Cloning and Purification of a Glycerol-Specific Alditol Oxidase for Biosensor Construction

Chrissa Barroma, Amanda Kryjak, Nina Bernstein, MacEwan University

Abstract

Wine production is dependent on ethanol, but also on optimal glycerol concentrations, both of which are produced by *S. cerevisiae* fermentation. Wine characteristics like sweetness levels are influenced by glycerol concentrations. Additionally, elevated glycerol levels can be an indication of abnormal blood sugar levels. In both situations, close observations of glycerol levels are essential. One proposed method of measuring glycerol concentrations is through enzymatic oxidation with a glycerol biosensor. Alditol oxidase (AldO) is a recently discovered carbohydrate oxidase in *S. coelicolor*. Despite specificity for longer-chained polyols, studies have proposed that AldO can be used as a glycerol oxidase. Using random point mutations, an AldO mutant was isolated and had increased specificity for glycerol. These results suggest that potential for AldO with glycerol biosensor development. This project aimed to produce a glycerol specific alditol oxidase to be used as a biosensor. A synthetic alditol oxidase (AldOG) gene was used to produce AldOG via cloning methods. We plan to overexpress and purify the AldOG protein to use in construction of a glycerol biosensor in collaboration with Dr. Samuel Mugo (MacEwan University).

Introduction

Carbohydrate oxidases are a specialized type of enzyme that harbours a variety of technical uses (1). For example, oxidases have been previously used as a biological sensor for blood sugar level readings (1) and quality control in wine production (2). Most carbohydrate oxidases have been discovered in fungal species, whereas only two have been found in the genome of bacterial species - one of which is an alditol oxidase.

Alditol oxidase is a flavoprotein enzyme that was initially discovered in the bacterial species *Streptomyces coelicolor* (1). Named AldO, this enzyme was determined to be a very promising carbohydrate oxidase for both technical and industrial purposes. Consisting of a single polypeptide chain with an FAD cofactor, AldO catalyzes oxidation reactions of multiple alditols to aldehydes (3). FAD gets reduced during this reaction then regenerated in downstream reactions. H_2O_2 is then produced as a by-product when electrons are transferred from oxygen.

Glycerol (a 3-carbon polyalcohol) is a poor substrate for AldO, as this enzyme strongly favours reactions with long chain polyalcohols, such as sorbitol (C6) or xylitol (C5). While other glycerol-specific oxidases have been identified from fungal species, the presence of copper prevents safe and desirable use in industrial purposes (3). Previous studies have utilized random point mutations to obtain an AldO mutant that demonstrated specificity towards glycerol and would improve on industrial applications (3).

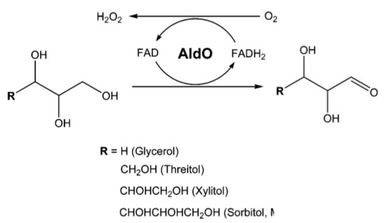

Figure 1: Catalysis reaction facilitated by AldO with the preferred substrates (4).

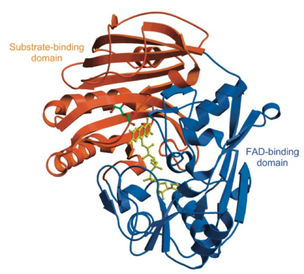

Figure 2: Model structure of AldO with xylitol bound within the active site (4).

As biosensor construction is important for technical applications including glycerol detection for blood sugar maintenance and quality control for wine production (2), the production of a glycerol-specific alditol oxidase became our primary goal. This study utilizes a pre-determined sequence from previous research (3). For our purposes, we named this potential biosensor AldOG. The results of this study will be used in the future to develop a senior-level laboratory course at MacEwan University and for the construction of a glycerol biosensor.

Methods

Cloning:

Synthetic AldOG and AldOWT gene sequence were purchased from Integrated DNA Technologies. The EcoRI and XhoI restriction sites were used for cloning the synthetic genes into a pGEX-6P-1 expression vector. An N-terminal GST affinity tag is included in the vector for protein expression and downstream purification. DH5-alpha competent *E. coli* cells (NEB) were used in the transformation protocol of the mutant and WT sequences as well as the pGEX-6P-1 vector.

Digestion Analysis:

Using EcoRI and XhoI enzymes, a restriction digest was conducted to determine the success of transformation of both the pGEX vector as well as the AldOG and AldOWT genetic sequences into the DH5-alpha competent *E. coli*. Gel electrophoresis was utilized to detect the presence of the digested fragment. Following success of digestion, the resulting sequences were PCR amplified for ligation preparation. Gel purification techniques were used to isolate the digested gene sequences after PCR amplification.

Ligation and Transformation:

The digested gene fragments are subjected to ligation in order to produce pGEX-6P-1 vectors with both AldOG and AldOWT. A pre-ligation analysis was conducted to ensure that the ligated products were properly assessed against pre-ligated fragments. Ligation products were then transformed into DH5-alpha *E. coli* and cultured on ampicillin plates.

PCR Screening:

Colonies were isolated from ampicillin plates and subjected to PCR amplification using pre-determined primers for the AldOG and AldOWT genes. These primers were tested on pre-ligated gene fragments. The successful ligated products were then isolated and used for transformation into BL21 (DE3) *E.coli* (NEB) which are optimized for protein expression.

Sequencing Analysis:

To further verify the success of ligation for the AldOG sequence and the pGEX-6P-1 vector, sequencing samples were prepared for analysis at the University of Alberta. DNA was extracted from isolated clones and incubated with synthesized primers. Primers were created for both the AldOG sequence, and the pGEX-6P-1 vector. Results were used to confirm if an AldOG and AldOWT clone were successfully obtained.

Induction Testing:

IPTG was selected for induction testing at different concentrations and varying incubation temperatures. Following overnight incubation at the varying IPTG concentrations (0.1 mM and 0.05 mM) and at temperatures of 18°C and 37 °C, cells were isolated and subjected to SDS-PAGE for analysis.

Results

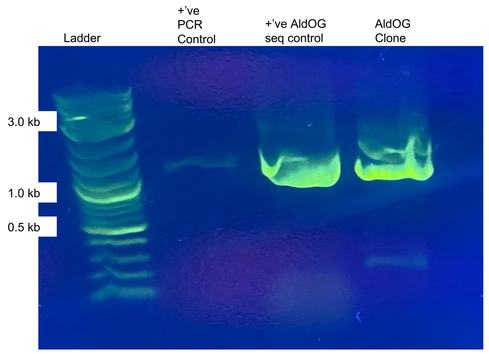

Figure 3: PCR Screening results of an isolated AldOG clone using primers created from the synthetic AldOG sequence. The positive PCR control indicates the success and integrity of the PCR reaction. A sample of the AldOG sequence within the initial vector and prior to ligation reactions was used as a positive control for the PCR reaction. As seen in the fourth lane, the AldOG clone within the pGEX-6P-1 vector was successfully amplified using the primer sequences. This demonstrates that the AldOG gene is likely found within the pGEX-6P-1 vector from the positive clone as it was able to be amplified.

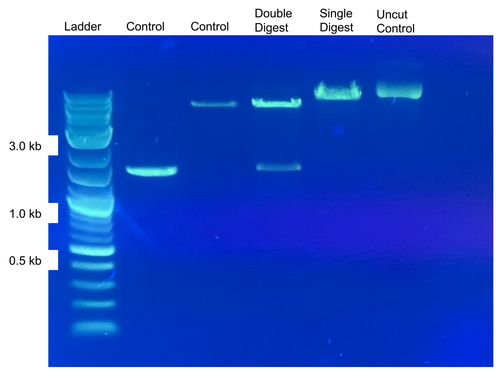

Figure 4: Diagnostic digest of the purified AldOG clone using EcoRI and XhoI restriction enzymes. DNA was extracted from the positive AldOG clone and subjected to diagnostic digests. As part of the pre-synthesized AldOG sequence, cut sites for EcoRI and XhoI were included to border the gene. DNA was incubated with EcoRI and XhoI and examined to determine the success of ligation and transformation. As compared to the positive controls of digested pre-ligated AldOG and the uncut control, the double digested samples of the isolated clone are the same size. This indicates that ligation of AldOG within the pGEX-6P-1 vector was successful. The clone was further verified from positive results from the sequencing results of the gene sequence.

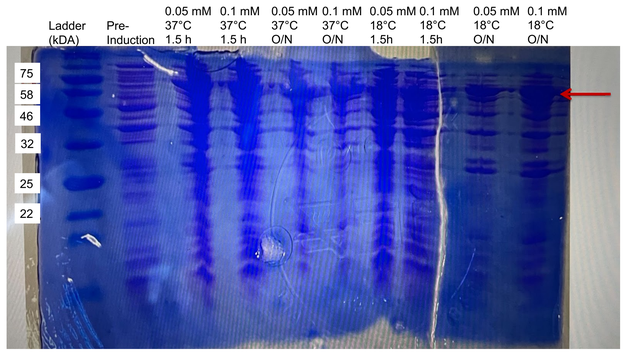

Figure 5: SDS-PAGE analysis of varying IPTG concentrations for optimal expression of AldOG for different incubation periods and temperatures. The DNA of the successful AldOG clone was transformed into protein optimized *E. coli* cells and subjected to IPTG treatments at different temperatures for optimal expression. As shown with the red arrow, optimal AldOG expression occurred under the conditions of 0.1 mM IPTG at 18°C for an overnight incubation period. A broad band can be seen at the corresponding protein size of 75 kDa for the AldOG protein.

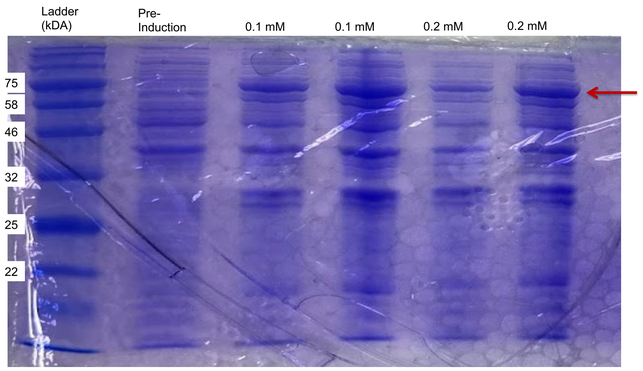

Figure 6: SDS-PAGE analysis of varying IPTG concentrations for optimal expression of AldOG. Using the results obtain from large scale induction, AldOG clones were incubated at 18°C overnight under 0.1 mM and 0.2 mM concentrations. At the 75 kDa marker, both 0.1 mM and 0.2 mM samples indicated high expression. Further testing to determine whether the concentration provides a substantial difference in expression is required.

Conclusions

Traditional ligation techniques produced the only successful ligation product. Additionally, only an AldOG clone was able to be isolated whereas our attempts to clone AldOWT were not successful.

IPTG concentrations at 0.1 mM and 0.2 mM showed the most promise for AldOG expression when incubated at 18°C overnight.

The possibility of AldOG as a glycerol specific oxidase is promising, however the cloning process appears to be the primary hurdle.

Future Directions:

Protein purification is needed to further apply this concept to a glycerol biosensor. Once protein purification is complete, enzymatic activity can be measured.

As glycerol biosensors are not widely available, development of one could be used in novel enzymatic methods of glycerol-level measurements in wine production (2)

Glycerol biosensors are also applicable to a wide variety of diseases. Elevated glycerol levels may play a role in type 2 diabetes and hyperglycerolemia (5).

The AldOWT clone is also needed in order to compare if there is an increase in function with the mutant version.

References

1. Heuts, D.P.H.M., van Hellemond, E.W., Janssen, D.B., and Fraaije, M.W. (2007). Discovery, characterization, and kinetic analysis of an alditol oxidase from Streptomyces coelicolor*. J. Biol. Chem. *282*, 20283-20291.
2. Šehović, D., Petravć, V., and Marić, V. (2004)i Glycerol and wine industry glycerol determination in grape must and wine. Kem. Ind. *50*, 505-516.
3. Gerstenbruch, S., Wulf, H., Mußmann, N., O'Connell, T., Maurer, K.-H., and Bornscheuer, U.T. (2012). Asymmetric synthesis of d-glyceric acid by an alditol oxidase and directed evolution for enhanced oxidative activity towards glycerol. Appl. Microbiol. Biotechnol. *96*, 1243-1252.
4. Forneris, F., Heuts, D.P.H.M., Delvecchio, M., Rovida, S., Fraaije, M.W., and Mattevi, A. (2008). Structural analysis of the catalytic mechanism and stereoselectivity in Streptomyces coelicolor alditol oxidase. Bicm. *47*, 978-985.
5. Ramonas, E., Ratautas, D., Dagys, M., Meškys, R., & Kulys, J. (2019). Highly sensitive amperometric biosensor based on alcohol dehydrogenase for determination of glycerol in human urine. *Talanta*, *200*, 333–339.

Acknowledgements

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