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学位の種類	博士(生命科学)
学位記番号	博　第９４号
学位授与の日付	平成２７年３月２０日
学位授与の要件	学位規則第５条第１項該当
学位論文題目	The characterization of ancestral lignin degrading enzyme
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論文内容の要旨

1. Background

Improving enzyme stability is one of the major subjects in protein engineering. In the early stage of the protein engineering, it was done by the method so called rational engineering: improving the packing of the hydrophobic core, introducing disulfide bond and extending ion pair network. Despite of many researches, it is still a challenging task to stabilize a particular enzyme of interest. The directed evolution is the alternative method. This method is based on the evolutionary process: mutation and selection. Later, the consensus approach was proposed, which is based on the dependence of the amino acid frequency in homologous amino acid sequences. These two methods do not need physical principle and tertiary structural information of the target enzyme. Our laboratory has developed ancestral mutation method, based on the phylogenetic analysis: (1) collecting amino acid sequences from database, (2) multiple sequence alignment, (3) phylogenetic tree construction, (4) estimation of ancestral sequence, (5) introduction of ancestral amino acid residue(s) into the target enzyme, (6) Enzyme expression and characterization. Some ancestral mutants have been created starting for isocitrate dehydrogenase (ICDH), 3-isopropylmalate dehydrogenase (IPMDH), glycyl-tRNA synthetase (GlyRS) and β-amylase. The dataset of ICDH, IPMDH and GlyRS contained archaea, bacteria and eukarya. That of β-amylase was constructed with bacteria and eukarya. The ancestral mutation method based on the dataset constituted only of eukarya hasn’t been investigated.

The lignin is the polymer in the cell wall and protects plant bodies from biodegradation.

In 1983, lignin degrading enzyme has been discovered from white rot fungi: lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). LiP oxidizes veratryl alcohol (VA) and the product $VA^{+\cdot}$ plays a role of mediator. MnP oxidizes manganese (II) to manganese (III) and manganese (III) forms complex with dicarboxylic acid. This complex attacks to lignin. VP has both activities. These enzymes have potential to be applied to the degradation of lignin. The application of these enzymes on degrading lignin is expected to contribute to lower the energy cost and by-products. However the low stability of these enzymes has been the obstacle to the industrial application.

The purpose of this study is to create stable lignin degrading enzyme by ancestral mutation method and resurrection of ancestral lignin degrading enzyme based on the dataset constituted only of eukaryal sequences.

2. Ancestral mutants of LiP

In chapter 2 we created stable LiP by ancestral mutation method.

2.1. Method

The amino acid sequences of lignin degrading enzymes were collected from NCBI database by Protein Blast search using wild-type lignin peroxidase from *Phanerochaete chrysosporium* strain UAMH3641 as a query sequence. The sequences were aligned by the program ClustalX with its default parameters and then manually adjusted. Well-conserved regions were collected by Gblocks 0.91. The maximum likelihood tree was constructed with Treefinder and PhyML 2.4.4. The WAG substitution model was used as the substitution matrix for amino acids. Ancestral sequence was estimated by CODEML in PAML 3.14. Comparing amino acid sequences of ancestral and wild-type, eleven mutation sites were selected. The point mutations were introduced by QuikChange Lightning Site-Directed Mutagenesis Kit. Enzymes were expressed in *Escherichia coli* BL21 (DE3) and were accumulated as inclusion body. After washing inclusion body, the enzyme was unfolded with urea and refolded. Crude sample was purified with DEAE-sepharose and HiTrapQ columns.

The enzyme activity was measured by monitoring the oxidation of veratryl alcohol to veratryl aldehyde (veratryl alcohol + H_2O_2 → veratryl aldehyde + H_2O). The thermal inactivation of LiP was done by incubating enzyme at 37 ^oC. The resistance for H_2O_2 was estimated by incubating enzyme with 0.1 mM H_2O_2 at 25 ^oC. The structural stability was estimated by measuring circular dichroism at 222 nm.

2.2. Result

The phylogenetic tree was constructed from 198 positions of the 49 fungal peroxidase sequences. LiP from Ascomycota was used as outgroup. The sequences at the branching point of Basidiomycota and Ascomycota, two ancestors of LiP and MnP were estimated. Eleven

ancestral mutants were created.

The enzyme activity of six ancestral mutants was increased. Especially the activity of H239F/T240L/I241L was 2.3-fold higher than the wild-type. The optimum temperature of H239F/T240L/I241L was increased by 10 ^{o}C than the wild-type. Five ancestral mutants showed high remaining activity than wild-type after thermal inactivation. Furthermore five ancestral mutants showed high H_2O_2 resistance than the wild-type. The T_m values, half-denaturation temperature, of H239F/T240L/I241L and the wild-type were 51.9 ± 0.7 ^{o}C and 50.1 ± 0.2 ^{o}C, respectively.

2.3. Discussion

We have extended the method to select the ancestral mutation site relying on the primary amino acid sequence. We estimated the relationship between thermal stability and the conservation of the neighboring amino acids within seven residues in the primary sequence. If a wild-type residue and the ancestral residue were identical, the likelihood value was taken as the conservation value. However, if the wild-type and ancestral residue differed then the conservation value was defined as 0. Finally, an averaged conservation value for neighboring residues on the primary amino acid sequence was calculated. This value is referred to linear ACV. The linear ACV values were plotted against the remaining activity after incubation at 37 ^{o}C or in 0.2 mM H_2O_2. When the linear ACV value is greater than 0.9, mutants with improved thermal stability were obtained at high efficiency. Three of four mutants whose linear ACV was >0.9 showed improved thermal stability. A similar trend was observed when the window size was increased to eleven residues. A similar relationship between the linear ACV value and the effect of ancestral mutation was found for the ancestral mutants of β-amylase and IPMDH reported previously. These results suggest that the mutants with higher linear ACV tend to show increased thermal stability. Thus, the linear ACV value can be used to select residues for mutation that will improve the thermal stability of the protein.

3. Ancestral lignin degrading enzyme

In chapter 3, we resurrected the ancestral lignin degrading enzyme whose amino acid sequence was entirely made of ancestral amino acids.

3.1. Method

LiP homologous 83 sequences were collected and aligned with MAFFT. The multiple sequence alignment was adjusted manually and well-conserved region was selected by Gblocks. The WAG+G+F model was selected as the amino acid substitution model by Prottest. Phylogenetic tree was constructed with PhyML. The ancestral sequence was estimated with CODEML in PAML and the gap position was estimated with GASP. Obtained ancestral sequence was named ancestral ligninase. Ancestral ligninase was expressed in *E. coli* BL21 (DE3). Enzyme was purified and the enzyme activities measured.

3.2. Result

The ancestral ligninase has two activities, MnP and LiP activities, although the former activity was lower than the counterpart from *P. chrysosporium*. The remaining LiP and MnP activities of ancestral ligninase were higher than LiP and MnP from *P. chrysosporium* after the 15 min heat treatment. The T_m value was defined as the half denaturation temperature. The T_m values of MnP, LiP and ancestral ligninase were 50 ^oC, 58 ^oC and 66 ^oC, respectively. The ancestral ligninase showed higher T_m value than LiP and MnP from *P. chrysosporium*.

3.3. Discussion

Most residues of ancestral ligninase at the glycosylation site were the same as those of extent glycosylated enzymes. Then ancestral ligninase probably must have been glycosylated in its nascent organism. Nie *et al*. reported that the glycosylation is contributing to enzyme stability (*Arch Biochem. Biophys*. 1999. **2**. 328). Because the stability of glycosylated LiP and MnP were higher than wild-type enzyme, glycosylated ancestral ligninase must also show higher stability than non-glycosylated ancestral ligninase.

In the previous studies of resurrecting ancestral enzymes, the high thermal stabilities were interpreted to represent the high environ temperature of the host organism. In the current study, the ancestral sequence represents the age around 270 million years ago (*Science* 2012. **336**. 1715), when the whole earth temperature is not very high. However, the stability of enzyme is often much higher than the growth temperature of the host organisms. For example, the T_m value of ribonuclease T1 from *Aspergillus oryzae* is 59.3 ^oC and the optimum growth temperature is 26 ^oC (*J. biol. Chem*. 1988. **24**. 11820). The ancestral ligninase was much more stable than the growth temperature of the host.

4. Conclusion

In the ancestral mutants of LiP, we introduced ancestral mutations into wild-type LiP from *P. chrysosporium* to improve its thermal stability. The recombinant ancestral mutant, m10 (H239F/T240L/I241L), showed improved thermal stability comparable to that of the glycosylated wild-type enzyme. Specific activity and k_{cat}/K_M of one of the ancestral mutants, m10, was improved by amino acid substitution. This is the first investigation to successfully improve enzyme stability by introducing ancestral residues inferred from the dataset constructed from eukaryotic sequences. The linear ACV value can be used to select ancestral residues to efficiently enhance the thermal stability of enzymes.

In the ancestral lignin degrading enzyme, we constructed dataset constructed with only Basidiomycota. By selecting position locating near peroxidases from *A. ramosus* and *C. cinerea* as ancestral node, our ancestral ligninase showed two activities. The ancestral ligninase showed high enzyme stability than modern enzymes. The resurrecting ancestral enzyme from limited dataset could be used to design thermally stable enzyme.

審査結果の要旨

本申請者は、祖先型耐熱化手法という技術を用いて、真菌由来のリグニン分解酵素から耐熱酵素を設計する方法に関する研究を行った。酵素の耐熱化は、タンパク質工学における重要な研究テーマの一つである。これまで、疎水コア内のパッキングを改善する方法やジスルフィド結合を導入する方法などによって、酵素の耐熱化に成功した例が知られている。申請者の所属する研究室においては、酵素のアミノ酸配列の一部を系統解析によって推定した祖先アミノ酸に置換する手法、祖先変異導入法が開発された。この手法を用いて、イソクエン酸脱水素酵素、3-イソプロピルリンゴ酸脱水素酵素、β-アミラーゼなどの耐熱化に成功している。また、酵素のアミノ酸配列全長を祖先アミノ酸で構成する手法、全祖先配列復元法も考案され、安定なヌクレオシド二リン酸キナーゼや DNA ジャイレースの設計に成功している。しかし、それらの酵素の祖先配列は、古細菌・真正細菌・真核生物のデータセット、あるいは真正細菌のみのデータセットから推定された。申請者は、真核生物の真菌のみが分泌するリグニン分解酵素、Lignin peroxidase (LiP)、 Manganese peroxidase (MnP)を用いて、祖先型アミノ酸導入法と全祖先配列復元法で安定酵素の設計を行った。その結果、真核生物のみのデータセットからでも安定な酵素の設計が可能であることを明らかにした。

申請者は真菌がもつ様々なリグニン分解酵素の系統樹を作成し、11 個の祖先型変異体と 4 種類の組合せ変異体を作成した。また、配列全長を祖先型アミノ酸で構成した全祖先型リグニン分解酵素も作成した。作成された 11 個の祖先型変異体の祖先変異体のうち、 m10 変異体は、至適反応温度が PcLiP よりも上昇し、かつ高活性化した。解析された祖先型変異体の耐熱性を評価する事から、変異導入箇所に一次配列上で近接するアミノ酸が祖先型配列である場合には、高い耐熱性となる確率が高いことを明らかにした。

申請者はさらに、全祖先型リグニン分解酵素を解析した。全祖先型リグニン分解酵素は LiP が持つ活性と MnP が持つ活性の両方を保持していた。その活性の温度依存性を測定した結果、全祖先型は、PcLiP や PcMnP よりも高い至適温度を示した。また、失活中点温度は PcLiP が約 45 ºC、PcMnP が約 45 ºC、全祖先型はどちらの活性においても約 60 ºC であり高い耐熱性を示した。円二色性スペクトル解析によって評価した変性中点温度 T_m 値も、PcMnP が 50 ºC、PcLiP が 58 ºC、全祖先型が 66 ºC であり高い変性温度をもつリグニン分解酵素の設計に成功した。

本申請者は、祖先変異導入法によって LiP の耐熱化、そして全祖先配列復元法によって安定なリグニン分解酵素の設計に成功した。そして祖先変異導入法と全祖先配列復元法をもちいて、真核生物のみのデータセットからでも酵素の耐熱化と安定な酵素の設計が可能であることを示した。博士論文、その発表と質疑、公開発表後の個別審査会での質疑と一般知識の口頭試問の結果、本申請者は博士の学位授与に値すると判定した。