Kinetics and NMR Studies on Xylanase A Mutant to Elucidate Thumb Flexibility in Enzymatic Mechanism

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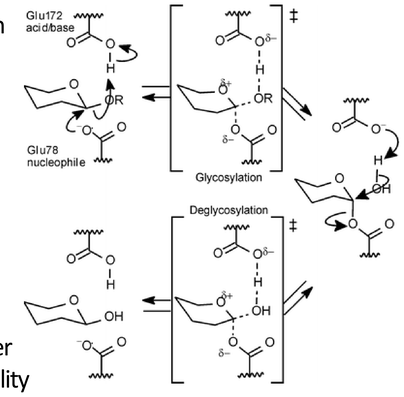
XylA Background

Protein activity is traditionally studied with major focus on the active site and protein dynamics which contribute to this. However, enzymes, such as Xylanase, have shown changes in activity when studying flexibility of adjacent regions, warranting more exploration into the dynamics of this mechanism.[1,2,3]

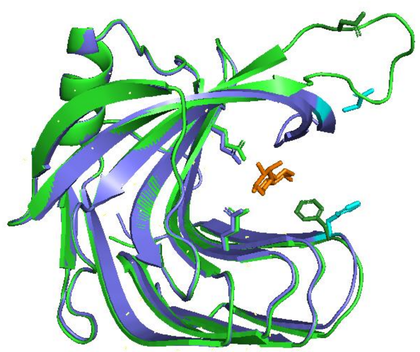
Xylanase A (XylA) from *Bacillus subtilis* cleaves xylan polymers or hemicellulose found in tree bark to xylose or heteroxylans by hydrolysis at internal β-1,4-xylosidic linkages as shown below.[4]

XylA contains a "thumb" region, correlated in prior studies to affect the activity of the enzyme through substrate binding and product release.[1,2,3] Using a double mutation D11F/R122D, already documented to affect the activity and to change the flexibility of the thumb, Michaelis-Menten enzyme kinetics was studied to evaluate activity perturbation.

Following, protein dynamics occurring in mutants relative to wild-type is being observed using NMR techniques, revealing the mechanism of activity perturbation by mutations on a fully-atomic level. NMR order parameter studies will be used to evaluate the enzyme's dynamics, especially as the active site is affected, in the presence of the two mutations. These as well as kinetics studies and MD will aid in further characterizing necessity of thumb flexibility in the enzymatic mechanism.

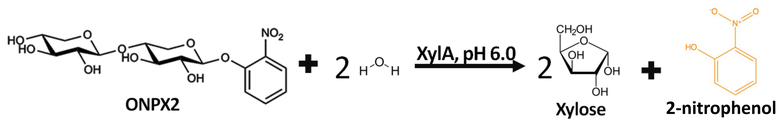


D11F/R122D Crystallizes in Two Conformations



Double mutant D11F/R122D has documented activity perturbations from wild type with focus on deviations in V_{max}, k_{cat}, and K_m values.[1,2,3] Structural studies in the presence of the two mutations have found two competing structures, one holding the thumb in a rigid "open" position and one allowing the thumb to be "closed". The structure above shows PDB 2B46 (blue) overlay with 3EXU (green) with D11F, R122D, and catalytic residues E78 and E172 shown as well as xylobiose ligand (orange).

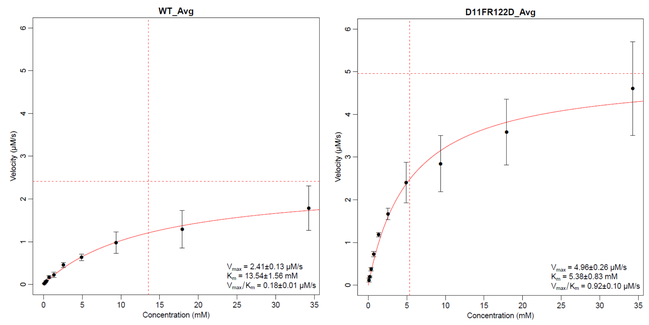
D11F/R122D Unexpectedly Enhances Hydrolysis of ONPX2

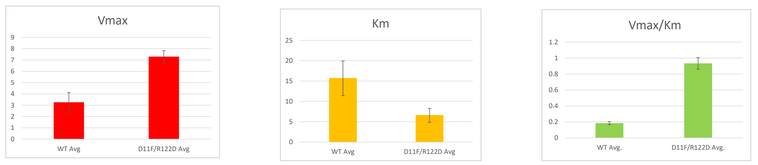


Hydrolysis of ONPX2 (Megazyme) by XylA yielding xylose and free 2-nitrophenol (pKa 7.2). Absorbance of 2-nitrophenol at 400nm read throughout the course of the reaction gives a direct measure of XylA activity.[6] Reaction was performed for varying ONPX2 concentrations and fit to a Michaelis-Menten kinetics model.

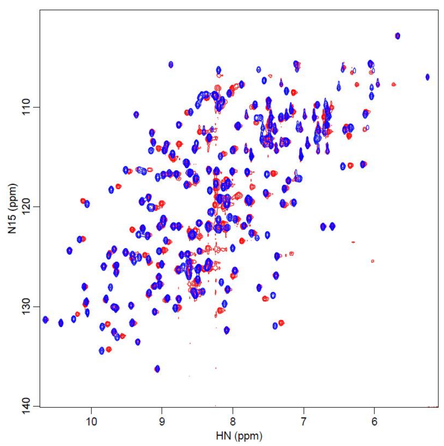
$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

$$k_{cat} = \frac{V_{max}}{[E]_T}$$



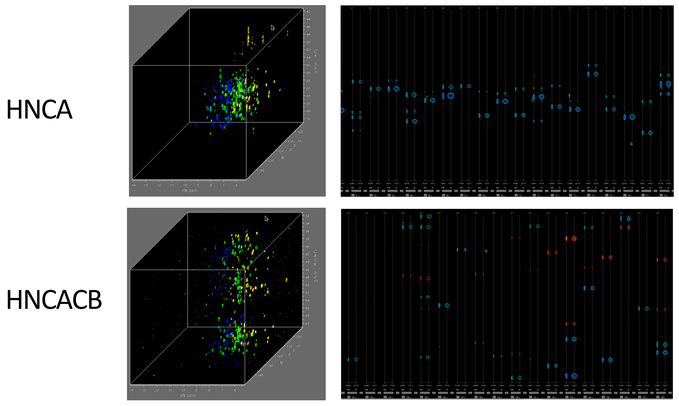


Backbone Dynamics

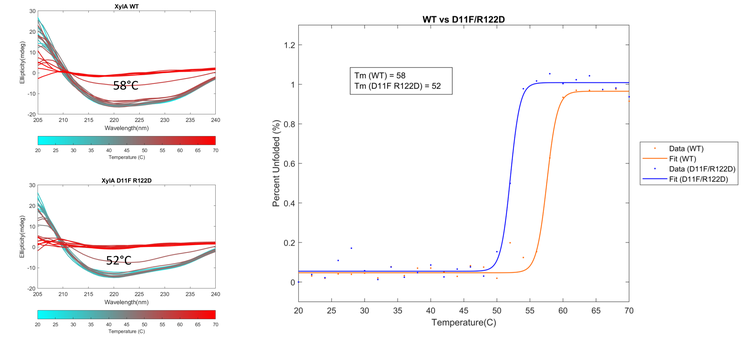


Overlay of WT (blue) and D11F/R122D (red) HSQC spectra. Overlapping peaks show good agreement between spectra, however, peaks that differ in chemical shift between the two indicate structural and potential dynamics differences between WT and D11F/R122D. HSQC on 600MHz was performed at 30°C to observe chemical shift perturbation relative to WT.[9,10] T_1, $T_{1\rho}$, and NOE on 800MHz will be analyzed to determine S^2 order parameters.

Peak Assignment for D11F/R122D



D11F/R122D Destabilizes Secondary Structure



Future Directions

- Kinetics studies using native xylan polymer to evaluate native activity
- Analysis of T_1, $T_{1\rho}$, and NOE to evaluate order parameters, especially those in the thumb region and the active/binding site
- Chemical shift perturbation analysis
- Assignment experiments to assign chemical shifts to residues
- Molecular dynamics simulations to establish ΔΔG

References

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[10]This study made use of NMRbox: National Center for Biomolecular NMR Data Processing and Analysis, a Biomedical Technology Research Resource (BTRR), which is supported by NIH grant P41GM111135 (NIGMS).