Vector

A λML-1 fragment containing the ligninase-H8 gene, obtained from *Eco*RI digestion of the pAT153-λML-1 plasmid *(7)*, was subcloned into the *Eco*RI site on the pBluescript-IIKS+ plasmid, and was then transformed

into competent *E. coli* DH5α cells. Orientation of the insert was determined by *SmaI* digestion. A clone containing the H8-gene insert in the right orientation was named pBL-λML-1 (Fig. 1) and was used for the crossover-linker mutagenesis.

## Construction of pBL-Mu-H8 by Crossover-Linker Mutagenesis

Large amounts of purified H8 must be available to facilitate our structure and function analysis of the ligninase H8. We decided to delete the 5′ noncoding region of the H8 gene and to add a putative RBS directly in front of the initiation-codon ATG of the H8 gene by crossover-linker mutagenesis. Success in this approach should increase the translational efficiency of the H8 mRNA, resulting in a higher level of production of the H8 isozyme than the previous recombinant baculovirus pEV11-1A.3.

We first designed an oligonucleotide crossover linker p24, with the following unique sequence: 5′-CTAGATATAAATATGGCCTTCAAG-3′. The 5′ end of this 24-base linker contains a sequence of the restriction site of *Xba*I (CTAGA) which is followed by the putative RBS (TATAAAT) *(16)*. The 12 bases (ATGGCCTTCAAG) at the 3′ end are identical to the first 12 nucleotides of the 5′ end of the H8 gene, which should facilitate crossover recombination. Thus, through recombination, the 5′ non-coding region of H8 gene would be deleted and the TATAAAT sequence would be inserted directly in front of the ATG codon (Fig. 1).

The pBL-λML-1 vector, after digested with *Xba*I and *Pst*I, was dephosphorylated by CIP. The p24 linker was phosphorylated with T4-polynucleotide kinase and purified with a Sephadex G-50 mini-column. The 5′-phosphorylated p24 was ligated to the abovementioned linearized pBL-λML-1 DNA at an 800:1 molar ratio for 22 h at 12°C. After ligation, the mixture was purified with the GeneClean Kit. The ligated-plasmid DNA was further digested with *Pst*I. This simple manipulation decreased the background of transformation and increased the yield of positive recombinant H8 clones. The linearized DNA was transformed into competent JM101 cells. A total of 26 clones were selected, of which 8 were found to be recombinant mutants. This was confirmed by digestion of the plasmid DNA of these 8 clones with either *Xba*I or *Eco*RI. Upon digestion, the plasmid DNA was linearized as expected and only one band of 4.3 kb was detected. However, when the DNA was digested with both *Xba*I and *Eco*RI, two bands at 3 and 1.3-kb were observed (data not shown). Furthermore, none of the plasmid DNA from these 8 clones could be digested by *Pst*I, a good indication that recombinant mutation had occurred in the pBL-Mu-H8 plasmid. DNA sequencing of the 5′ end of one of the 8 clones (pBL-Mu-H8) using a T-7-promoter primer showed that the 40 nucleotides in front of the ATG codon at the 5′ end of the H8 gene and 30 bases after the *Xba*I site (containing five restriction-enzyme sites) had been deleted. Furthermore

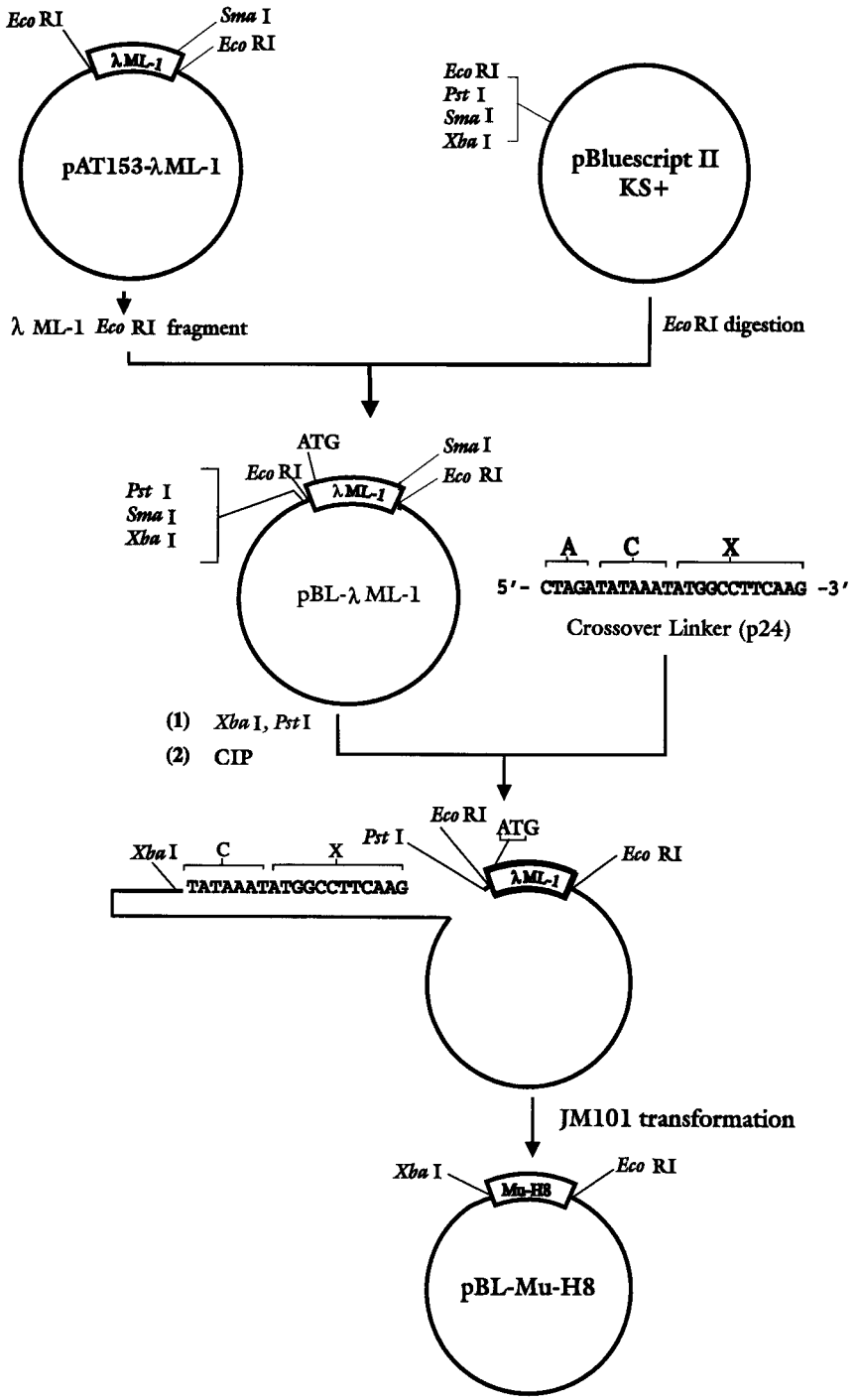


Fig. 1. Strategy summary for the construction of the plasmid pBL-Mu-H8 by crossover-linker mutagenesis. The sequence of the p24 linker has three regions: Region A contains the restriction enzyme *Xba*I cutting site; Region C represents the putative *Spodoptera frugiperda* RBS TATAAAT, and Region X is homologous to the DNA sequence immediately adjacent to the sequence to be deleted, providing the sequence homology required for the crossover event to take place.

```
pBL-λML-1

      XbaI  SpeI  Bam HI  Sma I  PstI  Eco RI
5'...TCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCTTTTTCTTCAGTCCCACTCA
     GCACCAGCAACACAGCGGAC ATG GCC TTC AAG CAG CTC TTC GCA
     GCT ATC ...3'



pBL-Mu-H8

      Xba I
5'...TCTAGATATAAAT ATG GCC TTC AAG CAG CTC TTC GCA GCT
     ATC...3'
```

Fig. 2. Sequence determination and analysis of the ligninase-H8 gene junction after crossover-linker mutagenesis. The sequences of the 5′ end of the pBL-λML-1 and pBL-Mu-H8 plasmids are shown. Note that the pBL-Mu-H8 plasmid has the insertion of the TATAAAT sequence and the deletion of five restriction-enzyme sites as well as 41 noncoding nucleotides present in pBL-λML-1.

the putative RBS TATAAAT was also correctly inserted between the *Xba*I site and the ATG codon (Fig. 2).

## Construction of the Plasmid pVL-Mu-H8

To produce recombinant baculovirus which can express higher levels of the H8 isozyme, the pBL-Mu-H8 plasmid DNA was linearized by *Xba*I digestion and the Klenow fragment of DNA polymerase was used to generate a blunt end *(12)*. After phenol/chloroform extraction and ethanol precipitation, the plasmid was again digested with *Eco*RI. A 1.3-kb fragment containing the H8 gene was obtained and then cloned into the pVL1393 baculovirus transfer plasmid pre-digested with both *Sma*I and *Eco*RI. This recombinant plasmid was transformed into competent DH5α. Positive clones containing the plasmid with the H8 gene were screened and confirmed by restriction-enzyme digestion and agarose-gel electrophoresis.

## Production of Recombinant Baculovirus Expressing the H8 isozyme

The pVL-Mu-H8 plasmid DNA and the wild-type AcNPV viral DNA were used to cotransfect log-phase Sf-21 cell cultures. Ten plaques with inclusion body-negative recombinant baculovirus were picked and further purified through three rounds of plaque purification. Recombinant baculovirus containing the H8 gene was identified from total DNA isolated from Sf-21 cells infected with recombinant baculovirus pVL-Mu-H8, wild-type AcNPV, and mock infection by dot-blot hybridization. Results of two such experiments are shown in Fig. 3. Only the DNA from the recombinant baculovirus-infected cells could hybridize with the non-isotopically labeled

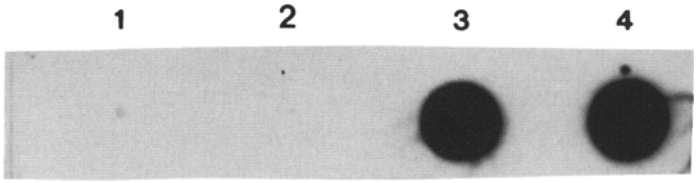


Fig. 3. Dot-blot hybridization analysis for the presence of the H8 gene using a non-isotopic probe. DNA samples (10 μL each) from total DNA obtained from Sf-21 cells infected by wild-type AcNPV (spot #1), mock-infected (spot #2), and pVL-Mu-H8-recombinant baculovirus (spots #3 and 4).

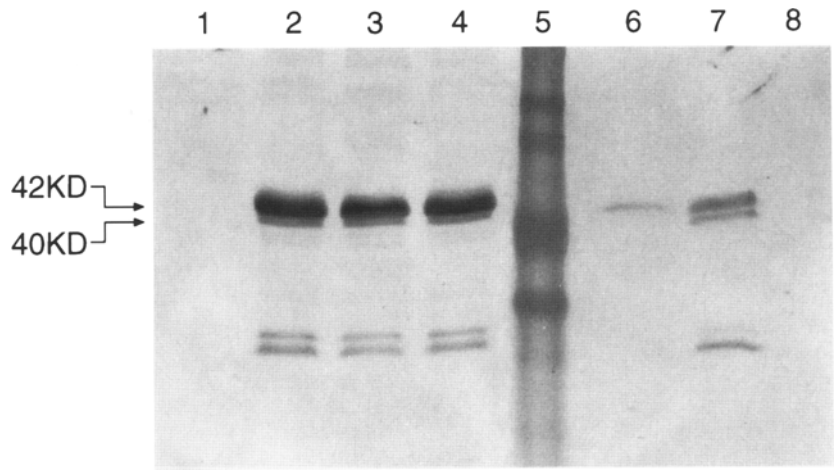


Fig. 4. Western immunodetection of the expressed-ligninase H8 using anti-H8 MAb 2D4.9. Sf-21 cells ($2 \times 10^6$) were infected with pVL-Mu-H8- and pEV11-1A.3-recombinant baculoviruses at a MOI of 10. Lane 1: wild-type AcNPV infected cells; Lanes 2–4: different infected-cell sample preparations from three pVL-Mu-H8-recombinant virus plaques; Lane 5: prestained protein markers, MW 58, 48, 36.5 and 26 KD, from top to bottom; Lane 6: 30 μL of culture-medium concentrate of the recombinant virus-infected cell culture of lane 3; Lane 7: recombinant pEV11-1A.3 baculovirus-infected cells; Lane 8: mock-infected cells.

H8-gene probe (Fig. 3, lane 3 and 4). DNA from the wild-type baculovirus AcNPV-infected cells and mock infection showed no hybridization (Fig. 3, lane 1 and 2). This indicated that the recombinant baculovirus containing the H8 gene had been successfully produced through recombination in vivo.

## Immunodetection of the H8 Isozymes Expressed by Recombinant Baculoviruses

Sf-21 cells ($2.5 \times 10^6$ cells/25 $cm^2$ flask) were infected with both recombinant baculoviruses, pVL-Mu-H8 and pEV11-1A.3, at an MOI of 10

for 72 h. The wild-type baculovirus AcNPV-infected and mock-infected cells were used as controls. After infected cells were harvested and washed, cell lysates were prepared and subjected to Western-blot analysis using anti-H8 MAb. As shown in Fig. 4, both recombinant pVL-Mu-H8 and pEV11-1A.3 baculovirus-infected cell lysates contained the 40 and 42 kD proteins, which reacted specifically with MAb 2D4.9. The molecular weights of the protein bands at 40 and 42 kD are similar to those of the H8 isozyme as previously reported *(4,7)*. There were also two minor protein bands, which might represent degradation products or proteins produced from downstream-start codons present within the H8 mRNA *(7)*. No lignin-ase-protein bands could be detected in the cell lysate of the mock-infected cells, not in the wild-type virus-infected cells (Fig. 4, lane 1 and 8). When the color intensities of the 42 kD protein bands were compared using a Molecular Dynamic densitometer, it was evident that the expression and yield of the H8 isozyme from the newly generated pVL-Mu-H8 recombinant baculovirus-infected cells (Fig. 4, lanes 2, 3, and 4) were 18.8 ± 3.4 (mean ± SE)-fold higher than that from the previous pEV11-1A.3 recombinant virus-infected cells (Fig. 4, lane 7). Whether the higher level of H8 expression was due to the deletion of the 5′ noncoding region and/or the correct insertion of the RBS TATAAAT remains to be determined. However, the latter hypothesis seems most likely.

## Determination of the Enzyme Activity of the Expressed-Ligninase H8

The first 84 nucleotides at the 5′ end of H8 gene encode a signal peptide of 26 amino acids *(17)*, which permits the gene product to be secreted extracellularly after expression. The crude enzyme was obtained from the media of the recombinant-virus-infected Sf-21 cells 52–76 h p.i. as described by Johnson and Li *(7)*. Enzyme activity was determined by the oxidization of 3.33 m*M* veratryl alcohol in 0.22*M* sodium tartrate, pH 2.5 at 25°C, with different amounts of crude enzyme in the presence of $H_2O_2$. The formation of the oxidized product, veratryl aldehyde (VAH), was immediately determined at $OD_{310}$. The yield of the reaction product increased proportionally to the amount of the enzyme added. According to $\lambda = 9.3/\mu$mol VAH, the crude-enzyme activity of the expressed H8 is 0.015 unit/mg of total protein in the culture medium. After one step of ionic exchange chromatography, as shown in Fig. 5, the specific activity of the enzyme was enhanced 7.5-fold. No enzyme activity was detected in the media of either wild-type virus infected or the mock-infected Sf-21 cells. These results confirmed that the pVL-Mu-H8 recombinant baculovirus contained the H8 gene, and the expressed product which was secreted to the media after synthesis, as shown in Western-Blot analysis (Fig. 4, lane 6), had the expected enzyme activity.

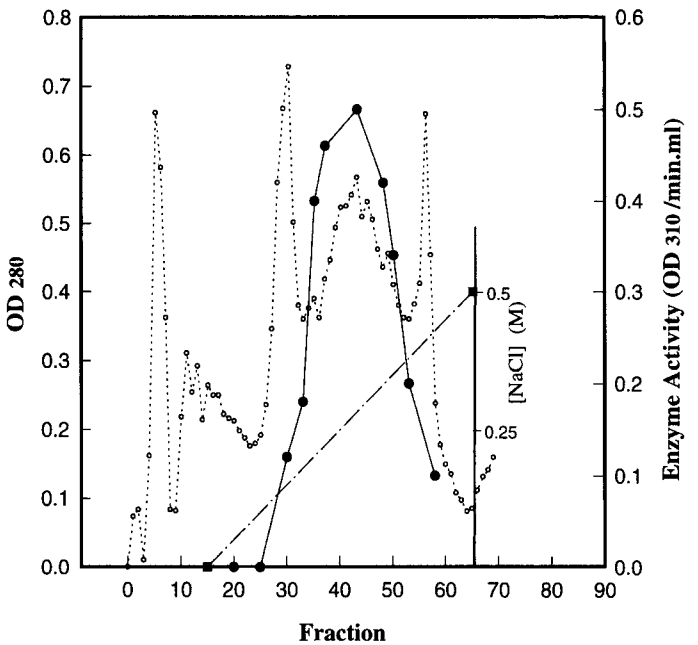


Fig. 5. DEAE-Sephadex A50 column chromatography and veratryl alcohol peroxidase activity of the recombinant ligninase-H8 product. Each fraction contained 6 mL of eluate. Procedures for the enzyme-sample preparation and assay were described in the Materials and Methods section. Open circles represent the concentration of total protein (OD 280); filled circles represent the enzyme activity (OD 310); filled squares represent the concentration of NaCl used in the elution gradient.

In summary, we successfully constructed a new recombinant-baculovirus pVL-Mu-H8 using the crossover-linker mutagenesis method, which enabled us to express heterologously the extracellular H8 isozyme at levels about 19-fold higher than the previous recombinant baculovirus in Sf-21 cells. Our results indicated that the putative RBS (TATAAAT), when directly cloned in front of the gene controlled by the polyhedrin promoter, will lead to overexpression of the gene product. We also confirm that the crossover-linker mutagenesis method was a very convenient way to manipulate and insert DNA sequences of interest into any genes. The higher yield of this ligninolytic enzyme will facilitate our study of the structure and function of this unique fungal protein and will allow us to further explore its potential(s) in the biodegradation of many environmental pollutants *(18)*.

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## REFERENCES

1. Tien, M. and Kirk, T. K. (1983), *Science.* **221,** 661–663.
2. Glenn, J. K. and Gold, M. H. (1985), *Arch. Biochem. Biophy.* **242,** 329–341.
3. Leisola, M. S. A., Kozulic, B., Meussdoerffer, F., and Fiechter, A. (1987), *J. Biol. Chem.* **262,** 419–424.
4. Farrell, R. L., Murtagh, K. E., Tien, M., Mozuch, M. D., and Kirk, T. K. (1989), *Enzyme Microb. Technol.* **11,** 322–328.
5. Paszcynski, A. and Crawford, R. L. (1991), *Biochem. Biophys. Res. Commun.* **178,** 1056–1063.
6. Tien, M. and Kirk, T. K. (1988), *Enzyme Microb. Technol.* **11,** 321–328.
7. Johnson, T. M. and Li, J. K.-K. (1991), *Arch. Biochem. Biophys.* **291,** 371–378.
8. Johnson, T. M., Pease, E. A., Li, J. K.-K., and Tien, M. (1992), *Arch. Biochem. Biophys.* **296,** 660–666.
9. Sung, W. L., Zahab, D. M., MacDonald, C. A. and Tam, C. S. (1986), *Gene.* **47,** 261–267.
10. Garson, K., Percival, H. and Kang, C. Y. (1990), *Virology* **177,** 106–15.
11. He, M., Wide, A. and Kaderbhai, M. A. (1990), *Nucleic Acid Res.* **18,** 1660.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, New York.
13. Summer, M. and Smith, G. E. (1987), *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedure.* Bulletin No. 1555. Texas Agricultural Experimental Station, Texas.
14. Maede, S. (1989), in *Invertebrate Cell System Application,* vol. 1, CRC Press, Boca Raton, Fl, pp. 167–181.
15. Groebe, D. R., Chung, A. E., and Ho, C. (1990), *Nucleic Acid Res.* **18,** 4033.
16. Luo, L., Li, Y. and Kang, C. Y. (1990), *Virology* **179,** 874–880.
17. Tien, M. and Tu, C. D. (1987), *Nature* **326,** 520–523.
18. Bumpus, J. A. and Aust, S. D. (1987), *Appl. Envir. Microbiol.* **53,** 2001–2008.