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A rational design for improving the trypsin resistance of aflatoxin-detoxifizyme (ADTZ) based on molecular structure evaluation

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ABSTRACT

The resistance of feed enzymes against proteases is crucial in livestock farming. In this study, the trypsin resistance of aflatoxin-detoxifizyme (ADTZ) is improved. ADTZ possesses 72 lys/arg residue sites, 45 of which are scattered on the outermost layers of the molecule (RSA ≧ 25%). These 45 lys/arg sites could be target sites for trypsin hydrolysis. By considering shape-matching (including physical and secondary bond interactions) and the "induced fit-effect", we hypothesized that some of these lys/arg sites are vulnerable to trypsin. A protein–protein docking simulation method was used to avoid the massive computational requirements and to address the intricacy of selecting candidate sites, as candidate site selection is affected by space displacement. Optimal mutants (K244Q/K213C/K270T and R356E/K357T/R623C) were predicted by computational design with protein folding energy analysis and molecular dynamics simulations. A trypsin digestion assay was performed, and the mutants displayed much higher stability against trypsin hydrolysis compared to the native enzyme. Moreover, temperature- and pH-activity profiles revealed that the designed mutations did not affect the catalytic activity of the enzyme.

1. Introduction

Aflatoxin is a mycotoxin widely found in grain, food and feed ingredients. The molecular structures of aflatoxin have been classified as B1, B2, G1, G2, M1, M2, GM1, and GM2. Of these classifications, the B1 classification has the highest toxicity. Aflatoxin contamination in feeds can cause weight loss or diseases in livestock and poultry. These toxins then enter humans indirectly through the food chain and can be a serious threat to human health [1]. Traditional chemical and physical detoxification methods for removing aflatoxin have drawbacks, including lower processing efficiencies due to aflatoxin's relatively high thermal stability. Aflatoxin is released gradually into the alimentary tract during digestion [2]. In comparison, biological enzymolysis of aflatoxins is ideal for degrading aflatoxins, as enzymatic detoxification processes are specific and precise. Aflatoxin-detoxifizyme (ADTZ) ruptures the furan double-bond in the bisfuran-ring structure of aflatoxin in vitro. Moreover, the final products of the ADTZ reaction are non-toxic [3]. Cao and Yao [4] reported the use of ADTZ products as feed additives for aflatoxin removal.

For biological enzymolysis, it is necessary to prolong the bioactivities of feed enzymes within animal intestines by improving the tolerance of feed enzymes for intestinal digestive enzymes (e.g., trypsin). Enzymes are ingested by animals during feeding and activated in the intestinal tract. During this process, a portion of the feed enzymes are destroyed by the animals' intestinal digestive enzymes, limiting total detoxification efficiencies. Although directed evolution and rational design methods have been used to enhance the catalytic activity and thermal stability of various feed enzymes, little work has been reported to improve the trypsin resistance of these enzymes [5–7].

Li et al. improved the trypsin resistance of β-mannanase (which possesses six lys/arg sites scattered on the outermost layers of the molecule, RSA ≧ 25%) using a set of criteria to select mutation sites and to evaluate molecular structures. These criteria included: (1) the candidate amino acid sites should be trypsin cleavage sites; (2)

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the mutation sites should have maximum surface exposure areas, or be close to the surface; (3) the mutation sites should not be close to the active site. This last criteria aimed to minimize the effect of potential mutations on the overall structure and catalytic activity of the targeted enzyme [8]. Homology modeling of ADTZ indicated that the protein possesses 72 lys/arg residues sites, with as many as forty-five lys/arg sites scattered on the outermost layers of the molecule (RSA ≧ 25%) [9]. The key to the effectiveness of this strategy is to limit: (1) the amount of computational power required to obtain the trypsin-resistant ADTZ; and (2) "induced fit effect" changes to the molecular structure of ADTZ during reactions between the enzyme and substrates. Such changes result in residues that do not match the catalytic pocket in trypsin, preventing trypsin hydrolysis at these positions. However, some residue changes could have the opposite effect, becoming capable of matching the trypsin catalytic pocket [10–12]. This situation affects the selection of mutation sites.

Protein–protein docking simulations can predict protein complex interaction interfaces. Following optimization of the docking results, the interaction interface between the enzyme's transition state complex and the substrate can be analyzed. From this interface, the side groups of the substrate protein that interact with the trypsin active center can be determined [13,14]. The degrees of exposure for cleavage sites located at the optimal interaction interface can then be analyzed [15,16]. Therefore, based on the strategy reported by Li et al. [8], the interaction interface of the transition state complex can be predicted by the protein–protein docking method. This approach simplifies the required calculations, and prevents structural changes in selected candidate sites due to the "induced fit effect".

The objectives of this study are: (1) to predict the interaction interface between ADTZ and trypsin based on a computational chemistry method; (2) to calculate solvent accessibilities and relative solvent-accessible surface areas; and (3) to evaluate the molecular structures of ADTZ mutants. Two aflatoxin-detoxifizyme mutants were designed, cloned, expressed and screened via enzyme activity and trypsin-resistance assays.

2. Materials and methods

2.1. Strains, plasmids, enzymes and reagents

Plasmids PB, PB-T*aox*, PB-P*aox-SS1*, and PB-T*aox-Ptgh* were previously constructed by our research group. *Pichia pastoris* SMD1168 and *Escherichia coli* DH5α were purchased from Invitrogen (USA). KOD-Plus DNA polymerase, T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs (NEB, UK) and TOYOBO (Japan). Plasmid isolation kits and DNA purification kits were obtained from Omega Bio-tek (Omega Bio-tek, Doraville, GA). Trypsin T4799-10G was purchased from Sigma–Aldrich (St. Louis, MO, USA), and electrophoresis reagents were purchased from Bio-Rad. All other chemicals were analytical grade.

2.2. Construction of a structural model of aflatoxin-detoxifizyme

A three-dimensional structural model was prepared based on dipeptide peptidases from human (PDB ID code: 3FVY) and yeast (PDB ID code: 3CSK) [17]. The human and yeast dipeptide peptidases share 42% and 37% identity with the aflatoxin-detoxifizyme sequence, respectively. The amino acid sequence of ADTZ was downloaded from NCBI. An initial model was constructed and optimized using MODELER [18,19], a protocol contained within Discovery Studio 3.0. The final model was validated using the Ramachandran and Profile-3D methods in Discovery Studio 3.0.

2.3. Prediction of interface residues via protein-docking

Protein-protein docking was performed with the ZDOCK module in Discovery Studio. ADTZ was set as the receptor, and the crystal structure of bovine trypsin (PDB ID code: 418G) was set as the ligand [20]. Trypsin His57, which lays at the interface of ADTZ exposure to trypsin, has been used as the constraint condition to run the candidate-screening after ZDOCK was performed. Docking results were optimized using the RDOCK algorithm. The docked structures predicted by ZDOCK were refined and re-ranked to select for near-native structures.

2.4. Selection of proper trypsin cleavage sites

All of the trypsin cleavage sites (K and R residues) in ADTZ were found in the 3D structural model. Solvent accessibilities, which reflect the amount of exposure to solvent, were investigated for these cleavage sites using Discovery Studio 3.0 [15]. Solvent-accessible surface areas of trypsin cleavage sites found at the interaction surface of the dominant conformation of ADTZ were calculated. Relative solvent accessibilities of corresponding trypsin cleavage sites were then calculated according to the formula:

$$\mathrm{RSA}(k,i) = \frac{\mathrm{ASA}(k)}{\mathrm{Max}_{\mathrm{ASA}(i)}} \times 100\% k = 1, \ldots, N, i = 1, \ldots, 20$$

In this work, the solvent-accessible surface area of residue "X" in Gly-X-Gly is the theoretical maximum [14].A larger RSA implies more solvent exposure. The most solvent-exposed trypsin cleavage sites at the ADTZ protein interface were identified as candidate mutant sites.

$$\mathrm{Max}_{\mathrm{ASA(Arg)}} = 312 \mathrm{A}^{\circ 2}$$

$$\mathrm{Max}_{\mathrm{ASA(Lys)}} = 290 \mathrm{A}^{\circ 2}$$

2.5. Mutation simulation and prediction of optimal mutant

To avoid changing the properties and structure of ADTZ as much as possible, selected K and R residues were mutated to eight other polar amino acids (H, N, D, E, Q, S, T, C) instead of using saturation mutagenesis. Three-dimensional models were constructed for all of these mutants using the standard mutation protocol provided by Discovery Studio 3.0 [21]. Optimal mutants were obtained via predictive stability predictions [14] and by evaluating molecular dynamics simulations. The structural stabilities of mutants were evaluated based on their calculated folding free energy: $\Delta\Delta G_{mut} = \Delta\Delta G_{fold}$ (mutant) − $\Delta\Delta G_{fold}$ (wild type). Mutants with the lowest $\Delta\Delta G_{mut}$ (folding free energy) values were selected for molecular dynamics simulations (100 ps of simulation heating steps, 500 ps of simulation equilibration steps, 400 ps of simulation production steps; 1×10^{-6} ps of time steps with the particle-mesh Ewald of method for electrostatics and constant pressure and temperature dynamics) [22,23]. Mutants with stable structures were determined by simulation and verified in subsequent biological experiments. If a candidate structure was not stable, another mutant structure with a low free energy was chosen for dynamics simulations.

2.6. Site-directed mutagenesis and vector construction

Plasmid PSA, which consists of a PB plasmid backbone and a gene encoding full-length wild-type ADTZ, was used as the template for site-directed mutagenesis. Site-directed mutagenesis was performed using the overlap-extension PCR method. All primers