TESTING FOR ESTERASE ACTIVITY IN ENZYME 4Q7Q

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Abstract

Enzymes are vital for life and all of life can be explained and better understood through enzymatic studies. The purpose of this project is to study and predict the function of an enzyme by using computational analysis and enzyme kinetics. Among the numerous enzymes of unknown function the enzyme we are working with is a possible lipase from Chitinophaga pinensis DSM 2588 with the PDB ID 4Q7Q. We have expressed this enzyme in *E.coli* and purified it with the help of Ni-NTA affinity chromatography. Since protein function is often tied to structure, structural comparisons and studies were done using computational tools. Lipase function was predicted after performing both global and local structure alignments using Dali and Moltimate. This function was supported by sequence alignment data from BLAST and PFam. To test for esterase function, enzymatic assays were carried out using two short chained substrates, *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB). We will determine whether the enzyme has a preference for a longer or a shorter chain ester-containing molecule by comparing these results to hydrolysis of lipid-like substrates with longer carbon chains. We have duplicated our first set of results which show significant esterase activity with the *p*-nitrophenyl acetate substrate and p-nitrophenyl butyrate. The next step would be to test longer chain substrates, lipid substrates and vesicles.

Introduction

Enzymes are vital for life and all of life can be explained and better understood through enzymatic studies. They assist in metabolic processes, break large molecules into smaller pieces that are more easily absorbed by the body, and help bind two molecules together to produce a new molecule. Enzymes allow all reactions to occur at the rate necessary for life. Understanding how enzymes work and what unique function each enzyme has can help us make major headway in the study of evolution, medicine, and just life and life processes in general. The roles of enzymes are identified by identifying what type of enzymes they are and what processes or reactions they aid in. For the preliminary studies, various computational tools can be used.

We performed computational analysis using several different programs as shown in Figure 1. The Protein Data bank (PDB[1]) first tells us about the quantitative properties and the structure of the enzyme. Each structure in the PDB is assigned a four-character alphanumeric identifier, called the PDB ID, and the focus of our current study is the structure with PDB ID 4Q7Q (Figure 2).

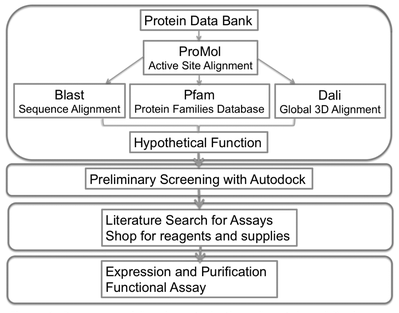

Figure 1. Process used for characterization of proteins of ''unknown function''. The top two boxes focus on *in silico* methods, followed by *in biblio* and *in vitro* steps [1, 2].

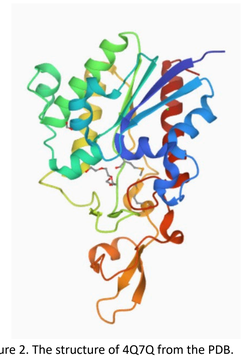

Figure 2. The structure of 4Q7Q from the PDB.

The active site of an enzyme is the region where the substrate molecules bind and undergo a chemical reaction. This active site consists of various amino acid residues that interact with the substrate forming temporary bonds and residues which catalyze the reaction of the substrate. The Enzyme Commission (EC) number is a numerical classification scheme for the enzyme, and this can tell us a lot about the given enzyme. The program Moltimate[2] can be used to compare 3D protein structures with a library of enzyme active sites, to predict whether the query structure (often a protein of unknown function) aligns with an enzyme of known function. These active sites can be better visualized with visualization software such as PyMOL[3]. The protein sequence can be run through BLAST[4] to find any other protein of known function which may help predict the potential function of the unknown protein. Similarly, more information can be found with the help of other tools such as PFam[5] and Dali[6]. PFam is a database of protein families, where families are sets of protein regions that share a significant degree of sequence similarity, thereby suggesting homology. Dali is used to align the backbone for entire protein structures. Finally, SwissDock[7] a protein-ligand docking program can be used to predict the position and orientation of a ligand or substrate of our choice when it is bound to a protein receptor or enzyme. Our computational analysis led us to conclude that the enzyme 4Q7Q was a possible lipase with specificity to ester substrates.

Kinetic testing was done to test this hypothesis. Enzyme kinetics is the study of the rates of enzyme-catalyzed chemical reactions. A measured rate can be used to derive information about the amount of enzyme present in a reaction sample, or about the effects of different experimental conditions on rate. The Michaelis-Menten model for enzyme activity treats an enzyme catalyzed reaction as a simple two-step process; the first, association of substrate and enzyme to form enzyme-substrate complex, and the second, conversion of substrate to product. Plotting the Michelis Menten graph will help us verify whether the enzyme has an affinity for a certain substrate.

Methods

The enzyme was analyzed with the help of structure viewing and alignment programs, and the alignments compared to various known enzymes in order to classify it and predict the potential functions. Computational analysis revealed it to be a potential lipase with specific affinity towards ester substrates. A plasmid containing the gene for 4Q7Q was successfully transformed in *E. Coli* by creating a surface that only bacteria with ampicillin resistance can survive and reproduce upon. Overnight Expression TB was then used for protein expression. Bacteria grew overnight by the accelerated process of TB. The cells were then spun down and the His-tagged protein was purified by metal-affinity chromatography after cell lysis. SDS-PAGE was used to verify the presence of protein. Buffer exchange was performed to reduce the salt content and remove the imidazole and obtain as high a concentration of protein as possible. The Bradford assay was then carried out to quantify the amount of protein in samples. Enzyme kinetics experiments were performed using a chromogenic substrate. Trial and error was used to determine conditions for carrying out assays to verify whether 4Q7Q follows Michaelis-Menten kinetics for any substrate.

Results

Computational Analysis:
All of the alignments performed indicate that 4Q7Q is a hydrolase with specificity for ester bonds. The 4Q7Q amino acid sequence and structure align with some acetyl esterases and some acyl esterases. The best active site alignment was to the 1BWR structure. 1BWR is described as an acetylhydrolase on the PDB (Figure 3). SwissDock was used to identify a possible binding site for the ligand propanoic acid (PPI) in 4Q7Q (Figure 4). This ligand is bound to various esterase structures on the PDB.

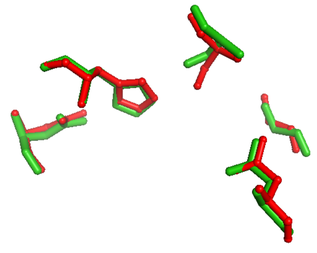

Figure 3. Active Site Alignment with 4Q7Q in green and 1BWR in red. The RMSD values for 5 residues with 35 atoms is 0.496, 5 residues with 0.459, and 5 residues with 9 atoms is 0.509. The Levenshtein distance is 0-5 and the EC class of 1BWR is 3.1.1.47.

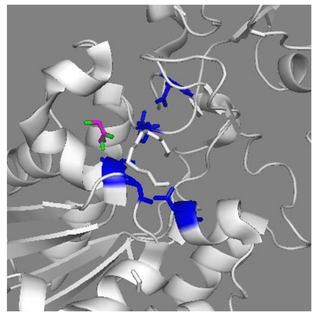

Figure 4. The active site of 4Q7Q is shown in blue and PPI from 5AOA is shown in purple. This ligand has a binding affinity of -4.1, a RMSD/ub of 2.437, and a RMSD/lb of 2.027. The PPI from 5LY1 is shown in green and this ligand has a binding affinity of -4.1, a RMSD/ub of 2.49, and a RMSD/lb of 2.114.

Purification of 4Q7Q:
4Q7Q was successfully purified using NiNTA agarose as verified by SDS-PAGE analysis (Figure 5).

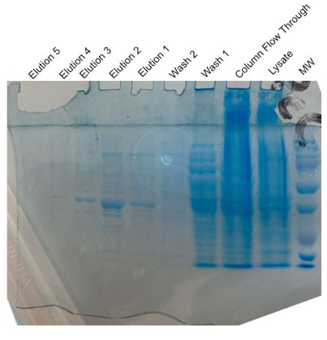

Figure 5. SDS-PAGE analysis of the purification of 4Q7Q by metal-affinity chromatography. The His-tagged 4Q7Q was separated from other proteins by washing the column with buffers containing increasing amounts of imidazole. Elution fraction 2 and 3 were combined and kept for kinetic testing.

Results

Kinetic Analysis:
The initial kinetic testing has revealed that 4Q7Q hydrolyzes both the *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate substrates. These substrates both produce a yellow product, *p*-nitrophenolate ion, when hydrolyzed, so the reactions can be followed by monitoring the absorbance at 405 nm (Figure 6). Trials were carried out to determine what quantity of enzyme was needed to demonstrate hydrolysis. Following this, the 4Q7Q concentration was kept at 0.05 mg/mL while the substrate concentration was varied in order to establish whether 4Q7Q follows the Michaelis-Menten kinetic model and attains saturation at high concentrations of enzyme.

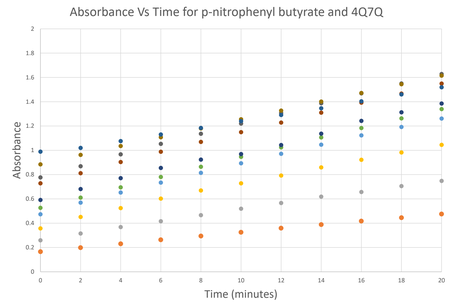

Figure 6: Absorbance Vs Time plot for p-nitrophenyl butyrate hydrolysis. The increase in absorbance as a function of time indicates that the yellow-colored product is being produced upon hydrolysis of the substrate.

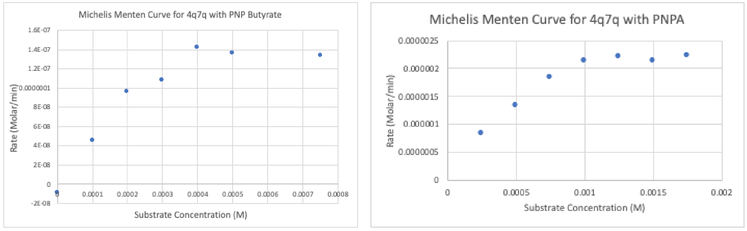

Figure 7: Michaelis-Menten plots (V_0 vs [S]) for reaction of enzyme 4Q7Q with *p*-nitrophenyl butyrate (left) and with *p*-nitrophenyl acetate (right).

Further Testing

The initial testing has shown promising results but the immediate next step will be replicating the results for consistency. Once that is completed, the Michaelis-Menten parameters of V_{max} and K_M can be determined and compared for the different substrates.

There are also further substrates to be tested including longer chain substrates like *p*-nitrophenyl decanoate and *p*-nitrophenyl dodecanoate. Since computational analysis showed similarities in the sequence of 4Q7Q with known lipolytic proteins lipid substrate testing is also going to be another goal.

References

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