Brief report

Preparation and Application of Polyclonal Antibody against a Recombinant Laccase

Yinghai Xu[1], Yuzhi Hong[1], Yazhong Xiao[1, 2] and Wei Fang[1]

A laccase gene from *Trametes* sp. 420 was recombinantly expressed in *Pichia pastoris*, producing the enzyme rLacD. Six mutant enzymes were produced by site-directed mutation at six potential glycosylation sites in the enzyme rLacD respectively. To probe the mutants with lower activities sensitively and specifically, the antiserum containing specific polyclonal antibodies were prepared by immunizing healthy male rabbits, about 4-month-old and 2 kilogram weight, using pure rLacD as an immunogen. Antibodies were collected after the fifth immunization injection. The antiserum had titres of 1:32 in double immunodiffusion test and of 1:128,000 in enzyme-linked immunosorbent assay (ELISA). The results obtained by Western blot analysis showed that the antiserum could react with rLacD and its mutants with highly specific and sensitive affinities. *Cellular & Molecular Immunology*. 2007;4(4):315-317.

Key Words: antiserum, laccase, polyclonal antibody

Introduction

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), a family of blue copper-containing oxidases, is commonly found in fungi, plants (1) and bacteria (2). The enzyme is capable of oxidizing a variety of aromatic compounds and other organic compounds with concomitant reduction of oxygen to water (3). Laccase has been well demonstrated to possess potential in several industrial and environmental applications including paper pulping/bleaching, bioremediation, and degradation and detoxification of textile dyes (4-6), etc. Due to these potentials, heterologous expression (6-8) and protein engineering (9) of laccase have been carried out for higher production and more excellent characters. However, laccase activity usually declines or even is lost when they are heterologously expressed in various hosts or when mutagenesis occurs in the genes. In these cases, the corresponding polypeptides may not be easily detected using spectrophotometry method. Thus, immunoassay method is an alternative for the sensitive and specific detection of a mutant enzyme.

[1]School of Life Science & Modern Experiment Technology Center, Anhui University, Hefei 230039, China;

[2]Corresponding to: Dr. Yazhong Xiao, School of Life Science, Anhui University, Hefei 230039, China. Tel: +86-551-510-8509, Fax: +86-551-510-7408, E-mail: yzxiao@ahu.edu.cn

Received May 18, 2007. Accepted Jul 31, 2007.

Copyright © 2007 by The Chinese Society of Immunology

Previously, a laccase gene (*lacD*) from *Trametes* sp. 420 was expressed in *P. pastoris*, producing a large amount of recombinant laccase (rLacD) (8). Furthermore, site-directed mutagenesis of the six potential glycosylation sites (Asn-X-Ser/Thr) in rLacD was carried out by mutating the amino acid residue Asn to Gln, resulting in six mutant laccase, orderly designated as rLacDm1 to rLacDm6 according to their mutation locus in *lacD* (will be reported in other papers). In this paper, we reported the preparation of the polyclonal antibody against rLacD and its application in detecting the mutant enzymes with lower activities than rLacD.

Materials and Methods

Strains, reagents and media

The recombinant *P. pastoris* strain GS-lacD-1 secreting rLacD and its six mutants secreting the above mentioned mutant laccase were kept in the culture collection of the School of Life Science, Anhui University, China. Rabbits were purchased from the Experimental Animal Center, Anhui Medical University, China. Complete Freund's adjuvant and incomplete Freund's adjuvant were purchased from Sangon Co. (China). Goat anti-rabbit IgG-HRP and DAB (3,3'-di-dminobenzidine) kit were purchased from Solarbio Co. (China). Unless otherwise stated, other chemicals were at least of analytical grade. The media used for yeast culture were prepared as described previously (7, 8).

Production and purification of recombinant laccase

GS-lacD-1 and its six mutants were cultured in BMM media as described to produce rLacD and rLacDm1 to rLacDm6,

respectively (8). The purification of laccase was performed as follows. Firstly, yeast culture was centrifuged at 12,000 g for 20 min and the supernatant was concentrated by 20 fold in volume. Secondly, the concentrate was dialyzed twice against buffer A (10 mM citrate-Na_2HPO_4, pH 5.8), 2 h once, and centrifuged again. Thirdly, the dialyzed product was applied to a DEAE-Sepharose FF column (1 × 20 cm, Amersham Pharmacia, Sweden) pre-equilibrated with buffer A, and then eluted with a linear gradient of ammonium sulfate (0 - 1 M) in buffer A. Finally, the fractions containing laccase activity were pooled. Enzyme homogeneity was assessed by SDS-PAGE, and protein concentration was tested using a BCA assay kit (Hyclone, USA).

Immunization of rabbit
Four male rabbits, about 4-month-old and 2-kilogram-weight, were chosen for preparing antibody against rLacD. A basic immunization was done by injecting 0.5 mg of pure rLacD as the immunogen, emulsified with complete Freund's adjuvant, into each rabbit by a multipoint subcutaneous implantation. After a 20-day growth, four enhancing immunization was further carried out as described above but using incomplete Freund's adjuvant instead of complete Freund's adjuvant, with a 10-day interval between each implantation.

Agarose-gel double immunodiffusion and ELISA
Double immunodiffusion and ELISA were used to assess the titre of antiserum against rLacD. For double immunodiffusion, the antiserum were diluted at 1:1, 1:4, 1:8, 1:16, 1: 32 and 1:64, respectively, and were incubated with rLacD at 37°C for 24 h. ELISA analysis of the antiserum against rLacD (4 pmol) was performed and the absorbance at 490 nm was measured with an EL-311SX ELISA Reader (Bio-Tek Instruments Inc., USA). The dilutions of sera were at 1: 2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000 and 1: 128,000, respectively. Horseradish peroxidase conjugated goat anti-rabbit IgG (IgG-HRP) fraction and *o*-phenylenediamine were used as the secondary antibody and the chromogenic reagent, respectively.

Western blot analysis
Proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore) at 1.0 mA/cm at 4°C for 4 h. The PVDF membrane was blocked with 5% (m/v) solution of nonfat milk powder, washed using PBST (phosphate-buffered saline, 0.05% Tween 20) buffer, and subsequently incubated with rabbit antiserum and IgG-HRP. Horseradish peroxidase was then colored using DAB assay kit.

Results and Discussion

Preparation of immunogen
The mutants of rLacD, rLacDm1 to rLacDm6, were different from each other only at one potential N-glycosylation site where Asn was replaced with Gln. The mutagenesis occured at the amino acid residue positions of 54, 189, 217, 333, 377 and 436, respectively. The seven enzymes were obtained from the individual cultures of their recombinant *Pichia* strains by a one-step ion exchange chromatography. The molecular masses of the seven enzymes, estimated by SDS-PAGE, were nearly identical (76 kDa, Figure 1). However, the specific activities of the six mutant laccase (will be reported in other paper) decreased by different degrees in comparison with that of rLacD.

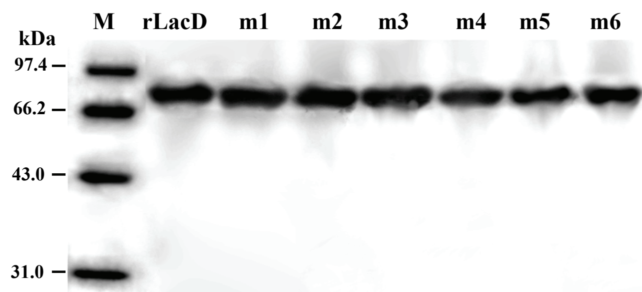

Figure 1. SDS-PAGE analysis of pure laccase. M, standard molecular mass markers; m1 to m6, rLacDm1 to rLacDm6 orderly.

Titre and specificity of polyclonal antibody
Rabbits are usually used for antibody preparation due to some traits, such as good immune characteristics, high affinity of antibodies and enough amounts of sera (10). Meanwhile, the amount of immunogen and the approach of injection might have influences on antibody production. In this work, a final 350-ml volume of antiserum against rLacD was obtained from four rabbits.

A precipitation line, formed by a reaction of rLacD with the antiserum at a maximum dilution of 1:32, could be obviously observed in agarose gel in double immunodiffusion. The titre of the antiserum in ELISA test was 1:128,000 (Figure 2), which is quantitatively similar to those of other rabbits' antiserum (10, 11). On the other hand, the

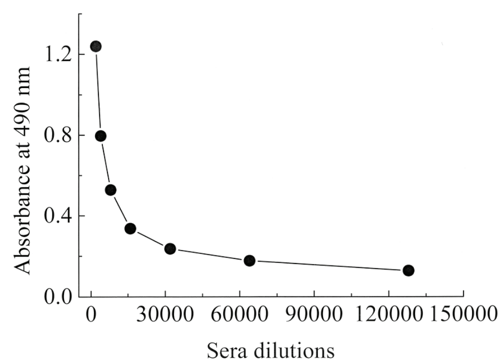

Figure 2. ELISA of the antiserum against rLacD. The titre was measured by ELISA while the dilutions of sera were at 1:2,000, 1:4000, 1:8,000, 1:16,000, 1:32,000, 1:64,000 and 1:128,000, respectively.

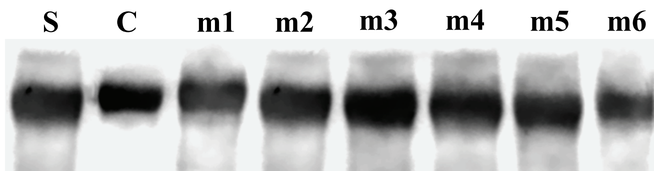

Figure 3. Western blot analysis of rabbit polyclonal antibody against laccase. S, the culture supernatant of rLacD without purification; C, control of pure rLacD; m1 to m6, the culture supernatants of rLacDm1 to rLacDm6, respectively.

antiserum obtained were specific to its immunogen, rLacD, according to the results of Western blot (Figure 3).

Immunological detection of mutant laccase

The specificity of the polyclonal antibodies to the six mutant laccase, rLacDm1 to rLacDm6, was also assayed. The fermentation supernatants of these mutants, instead of pure enzymes, were directly used. The results of Western blot showed that the antibodies were able to react specifically to these mutant enzymes (Figure 3).

Laccase activity may decrease or lose when modification/ mutagenesis of the polypeptides occurs, making it undetectable by some methods such as spectrophotometry. The aim of this paper is to find a feasible approach to prepare polyclonal antibodies that can be used for the sensitive and specific detection of mutant laccase with lower or without activity. The results obtained from the Western blot analyses showed that the polyclonal antibodies in sera were able to react with not only pure rLacD and its supernatants without purification but also the mutant forms of rLacD (Figure 3) with specific and sensitive affinities. Therefore, this work proposed an effective detection means for further studies on the protein engineering of laccase. The effective affinities of antibodies to these mutant laccase could be explained by the multi-epitopes on rLacD, which resulted in a complex of polyclonal antibodies that are capable of recognizing several different anti-genic determinants regardless of the changed epitopes such as glycosylation sites.

In conclusion, an antiserum was obtained from immunized rabbits by using rLacD as the immunogen. The polyclonal antibody obtained was proved to possess highly specific and sensitive affinities to not only rLacD but also its mutants with lower activities.

Acknowledgements

The authors thank Dr. Wei Jiang for her careful reading of this paper. This work was supported by grants from the National Natural Science Foundation of China (30370045, 30470056, 30670069), the Natural Sciences Foundation of Anhui Provincial Education Department (2006KJ049A) and the Innovative Research Team of 211 Project in Anhui University (02203109).

References

1. Thurston CF. The structure and function of fungal laccases. Microbiology. 1994;140:19-26.
2. Alexandre G, Zhulin IB. Laccases are widespread in bacteria. Trends Biotechnol 2000; 18:41-42.
3. Ullah MA, Bedford CT, Evans CS. Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. Appl Microbiol Biotechnol. 2000;53:230-234.
4. Abadulla E, Tzanov T, Costa S, et al. Decolorization and detoxification of textile dyes with a laccase from Trametes hirsute. Appl Environ Microbiol. 2000;66:3357-3362.
5. Gianfreda L, Xu F, Bollag JM. Laccases: a useful group of oxidoreductive enzymes. Biorem J. 1999;3:1-25.
6. Li JF, Hong YZ, Xiao YZ, et al. High production of laccase B from *Trametes* sp. in *Pichia pastoris*. World J Microbiol Biotechnol. 2007;23:741-745.
7. Hong YZ, Xiao YZ, Zhou HM, et al. Expression of a laccase cDNA from *Trametes* sp. AH28-2 in *Pichia pastoris* and mutagenesis of transformants by nitrogen ion implantation. FEMS Microbiol Lett. 2006;258:96-101.
8. Hong YZ, Zhou HM, Tu XM, et al. Cloning of a laccase gene from a novel basidiomycete *Trametes* sp. 420 and its heterologous expression in *Pichia pastoris*. Curr Microbiol. 2006;54:260-265.
9. Xu F, Berka RM, Wahleithner JA, et al. Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. Biochem J. 1998;334:63-70.
10. Ma XY, Cao YC, Ma JY, et al. Preparation and identification of polyclonal antiserum against myostatin. Hua Nan Nong Ye Da Xue Xue Bao. 2005;26:89-92.
11. Zhang YY, Li FY, Zhang WH, et al. Expression and antibody preparation of a novel serine protease ESP30. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2005;21:334-339.