EXPRESSION OF BARLEY OXALATE OXIDASE ENZYME USING A PICHIA PASTORIS SECRETION SYSTEM

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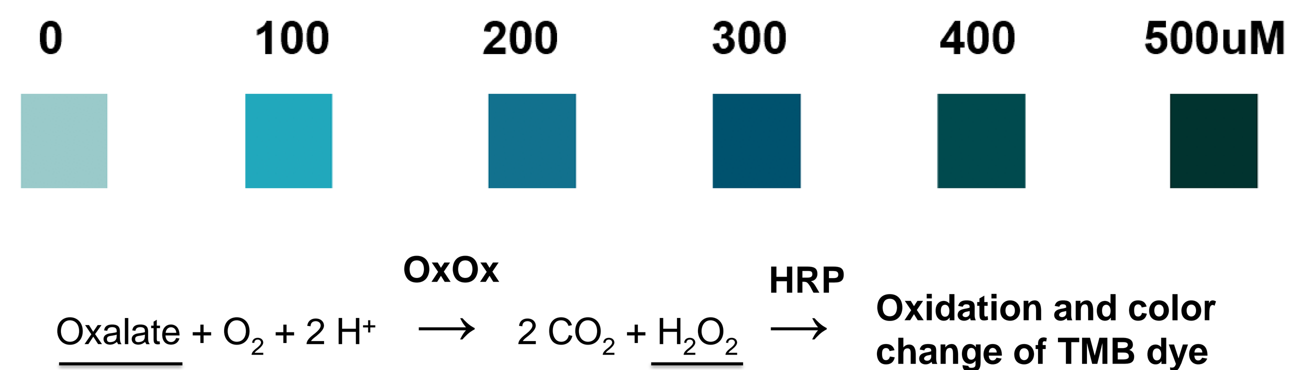
INTRODUCTION

- Roughly 80% of kidney stones contain calcium oxalate
- Urinary oxalate is a key factor in estimating kidney stone risk -Part of the 24-hour urine stone profile
- There is no simple and quick point-of-care oxalate test for clinicians and patients

Hypothesis: Routine monitoring will enable patients to become aware of oxalate levels and to take measurements on a daily basis to reduce possible recurrence of kidney stones or other oxalate-related diseases through changes in diet and fluid intake

Project Objective: To develop a dipstick test to measure urinary oxalate levels.



METHODS

A codon optimized synthetic OxOx gene derived from *Hordeum vulgare* (barley) was generated and cloned into the EcoRI/XbaI sites of the pPICZαA expression vector downstream of the N-terminal alpha mating factor secretion signal peptide sequence and used for expression in *Pichia pastoris* X-33 strain. This expression system is well suited for our purpose since it is relatively rapid, inexpensive, and capable of generating disulfide bonds and addition of glycans required for the functional expression of this enzyme. Another major advantage of using this secretion system is that it functions as a first step in the process of protein purification. Since the pI of this OxOx enzyme is predicted to be 5.5, anion exchange chromatography was used for purification (Q-sepharose: equilibration/binding with tris buffer pH 9.0 and elution with 1M NaCl). Kinetic assays were performed at optimal enzyme conditions as noted in the figure legend.

RESULTS

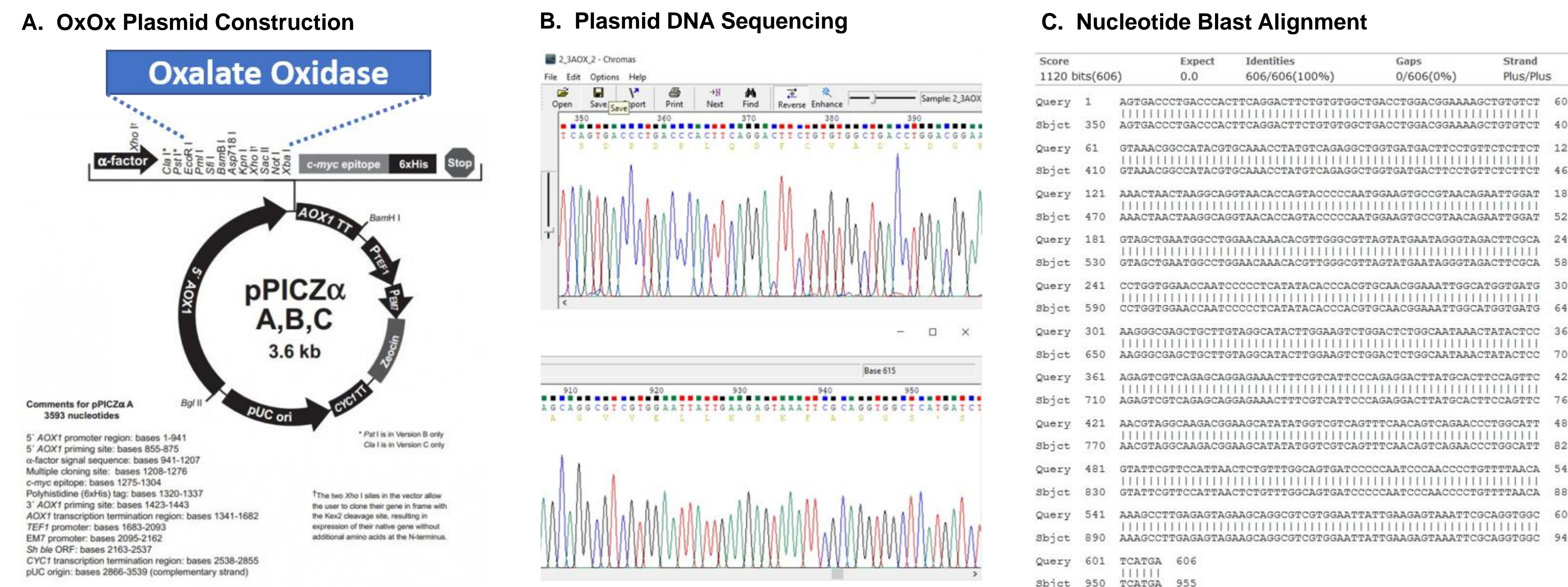


Fig 1. OxOx Plasmid Construction and Confirmation. A. We synthesized a codon optimized barley-derived oxalate oxidase gene and cloned into the EcoRI/XbaI sites of the pPICZαA expression vector. B. After transforming the OxOx plasmid into E. coli for amplification and purification, the OxOx gene insert was sequenced. C. The NCBI nucleotide blast alignment tool was used to confirm the integrity of the inserted OxOx gene.

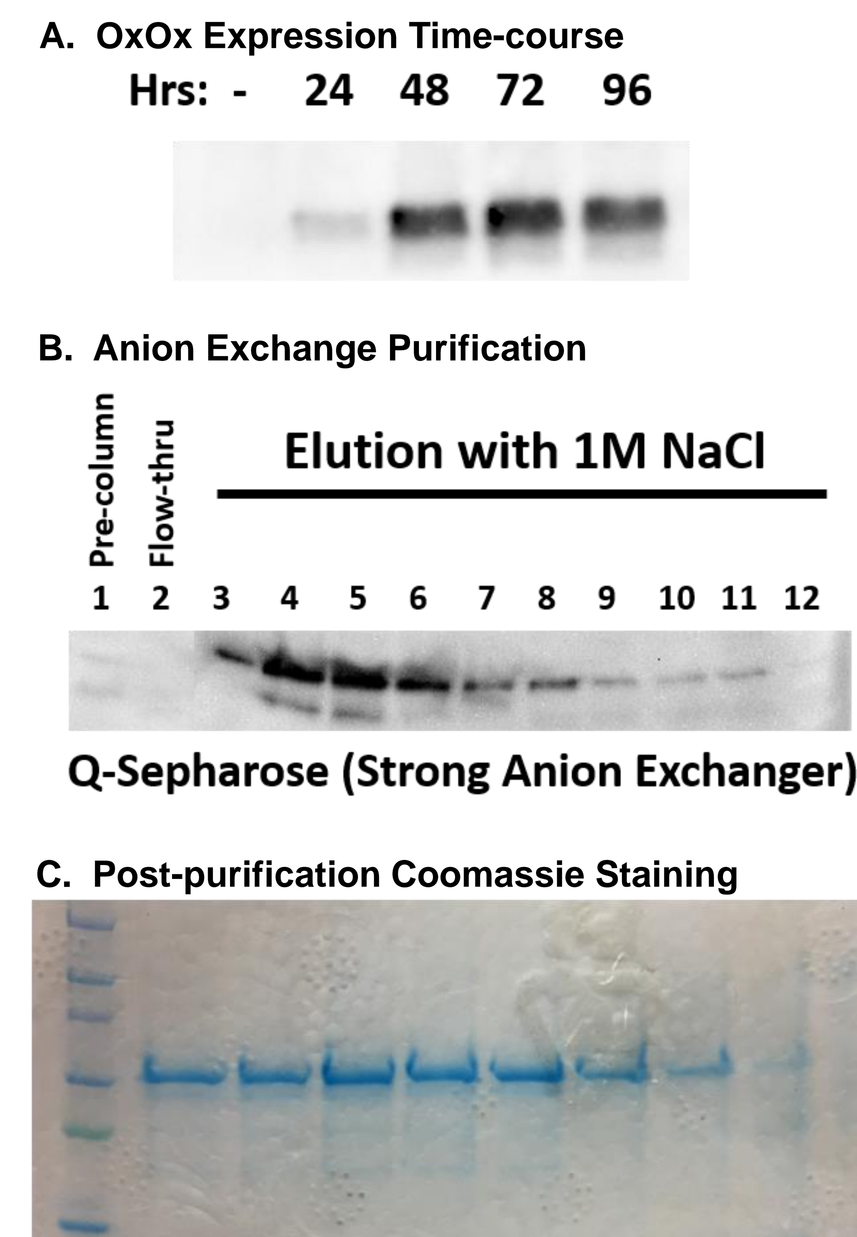


Fig 2. OxOx Expression and Purification. A. *P. pastoris* culture media samples were taken every 24 hours to evaluate expression by western blotting using an anti-OxOx antibody. B. Culture media was dialyzed overnight against distilled waster, the pH was adjusted to 9.0 using a tris buffer, applied to a Q-sepharose anion exchange column, and purified OxOx enzyme was eluted with 1M NaCl. C. Eluted fraction were separated by SDS-PAGE and total protein was visualized with Coomassie stain to determine purity.

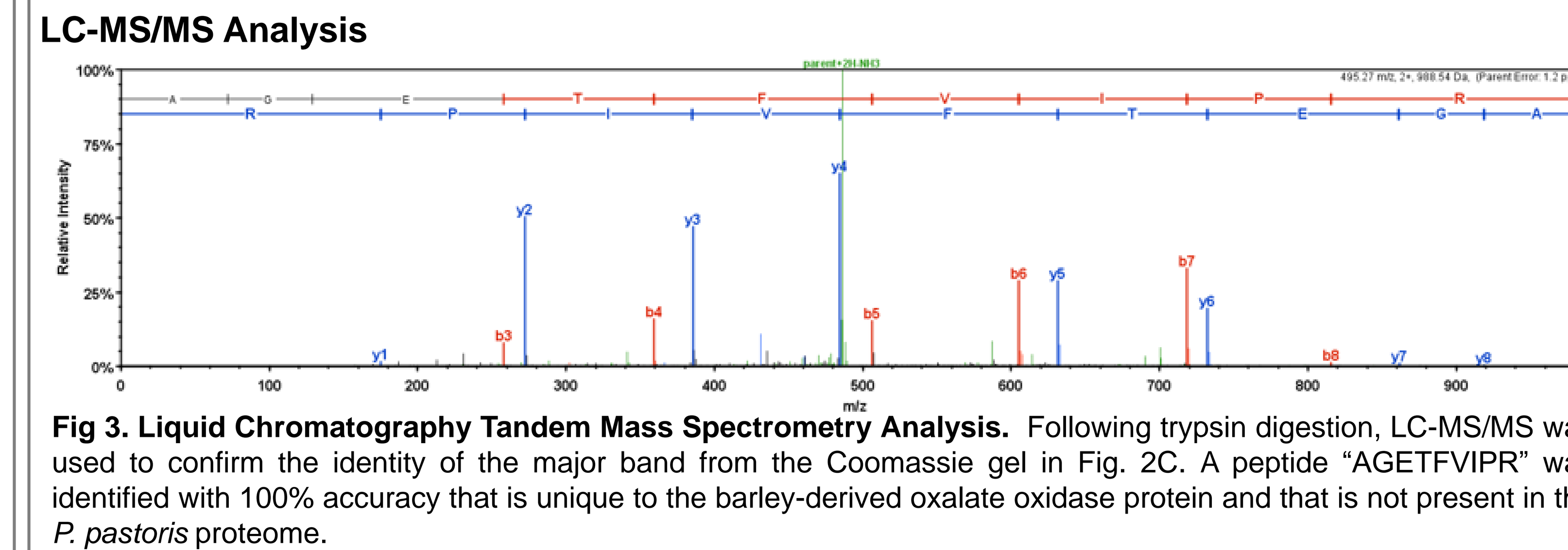


Fig 3. Liquid Chromatography Tandem Mass Spectrometry Analysis. Following trypsin digestion, LC-MS/MS was used to confirm the identity of the major band from the Coomassie gel in Fig. 2C. A peptide "AGETFVIPR" was identified with 100% accuracy that is unique to the barley-derived oxalate oxidase protein and that is not present in the *P. pastoris* proteome.

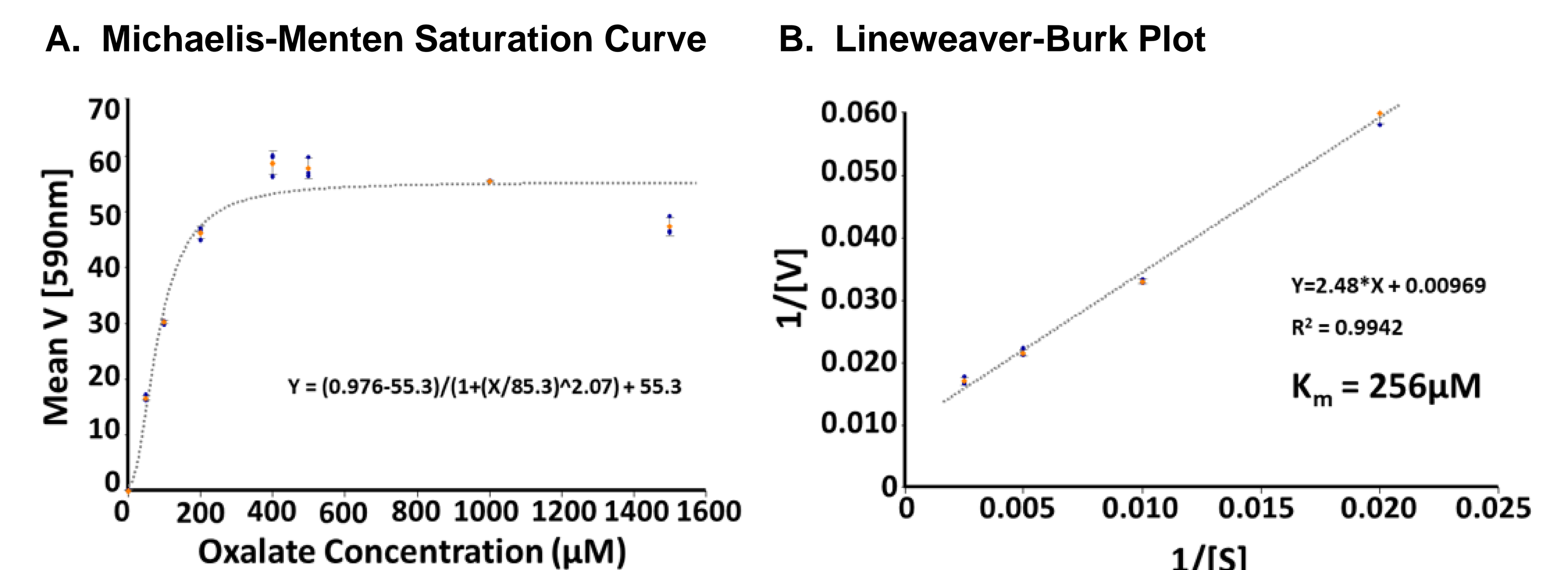


Fig 4. Reaction Kinetics of OxOx. A. Michaelis-Menten Saturation Curve. Kinetic analysis was performed in a 96-well plate using a plate reader at 37°C. Purified OxOx (400μg/ml) was added to buffer pH 3.1 containing (MBTH, 0.11mM, DMAB, 1.6mM, HRP, 0.1U/ml) at designated potassium oxalate concentrations. Absorbance at 590nm was measured every 30 sec for 30 min to determine the rate of reaction which was plotted against substrate concentration. B. Lineweaver-Burk Plot. Since the purified OxOx displayed Michaelis-Menten kinetics for concentrations up to 400μM, we plotted a linear transformation of the rate against substrate concentrations and estimate the enzyme Km = 256μM.

RESULTS SUMMARY

We identified an active oxalate oxidase sequence derived from barley and cloned a synthetic gene into the yeast expression plasmid pPICZαA. *P. pastoris* was transformed with the OxOx expression plasmid and induced to express active enzyme. Culture media was subsequently dialyzed overnight against distilled water. Active enzyme was purified to greater than 90% purity using Q-sepharose anion exchange chromatography. Our purified OxOx enzyme displayed standard Michaelis Menten kinetics at substrate concentrations up to 400μM (after which a well-known substrate inhibition occurs) and had an estimated Km value of 256μM based on linear regression analysis using a Lineweaver-Burk plot.

CONCLUSIONS

- **Generated an active Oxalate Oxidase yeast secretion expression system**
- **Purified OxOx enzyme using anion exchange chromatography**
- **Determined OxOx enzyme kinetics**
- **<u>Future Direction:</u> We plan to optimize the expression and purification protocols for this enzyme while developing a first-generation oxalate dipstick prototype.**

ACKNOWLEDGEMENTS

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