Determination of Potential Protein Binding Targets of Histatin 5 in *Saccharomyces cervisiae*

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Introduction

As more fungi become resistant to current antifungal treatments, there is a growing concern regarding the development of novel therapeutic agents. Histatins are a family of salivary antifungal peptides found in the parotid and submandibular glands of humans and some primates. There are 12 electrophoretically distinct members of the histatin family. Of the twelve, histatin 5 (H5) was found to have the highest antifungal activity against the known pathogen *Candida albicans*. Because of its high potency, and low toxicity, H5 is an excellent target for the development of novel antifungal therapeutics. However, the mode of action of H5 and the rest of its family members is still unclear.

Previous studies of H5 activity against *Candida albicans* determined that the antifungal activity was not through membrane disruption, a method that is predominantly used by other antimicrobial peptides. Instead, H5 was found to pass through the cell wall and membrane, migrating to mitochondria where it is believed that it interacts with cellular targets causing cell death.

Using *Saccharomyces cerevisiae* as a model organism due to its similar morphology to *Candida albicans*, a method was developed to determine possible protein binding targets to H5.

Method

H5 was synthesized with Fmoc chemistry and using solid phase peptide synthesis protocols. An aminooctylcarboxylic acid linker was coupled to the N-terminus of the H5 peptide, followed by coupling to a biotin molecule. A control peptide was prepared using a computer generated randomized sequence of H5. The peptides were cleaved, extracted and purified using semi-preparative Waters 600E HPLC with an SB-C18 column and a gradient of 5 to 35% ACN (+ 0.1 TFA) against H_2O (+ 0.1% TFA) at 4.0 mL/min. The peptide purity was checked by analytical HPLC and mass was verified by ESI/MS (Micromass®).

Using the protocols from Sigma, the avidin column was regenerated with 5 mg of biotin-linker-H5 or biotin-linker-column (1mg/mL) in phosphate buffered saline (PBS). The column was rinsed with 10 mL PBS and 10 mL H_2O.

Saccharomyces cerevisiae cells were grown to mid-log phase and lysed. Cell extract (1.0 mL) was loaded onto the column and incubated for 1 hour. The column was washed with PBS and H_2O.

The column was eluted with buffers made from volatile salts using a stepwise gradient of pH 11, 9, 7, 4, 2.5 and the final elution was with 6M guanidinium hydrochloride (pH 2.0). Briefly the column was incubated with 8.0 mL of the pH buffer. The column was eluted and washed with H_2O (2.0 mL) before proceeding to the next pH buffer.

Fractions were lyophilized and 50 µL of Milli-Q H_2O was added to each sample. The pH of each fraction was checked. A portion (20 µL) of each fraction was removed and the pH was adjusted to between pH 7-8. Porcine modified trypsin (Promega, 100 ng) was added to each sample. The samples were incubated overnight at 37°C and dried

on a Savant SpeedVac Plus® concentrator. Samples were rehydrated with 0.2 % FA (20 µL).

Enolase was purchased from Sigma and was loaded and incubated on the column. The column was eluted as previously mentioned. Samples were lyophilized and a portion (20 µL) was loaded onto a Criterion® gel and silver stained.

Digested fractions (1.4 µL) were identified using peptide mass fingerprinting (Micromass® MALDI-R) and peptide sequencing (Micromass® nano-LC/MS/MS).

Using the purchased enolase, preliminary inhibitory kinetics were attempted.

Results

Two proteins were identified: glyceraldehyde-3-phosphate (GAPDH) and enolase (ENO) which were not identified on the control column. GAPDH has three isoforms found in yeast. Using peptide sequencing and mapping we were able to determine that only GAPDH3 was present with a total coverage of 25%. Subsequent searches of the yeast genome database (http://www.yeastgenome.org/) on GAPDH3 demonstrate that deletion of the GAPDH3 gene or removal of its activity results in a mutant which is still viable.

The second protein, enolase has two isoforms in yeast. We were able to determine that ENO2 was the only isoform present in the sample with a total coverage of 57.6% using both peptide sequencing and peptide mapping. Searches of the yeast genome database on ENO2 resulted in the discovery that removal of this gene or removal of the activity of the protein results in the loss of cell viability. Because of the impact this protein has on the viability of *Saccharomyces cerevisiae*, we focused on further exploration of these results.

Purchased enolase was incubated and eluted from both columns as previously reported. Fractions were lyophilized and rehydrated in 50 µL of H_2O and a portion (10 µL) was used to run SDS-PAGE. Gel analysis of the fractions confirmed specific binding to H5 and no binding to the control column.

Preliminary kinetics did not show any inhibition of enolase by H5.

Conclusion

H5 was found to bind specifically to ENO2, which is an enzyme required for cell viability. The fact that preliminary kinetics do not show inhibition of ENO2 by H5 does not preclude any function. It may prevent essential protein/protein interaction. The inhibition assay must be repeated to confirm the results. The method should be attempted using *Candida albicans* cells to determine the binding targets of H5.

References

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