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The use of novel, fluorescent biosensors to measure *Aspergillus niger* prolyl-endoprotease activity and substrates in beer over time.

Trevor C. Cross; Peter B. Berget, PhD; Matthew J. Farber, PhD

University of the Sciences – Philadelphia, PA

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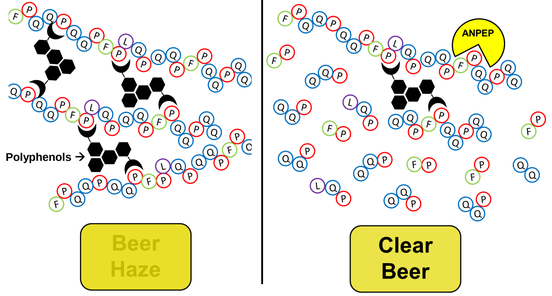
Abstract

Aspergillus niger prolyl endoprotease (ANPEP) is a proline-specific protease added by the brewer during fermentation to reduce chill haze and gluten content. Using technology developed in our lab, we have engineered fluorescent biosensors capable of measuring ANPEP activity in a single drop of beer. With this tool, we confirmed that ANPEP activity is stable over at least 6 months in commercial beer with no negative effect on foam. To further examine the relationship between ANPEP and foam, we designed several biosensors based on the 7 amino acids surrounding each Proline residue in the foam positive protein LTP-1. Surprisingly, many of the proline residues in LTP-1 are poor substrates. This data provides support for the protection of foam stability when using ANPEP and suggests ANPEP is not as general of a Proline-endoprotease as is often described.

The commercial use of ANPEP in Beer

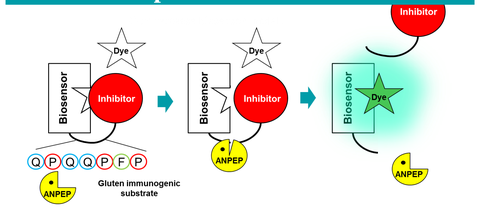
Hordein, a gluten protein in barley, forms a complex with polyphenols creating beer haze.

ANPEP non-specifically cleaves proteins at C-terminus of Prolines (P).



- Hordein and other Gluten fragments elicit an immune response in people with Celiac's Disease.
- When ANPEP is added during fermentation beer haze is reduced (1).
- ANPEP degrades all Gluten immunogenic peptides to <20ppm (2)

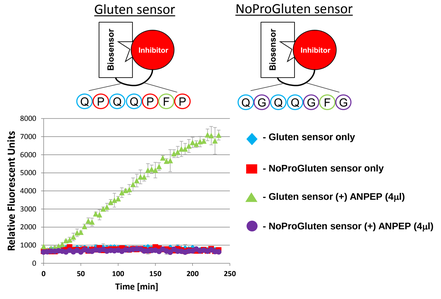
Selective protease biosensor model



The biosensor is an scFv-derived antibody which dimerizes around a cognate dye, inducing fluorescence. By connecting the scFv to an inhibitory scFv with a peptide linker containing a protease target sequence, we effectively generate a selective protease biosensor. **Because the system is genetically encoded on a plasmid, we can easily engineer the linker to be any protein sequence of interest.**

Figure 1

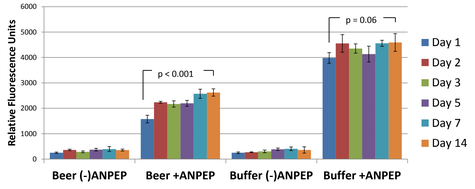
Kinetic measurement of ANPEP over time.



The responsible Hordein peptide in Celiac Disease is: QPFPQPQQPFPQ (3). A biosensor was constructed using the sequence in red (Gluten) and a sequence with P → G mutations (NoProGluten). Data represents kinetic reading of fluorescence over time (Ex 630nm / Em 660nm) in the presence or absence of commercial ANPEP (Brewers Clarex, DSM), reported as the average of triplicate samples with error bars representing standard deviation. Experiment conducted in 100µl total volume in Tris Buffered Saline, pH 7.4.

Figure 2

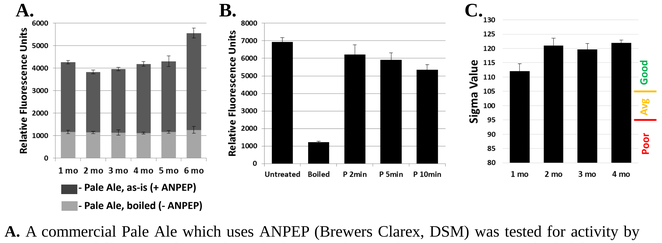
ANPEP activity is stable over time in beer.



Industry relevant concentrations of ANPEP at 2µl/100ml (Brewer's Clarex, DSM)) were tested for stability in an unhopped, 9°P light DME beer or McIlvaine's buffer, pH 4.0. Samples were incubated for 6 hours at 37°C. Represented data is the endpoint fluorescence reading after recovery in PBS, pH 7.0 and is reported as the average of triplicate samples with error bars representing standard deviation. Significance measured with the Student's T-test.

Figure 3

ANPEP activity persists for at least 6 months in a commercial beer with no effect on foam



A. A commercial Pale Ale which uses ANPEP (Brewers Clarex, DSM) was tested for activity by comparing 6 different production lots which spanned 6 months in age. Beer was boiled for 2 min to generate a no ANPEP control.

B. Pasteurization slightly reduces ANPEP activity. A commercial Pale Ale which uses ANPEP was either boiled (2 min) or Pasteurized (70°C) for the indicated time before analysis.

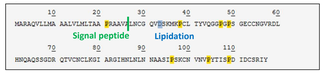
C. Foam stability of a commercial Pale Ale which uses ANPEP was tested by the Sigma value method as described in ASBC-Beer22. The same lot was tested over the course of 4 months. Typical Sigma values for poor foam, average (avg) foam, and good foam are indicated.

In all figures, data is reported as the average of triplicate samples with error bars representing standard deviation.

Figure 4

Most Prolines in LTP-1 are poor substrates.

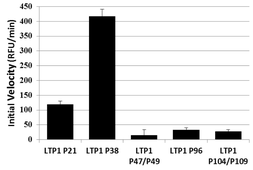
A. **Lipid Transfer Protein -1 (LTP-1):**



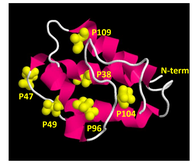
B. **Sensor cleavage sequences:**

P21	– TAAPRAA
P38	– KMKPCLT
P47/P49	– GGPGPSG
P96	– ASIPSKC
P104/P109	– VPYTISPD

C.



D.



A. Barley LTP-1 is a 117 a.a. protein with 7 Proline residues (highlighted in yellow).

B. Five biosensors were created to test cleavage of the indicated sequences. All 5 biosensors only differ by these 7-8 amino acids.

C. Kinetic cleavage of each substrate was tested with commercial ANPEP (Brewers Clarex, DSM) in PBS, pH7.0. Data is represented as the initial velocity (the increase in fluorescence per minute over the linear range). Data is reported as the average of triplicate samples with error bars representing standard deviation.

D. 3-D structure of Barley LTP-1 with Prolines in Yellow (4).

Conclusions

- We have constructed several fluorescent ANPEP biosensors based on Gluten and LTP-1 substrates which contain Proline.
- ANPEP is active for at least 6 months in commercial beer.
- ANPEP has no effect on foam stability in the beer tested.
- ANPEP demonstrates substrate selectivity and is not as general of a Proline Endoprotease as often described.
- Poor substrate specificity of foam positive proteins may underlie the neutral impact of ANPEP on beer foam.

References:

1) Lopez and Edens. (2005) *J. Agric. Food Chem.*
2) Akeroyd *et al.* (2016) *J. Am. Soc. Brew. Chem.*
3) Dromey *et al.* (2010) *Science Trans. Med.*
4) Heinemann *et al.* (1996) *Protein Sci.*

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