RESEARCH & EDUCATION FUND

MEASUREMENT OF PROTEASE SECRETED INTO BEER DURING FERMENTATION

American Homebrewers Association®

Measurement of protease secreted into beer during fermentation

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Statement of Purpose:

The purpose of this research was to examine the effect of yeast strain selection and fermentation temperature on protease activity in beer.

Background:

Proteases are proteins that degrade other proteins. Sometimes this cleavage is specific as in one protease targets one protein but other times recognition is broad with one protease cleaving multiple targets. One particular protease relevant to brewing falls into the later category.

Proteinase A (PrA) is a yeast protease which typically resides inside yeast cells. It nonspecifically degrades intracellular proteins during times of nutritional stress (*i.e.* post-fermentation) in order to support essential metabolism necessary for survival. During stressful brewing conditions such as nutrient deprivation or high gravity fermentation, PrA can be released into the beer where it will ultimately degrade beer proteins [1,2].

Proteins from barley are the major source of protein in beer. Those proteins with both hydrophobic and hydrophillic characteristics greatly contribute to beer foam stability, as they help maintain surface tension of a bubble within its surrounding aqueous environment [3]. PrA has been shown to degrade these foam-positive proteins in beer both specifically through degradation of Lipid Transfer Protein 1 (LTP1) and non-specifically through overall reduction of hydrophobic proteins [4,5]. Ultimately, PrA negatively affects beer quality through the degradation of beer proteins and reduction of foam. Therefore, avoiding PrA release into beer during fermentation is an important step in optimizing the quality and stability of homebrew.

As a postdoctoral scientist in a biotechnology research lab at the University of the Sciences in Philadelphia, I helped develop a technology to measure specific protease activity in a sample volume of less than a drop of liquid. Essentially we built a specific biosensor for a specific protease target. As a homebrewer, I came across a statement in George Fix's book *Principles of Brewing Science* that mentioned, in passing, that a yeast by-product called protease could be secreted into beer during fermentation. After reading more into the primary literature, I engineered a biosensor which can measure the activity of PrA in beer. This report is not a discussion of the technology (which is currently unpublished), but rather an analysis of preliminary results with implications for homebrewers.

Results:

The protease biosensor we engineered is based on a PrA-specific biosensor previously published [6]. In general, we used a similar approach to generate a biosensor which fluoresces after cleavage by a specific protease, in this case PrA. In this manner, if a beer sample does not contain protease, there is no cleavage of the biosensor and no fluorescence. If the beer sample contains protease, then as more biosensor is cleaved, more fluorescence will be generated (Figure 1). By plotting the relative fluorescence units assigned by a fluorescence detector, we can estimate the amount of protease in a sample by analyzing the slope of the line. More protease in a sample generates a steeper slope. Ultimately in the presence of protease and with sufficient incubation time, all of the biosensor will be cleaved resulting in a fluorescence plateau.

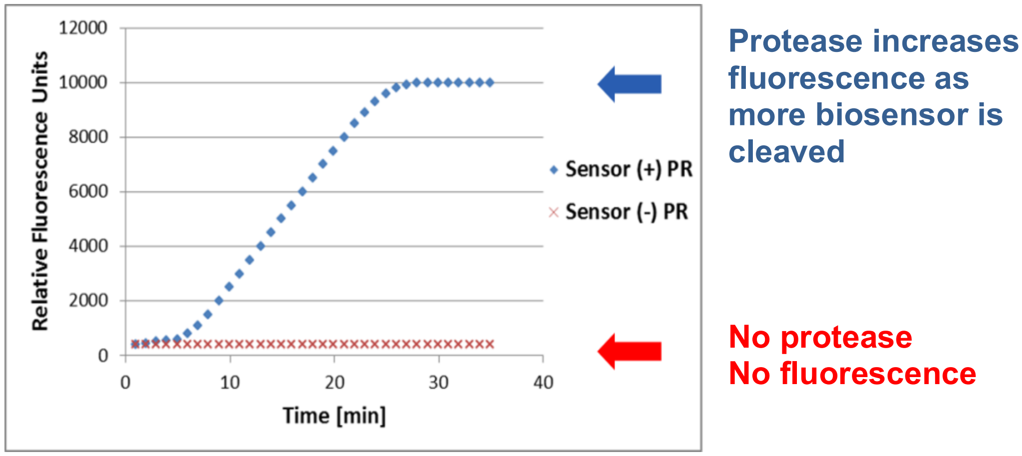

Figure 1: Mock data demonstrating how protease activity is measured. The PrA biosensor fluoresces only if it is cleaved by protease. Less than a drop of beer is used as the source of protease.

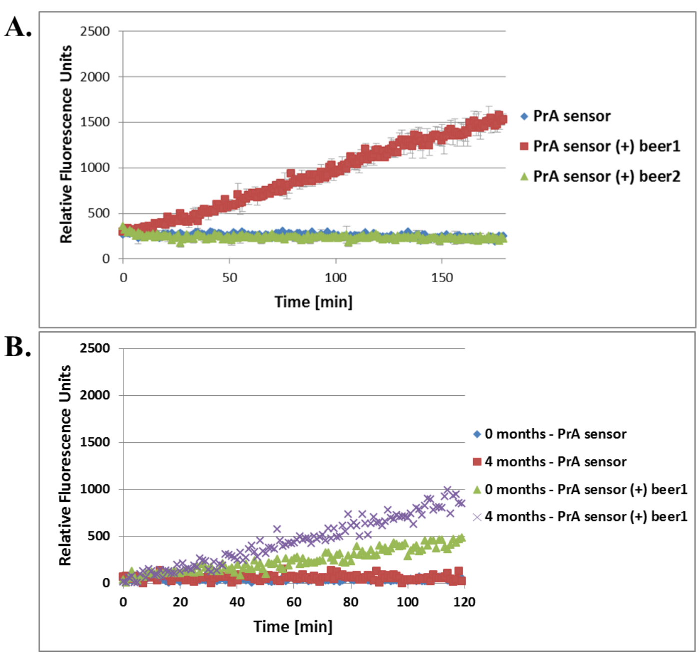

Figure 2: Measurement of protease activity in homebrew.
(A.) The beers of two different homebrews were tested for PrA activity. Beer1 shows activation of the sensor, indicating the presence of PrA. Beer2 has no detectable PrA. Error bars represent the standard deviation of three experiments. **(B.)** PrA activity increases with long-term cold storage of beer.

After making the PrA biosensor in my academic lab, I tested two homebrews recently made at home. Beer 1 was an IPA fermented with Wyeast American Ale II yeast and beer 2 was a porter fermented with Danstar Windor Ale yeast. Using less than a drop of beer as the sample for the experiment, beer1 demonstrated cleavage of the PrA biosensor and an increase in fluorescence over time, indicating the presence of protease in the homebrew.

Trüb had been collected from beer1 and was stored at 4°C in a refrigerator. As the beer had cleared via gravity and flocculation, beer samples were collected from the top and tested for protease activity at 0 months and 4 months of storage. Comparison of the rates of cleavage of the biosensor indicate 310% more protease in the sample stored for 4 months. Therefore, these results indicate that prolonged storage of beer on yeast, even at chilled temperatures, can cause an increase of protease in beer.

In an effort to generate a more controlled experiment, simple fermentations were conducted in the laboratory in 50mL conical tubes. Light dry malt extract (DME) from Muntons was used to generate a 1.04 gravity wört using 100g of DME in 1L of water. Three different commercially available yeast strains were purchased from a local homebrew supply shop for this experiment, Wyeast American Ale I, White Labs Super High Gravity Ale, and White Labs English Ale. Wyeast American Ale II was re-cultured from a previous stock. Starter cultures were set up in small Erlenmeyer flasks using 1.04 gravity DME wört and were grown at room temperature for 4 days before use. After boiling the wört for 5 minutes and cooling to room temperature, $1x10^6$ cells per mL per degree Plato was pitched from starter cultures for primary fermentation. Three static fermentations for each strain above were set up and incubated at 20°C (69°F), 25°C (77°F), or 30°C (86°F). Beer samples were collected once a week for 4 weeks, cleared by centrifugation, and analyzed for protease activity (Figure 3A). Interestingly, no protease activity was detected after one week of fermentation, even at stressful fermentation temperatures. However, by the second week of fermentation, noticeable protease activity was present in the English Ale fermentation at 30°C. The other yeast strains did not demonstrate protease activity until sometime between week 3 and week 4 at 30°C. By week 4, the English Ale strain at 25°C started to demonstrate protease activity. Importantly, no protease could be detected in any samples incubated at 20°C, the generally accepted fermentation temperature. The Super High Gravity strain never demonstrated measureable protease activity at any temperature or timepoint.

Before setting up the starter cultures for the experiment described above, beer samples were taken directly from the Wyeast smack pack or White labs culture vials. After clarification by centrifugation, each beer was tested for protease activity. As seen in Figure 3B, the Super High Gravity Ale and the English Ale contain measurable amounts of protease activity. These results demonstrate that commercial yeast products can contain significant amounts of active protease.

Pasteurization is a technique employed by most large breweries and an increasing number of microbreweries where, shortly after filtration, the beer is heated to ~70°C for a short period. Pasteurization is principally employed to denature any microbial contaminants which were not filtered, but as demonstrated in Figure 4, pasteurization can also be used to denature problematic proteins secreted by yeast into the beer. In order to examine the effects of pasteurization on PrA activity, a protease-containing beer sample (beer 1) was incubated at 70°C for 2 minutes. Compared to an unpasteurized sample, protease activity is completely eliminated following pasteurization (Figure 4).

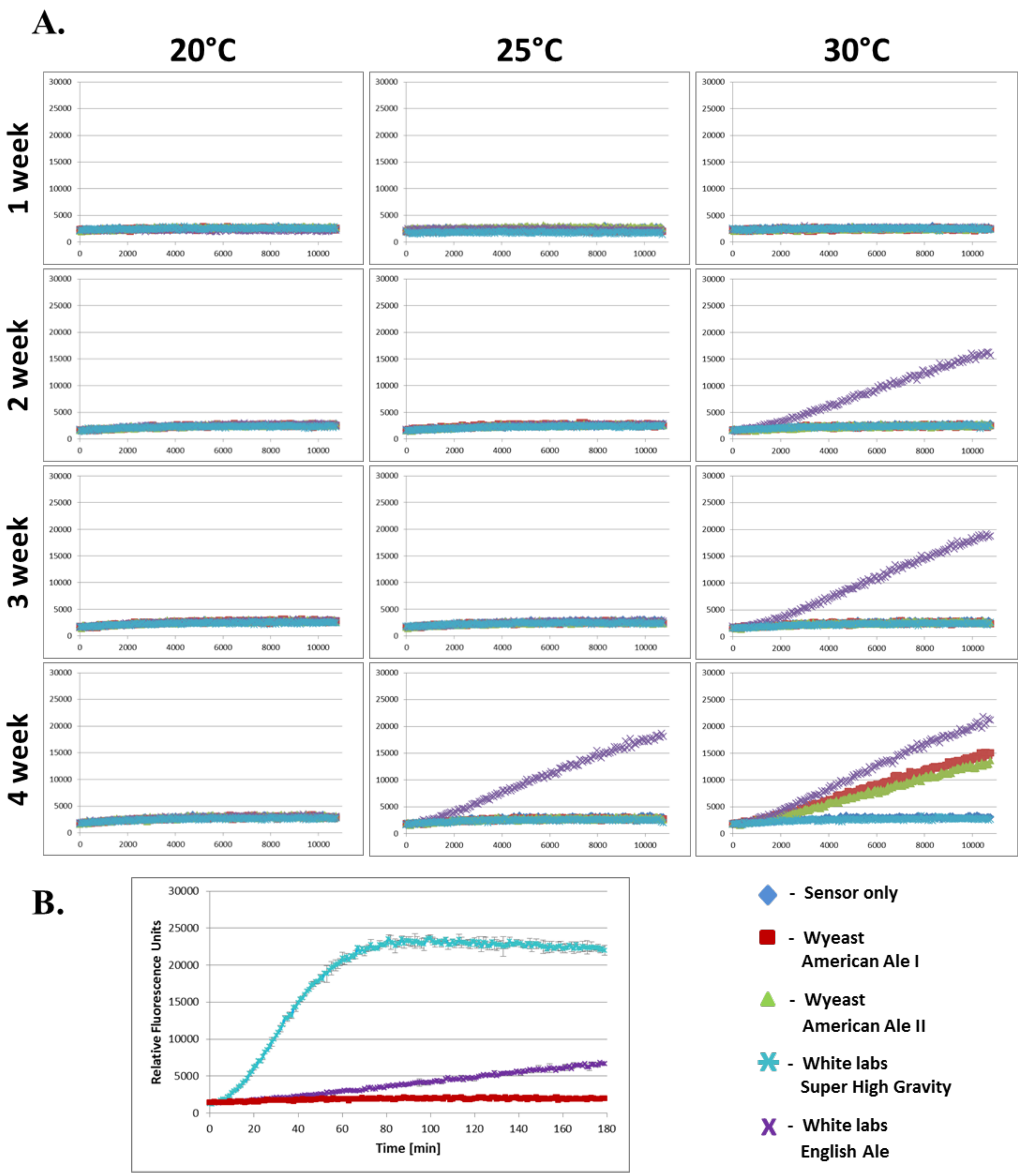

Figure 3: The effects of yeast strain and fermentation temperature on protease activity in beer. **(A.)** To standardize protease detection in beer, unhopped DME was used to make a 1.04 gravity wört which was inoculated with the indicated yeast strain. Measurement of protease activity is plotted as Relative Fluorescence Units vs. Time [sec]. Data is representative of at least 2 independent experiments. **(B.)** Protease activity in commercial yeast was tested by taking samples directly from the packaged starter packs. After clearing by centrifugation, protease activity was assessed. Error bars indicate the standard deviation of three experiments.

Discussion:

In summary, we have developed a novel technique to measure an established yeast by-product detrimental to beer. PrA can be secreted into beer during stressful conditions such as prolonged primary fermentation at elevated temperatures. Therefore, these results strongly advise against prolonged fermentation times, however the varying protease activity observed between different strains suggest that this is strain specific. These results also stress the importance of proper fermentation temperature control during fermentation as no protease could be detected in any strains fermented at 20°C.

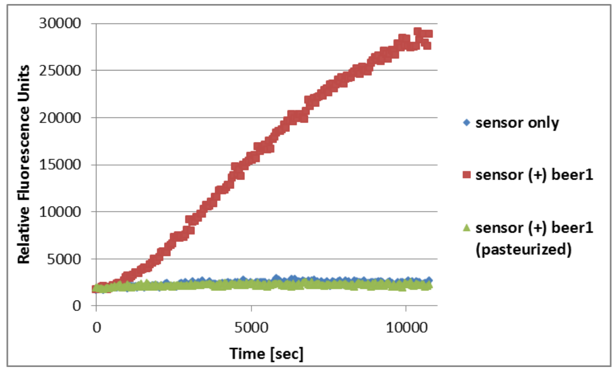

Figure 4: Pasteurization eliminates protease activity.
Unpasteurized or pasteurized beer1 (homebrew) was tested for protease activity.

In reviewing my homebrew notebook regarding the original beer 1 which was positive for protease (American Ale II IPA), I realized that this beer was fermented on a 90°F + summer week in an un-air-conditioned apartment. Therefore, my lack of fermentation temperature control likely led to the first detection of protease by our biosensor. It was with this in mind that I set up the simple fermentations at varying temperatures (Figure 3A) in order to re-capitulate my homebrew. And while I was able to detect protease activity in the experimental fermentations, these beers are simply unhopped DME-based brews. Additional experiments will be needed to test the effects of other parameters or ingredients on protease activity.

I was quite surprised to see high levels of protease activity in the commercial yeast samples. This suggests that the health of the yeast at the point of my assessment was sub-optimal. While I used yeast that were well within the "Best by" dates on the packaging, I have no knowledge of the conditions the packages were subjected to prior to their receipt, such as high temperature. One might worry that with high levels of protease present in commercial yeast designed for pitching directly into wört, transfer of the protease might be detrimental to the beer. However, when the Super High Gravity beer sample from Figure 3B was diluted 1:150 times in order to simulate the effects of pitching the vial volume into a 5 gallon wört, no protease activity could be detected (data not shown). This likely reflects the sensitivity of our biosensor, however, sufficient dilution of the protease may offset any negative effects. Regardless of the presence of protease in the commercial culture, by setting up a starter culture prior to fermentation, protease activity can be eliminated. For example, compare the significant amount of protease in the commercial Super High Gravity Ale strain (Figure 3B) to the results in Figure 3A. This protease-positive yeast sample was used to create a starter culture prior to primary fermentation, thereby eliminating protease activity.

While pasteurization is an effective technique for eliminating protease activity, most homebrewers should not attempt pasteurization in the absence of filtration. Pasteurization of unfiltered beer will cause yeast lysis and further increase the release of by-products into the beer. Based on these results, the best technique to avoid protease activity is proper fermentation temperature control.

Implications for homebrewers:

- Fermentation temperature control is the most practical way to avoid protease in homebrew.
- Strain selection may also be utilized where prolonged storage of beer on yeast is needed.
  - White Labs English Ale yeast seems particularly prone to protease release.
  - White Labs Super High Gravity Ale yeast may be a good strain for bottle or cask conditioning or for long term fermentation.
- If you filter your beer, pasteurization is the best option for preventing the negative effects of protease in your homebrew.
- Contaminating protease activity in commercial yeast products can be avoided by using starter cultures.

Acknowledgements:

As the principle investigator of my lab, Peter Berget set the foundation for this research through his development of the biosensor technology. A big thank you to him for supporting this research. It never hurts to be able to share the homebrew that generated the results! Funding for this research outside of brewing ingredients came from the University of the Sciences and an NIH National Technology Centers for Networks and Pathways (TCNP) grant U54 RR022241, P.I. Alan Waggoner.

I would like to acknowledge what I consider an amazing organization, this Research and Education Fund (REF). As federal research money continues to decline, it is public and private organization funding which will help drive research, education, and progress. The ability to share my research on this platform has been an exciting opportunity, and I hope others will do the same. Thank you to all of the AHA members involved in REF projects, especially D. Adam Lauver my REF liason.

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If you have any questions, comments, or concerns please email me at m.farber@usciences.edu

I also tweet nuggets of brewing science @HighBeerQ

I would be especially happy to hear from you if you are interested in collaborating on testing specific variables relevant to your brews. Or if you might need some PrA diagnostics done on your homebrew.

Cheers!

Matt