A Journey into the Hazy Beer Proteome: How Does Dry Hopping Alter the Proteomic Landscape of Beer?

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ABSTRACT

Purpose: For the past decade, brewers have been embracing a new twist on the classic India Pale Ale (IPA) beer style. The East Coast style IPA has exploded as a major contender in the worldwide craft beer scene. This style is characterized by its orange juice-like or "hazy" appearance (Figure 1). The haze is created to enhance aromas and impart a smooth mouthfeel while reducing the bitterness associated with conventional IPAs. The choice of yeast strain and hop variety are critical components. Certain yeast strains impart their own characteristic, fruity esters and other molecules suspended in the beer. In this study, high resolution, accurate mass (HRAM) mass spectrometry has been utilized in the evaluation of beer proteomics. We have evaluated the effects of a commercial yeast strain in combination with timed dry hopping on the proteome of hazy beer.

Methods: A label-free, relative quantitative proteomics methodology was used for this study. The Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer was utilized to analyze beer made from a single yeast strain (OYL-011, Omega Yeast), wheat, barley, and varying dry hopping addition times. A global proteomics approach allowed for the determination of proteins across the samples. In addition, the presence of hop-derived post-translational modifications targeted as a novel aspect of this study. Proteins from hazy beer samples were precipitated with cold acetone and incubated at -80C° overnight. Total proteins were quantified BCA assay. Proteins were digested with trypsin and desalted. For each ddMS2 run, 2ug of digest was loaded according to the peptide yields in order to facilitate equivalent amounts per analysis. Data was processed using Thermo Scientific™ Proteome Discoverer™ 2.5 software, which facilitated protein database searching, MS1 feature matching for label-free quantification, sample comparison and statistical analysis. In addition, software from Protein Metrics (Preview™ and Byonic™) was utilized orthogonally for interpretation of beer proteome digest complexity as well as searching for novel post-translational modifications.

Results: The proteomics of hazy beer based on varying hop addition times during fermentation are shown to generate unique protein expression patterns through label-free protein quantification. Data analysis confirmed the identification of 1430 proteins with high confidence. Data comparing proteins present in samples dry hopped at 168hr (maximal observed haze) vs 24hr show 51 proteins with a negative fold change and 88 proteins with a positive fold change. Finally, we have demonstrated evidence of polyphenolic PTM formation in proteins within the hazy beer proteome.

INTRODUCTION

The beer industry has been exploding with new trends and an urge to provide new experiences for beer drinkers. One major movement in the craft beer scene is the craze for the East Coast style IPA. These hazy or "juicy" IPAs are famous for their velvety smoothness that is imbued with a fruity, citrusy or even resiny character imparted by the choice of hops added by the brewer. Unlike bittering hops, which are added during or near the end of the boil, hops in hazy beers are added on what is called the "cold side". This occurs during and towards the end of fermentation, allowing the hops to add a flavor and character that is altogether different than what is achieved by bittering hops. This explosion of hazy beer popularity has also increased the demand for professional brewers as well as home brewers to develop the best hazy beer product on the market. In turn, this demand had driven the suppliers of yeast strains to step up production and start marketing new yeast strains. Omega Yeast is at the forefront of this movement and has been targeting yeast strains that produce beers extra hazy qualities for commercial purposes. They are also scientifically curious about what happens to the proteome of these new hazier beers. To date, very little is known from a proteomics and metabolomics standpoint as to what makes up the composition of the haze and if there are key components responsible for its characteristics. This project is a collaboration between Laura Burns and Lance Shaner at Omega Yeast, and Eric Tague at Thermo Fisher Scientific (Figure 2). To fully understand these hazy beers, Eric is analyzing from the same samples for metabolomics profiling (Poster MP072).

Figure 1 – The characteristic appearance of Hazy IPAs

Figure 2- Collaborators from Omega Yeast and Thermo Fisher Scientific

MATERIALS AND METHODS

Sample Preparation: For beer samples, a simple, wort consisting of 2 row pale (Rahr 2-row) malt with a target of 15° Plato was brewed. OYL-011 (haze positive yeast strain) was pitched at 10 million cells/mL and fermented at 70°F (21.1°C). Triple Perle hops were added at 8g/L (2lb/bbl) at 24, 48, 72, 96 and 168 hours into fermentation with an endpoint of 14 days. In addition, a control (no dry hops), knockout and double dry hop addition (DDH, ½ at 96 and ½ at 168 hours). There were 2 biological replicates created for each time point or treatment. Samples were collected upon completion and immediately frozen at -80°C until analysis. Protein precipitation was done with a 4x volume of cold acetone and held overnight at -80°C. Samples were centrifuged to pellet the precipitate and washed 3 times with additional acetone. Total proteins were quantified by BCA assay (Pierce™ Rapid Gold BCA Assay Kit). 100µg of protein per sample was digested and peptides were cleaned up using the Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit. The final yield of peptide post digestion was confirmed to facilitate equivalent peptide loading for mass spec analysis (Pierce™ Quantitative Colormetric Peptide Assay).

LC-MS Method: All samples were separated using Ionopticks™ AURORA™ Series nanoUHPLC columns (25cm x 75µm) and a Thermo Scientific™ Easy-nLC™ 1200 system. Column temperature was held at 45°C. Mobile phases consisted of [A] 0.1% formic acid in water, [B] 0.1% formic acid in 80% acetonitrile. The nanoLC gradient for separation is shown in Figure 3. In order to enhance sensitivity and offer further separation of ions in the gas phase, a Thermo Scientific™ FAIMS Pro™ interface was utilized with the nanospray source. For each analysis, 2µg of each digest was injected for analysis on a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer (Figure 4). In order to maximize proteome coverage, data was collected in high resolution full scan (240k) with data dependent MS2 configured for optimized, parallel ion processing in Orbitrap/Ion Trap Mode (Figure 5). Additionally, FAIMS CV voltages of -45V, -60V and -75V were applied the across three, experimental scans collected.

Figure 3- Gradient for nanoLC

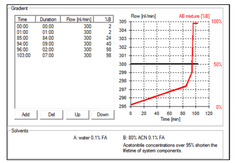
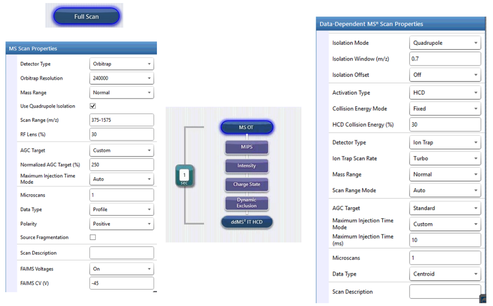


Figure 4- Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer

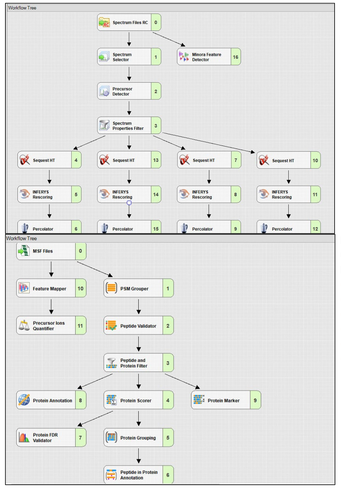
Figure 5- Mass Spectrometer settings for MS1 and Data Dependent MS2 analyses



PROCESSING METHODS

Data Analysis: Proteome Discover 2.5 software was used to process all data files. The proteomes of *Saccharomyces cerevisiae* (yeast), *Hordeum vulgare* (Barley), *Triticum aestivum* (Wheat) and *Humulus lupulus* (hops) were downloaded from UNIPROT and used for database searching. A branched configuration using indexed semi-tryptic proteome databases with Sequest™ HT INFERYS™ Rescoring and Percolator was used for database searching and peptide validation (Figure 6). MS1 features for label-free quantification were determined using Minora Feature Detector. In addition, software from Protein Metrics (Preview and Byonic) was utilized orthogonally for interpretation of beer proteome digest complexity as well as searching for novel post-translational modifications.

Figure 6- Proteome Discoverer 2.5 Workflows



Proteome Discoverer 2.5 Processing Workflow
Highlighted nodes
- Precursor Detector
- Spectrum Properties Filter
- Sequest HT
- INFERYS Rescoring (new)
- Percolator
- Minora Feature Detector

Proteome Discoverer 2.5 Consensus Workflow
Highlighted nodes
- Feature Detector
- Precursor Ions Quantifier
- PSM Grouper
- Peptide Validator
- Protein Scorer
- Protein FDR Validator

RESULTS

Our collaborators at Omega Yeast have determined through various fermentation trials that specific factors are critical for formation of a stable haze in beer. The strain specificity of the yeast as well as dry hop timing dramatically impact the degree of haze. Time dependent dry hopping and haze formation (pictures at 14 days fermentation) is illustrated in haze positive (OYL-011) and haze negative (OYL-004) yeast strains (Figure 7).

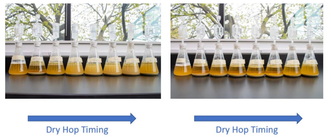


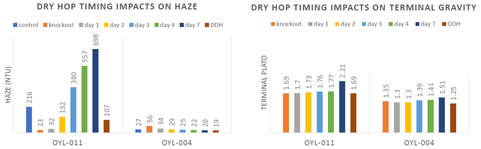
Figure 7- Haze formation in OYL-011 and OYL-004

Pictures at 14 days: (Left to Right)
Control (no dry hop)
Knockout
Day1
Day2
Day3
Day4
Day7
Double dry hop (Day1/7)

RESULTS (continued)

Visual appearance of haze as shown in Figure 7 was quantified via turbidity assessment using light scattering (nephelometric turbidity units, NTU) in dry hopped OYL-011 and OYL-004 fermentations (Figure 8). Terminal gravity measurement was also assessed in order to show completion of fermentation (Figure 8). The degree of haze was determined to be independent of terminal gravity.

Figure 8- Dry hop timing and yeast choice impacts haze independent on terminal gravity



Based on these fermentation trials, samples of OYL-011 with dry hop additions over the 1-to-7-day timing were assessed for protein identification and label-free quantification.

Rapid screening of digests to assess the completion of digestion and specificity of enzymatic cleavage using Preview revealed that digests were composed of greater than 50% semitryptic peptides.

Figure 9- Example of Protein Metrics Preview assessment of nonspecific cleavage.

Cleavage sites (C-side): RK
Missed cleavage: 6.3% (92/1459) of semitryptic peptides contain an internal K or R not followed by P
Semitryptic peptides (% of tryptic and semitryptic): 52.2% (761/1459) ragged-N, 7.5% (109/1459) ragged-C
Nontryptic peptides (% of all peptides): 9.9% (161/1619)

This unexpected observation is quite possibly due to the enzymatic processing of proteins during the various stages such as the malting of brewing grains, the mashing process and fermentation. Enzymes present may have led to the additional protein processing that was observed. To ensure that data analysis was done efficiently, semi tryptic FASTA files were pre-indexed for each proteome prior to Sequest HT database matching. In addition, the INFERYS Rescoring Node, a new node that is present in Proteome Discoverer 2.5 was utilized (Figure 6). It enables prediction of MS/MS spectra on-the-fly for peptides identified by Sequest HT using a Prosit-derived deep learning-based method. The predicted spectra are subsequently compared to the experimental spectra, providing additional figures-of-merit that Percolator uses for the FDR calculation.

Data analysis confirmed the identification of 1430 proteins with high confidence using the Processing and Consensus workflows shown in Figure 6. In addition, 18190 Peptide Groups and 240868 PSMs were observed across all samples. Proteins identified using the FAIMS Pro set at CVs of -45, -60 and -70V is shown in Figure 9. Each voltage setting correlated with overlapping as well as unique proteins identified. The distribution of the corresponding PSMs for each FAIMS Pro CV is highlighted in Figure 10.

Figure 9- Venn Diagram of Proteins and FAIMS Pro Compensation Voltages

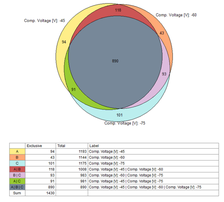
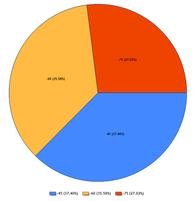


Figure 10- Pie Chart of PSMs and FAIMS Pro Compensation Voltages



RESULTS (continued)

Volcano plots were used to screen the data for proteins that show altered (>2x) and significant fold changes (<0.01). Due to the fact that haze was most prominent in the 168hr DH sample, a representative Volcano plot for this comparison is shown (Figure 13). Further examination showed that 47 unique proteins were identified only in the 168hr DH samples (Figure 14). An example of protein upregulation is shown for protein MMF1 (P40185) in Figure 15.

Figure 13- Volcano plot from the 168hr DH vs 24hr DH samples showing 51 proteins with a negative fold change (green), and 88 proteins with a positive fold change (red).

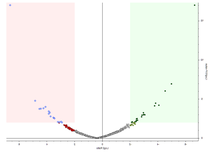


Figure 14-Distribution of proteins identified across samples (Control, 24hr DH and 168hr DH.

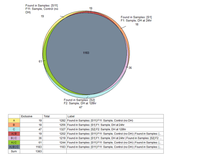
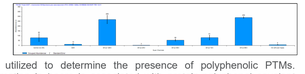


Figure 15- Examples of a protein showing time dependent upregulation due to dry hopping



Protein Metrics Byonic software was utilized to determine the presence of polyphenolic PTMs. Publications have shown that haze formation in beers is associated with protein-polyphenol covalent bonding.

Figure 16- Evidence of polyphenolic PTMs via Byonic search using Wildcard. Maximum mass of 400 was assessed on the168hr dry hopped samples to search for PTMs via polyphenolic compounds such as epicatechin (mw = 290.27).

CONCLUSIONS

This study on hazy beer has shown it is possible to use label-free quantification as a tool for understanding how the timing of dry hopping affects the complexity of the global beer proteome. We have compared five different dry hopping times, 24, 48, 72, 96 and 168 hours and determined that relevant changes can be determined in the beer proteome. Future work includes expanding this experiment to include more biological replicates for greater statistical power, comparison to haze negative strains and further elucidation of haze-related PTMs which have not been fully characterized in this study.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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