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The lowering of gushing potential from hydrophobin by the use of proteolytic enzymes

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Abstract

Why was the work done: Gushing caused by hydrophobin originating from field fungal contamination of grain remains a significant problem in certain regions. We sought to explore whether the use of added proteinase enzymes could overcome the problem and allow the use of problematic grain.

How was the work done: Four commercial proteinases were studied for their ability to digest hydrophobin and remove its foaming potential. One of those enzymes was then explored for its utility as an addition to mashing with a view to lowering the gushing potential of the ensuing beer.

What are the main findings: The four enzyme preparations (Smizyme LP-G, Thermoase PC, Bromelain and Papain) were all capable of digesting hydrophobin. Of these, papain was chosen to assess whether the use of such an enzyme in mashing could ameliorate the gushing potential in finished beers. It was demonstrated that this can be achieved and without apparent detriment to the foaming potential of those beers.

Why is the work important: It is now possible for brewers to consider an alternative approach to rectifying gushing risks if they are confronted with problematic grain. It must be stressed that this technique will not address the other main risk from infections of this type, namely the production of mycotoxins. Even in a context where gushing is not a problem, this work has indicated that there should be little concern with addition of papain at the mashing stage from a perspective of decreasing the foaming performance of beer.

Keywords

Gushing, hydrophobin, proteinase, papain, foam.

Introduction

In some beer markets, notably in Northern Europe, there continues to be a risk of gushing, i.e., the uncontrolled foaming of beer upon the opening of a can or bottle (Garbe et al. 2009; Shokribousjein et al. 2011; Lusk 2016). The primary reason for this is the presence of the low molecular weight protein hydrophobin, originating from the contamination of grain by *Fusarium* (Sarlin et al. 2005, 2007). This is a particular problem in high risk locations where barley is grown under damper and cooler conditions.

Substantial research has been reported on the science underpinning the causation of gushing by *Fusarium*. Quality Assurance approaches to avoiding the problem include the screening of grain for the presence of problematic mould (Garbe et al. 2009). As yet, there is no entirely reliable method for measuring hydrophobin, with ELISA-based procedures being at the forefront of consideration (Sarlin et al. 2005). Instead it has been customary to check for the presence of Fusarium by measuring deoxynivalenol, a toxin produced by this organism (Garbe et al. 2009). Its presence is taken as being indicative of increased risk of gushing if the grain is processed into beer.

Despite precautions, there still remain instances of the gushing of beer due to the presence of hydrophobin. The wisest course of action is to dump the problematic beer but there is an understandable reluctance to do this. Various suggestions have been made for ways to overcome the gushing tendency, including the use of PVPP and silica gel (Amaha and Kitabakate 1981; Garbe et al. 2009), xeorogel and isinglass (Leiper at al. 2002), gallotannin (Schneidereit et al. 2013) and foam-negative hop oils (Buffin and Campbell 2013).

We were drawn to the observations of Aastrup et al (1996) who showed that enzyme mixtures could be added to beer to reduce their tendency to gush. Here, we have explored the potential of four proteolytic enzyme products in reducing gushing induced by hydrophobin. Consideration has been given as to how this might be practically be achieved as it is more realistic to employ the enzymes in mashing rather than by addition to the finished beer. It is important to state that while

these approaches may prove a useful tool in preventing gushing in beer produced using mould-contmined grain, this palliative approach (like the others referred to above) will not obviate the food safety risks presented by contaminating organisms.

Materials and methods

Fungal strain

Fusarium graminearum (syn.*Gibberella zeae*) NBRC 7520 was from the National Institute of Technology and Evaluation (*www.nite.go.jp*). It was cultivated on a lactose growth medium (7 days) by a modification of the method of Khalesi et al (2013) (Figure 1).

Figure 1.

Growth of *Fusarium graminearum* (*Gibberella zeae*) NBRC 7520. The error bars show the range of values obtained in triplicate assays.
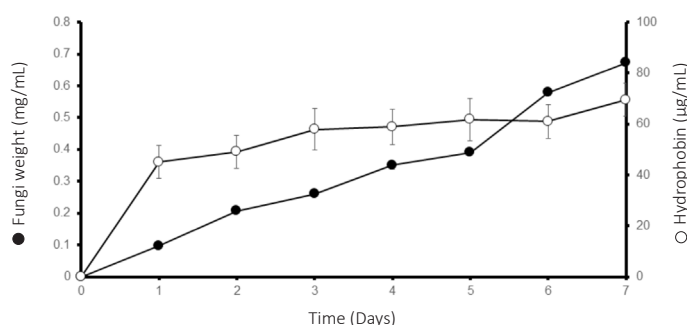
Extraction of hydrophobin

Hydrophobin was extracted from the mycelium by a modification of the method of Linder et al (2001). The cultivation medium (10 mL) was sampled at daily intervals. Samples were centrifuged (4800 x g for 10 min). Surfactant solution (5mL, Emulgen 1118S-70, Kao Corporation, Tokyo, Japan) was added to the precipitate and the mixture shaken for 1 h prior to adding acetate buffer (100 mM, pH 5.5, 1 mL) and isobutanol (1.5 mL). The mixture was re-centrifuged as above. Isobutanol was added to the supernatant and the procedure repeated twice to yield a final supernatant or 'crude hydrophobin'.

Assaying hydrophobin

The sample solution (100 μL) was mixed with 100 μL of 1mM TNS (6-p-Toluidino-2-naphthalenesulfonic acid) fluorescent probe dissolved in 10% DMSO

(Marker Gene Technologies, Eugene, Oregon, P/N M0704, acquired by Abcam, Inc. in 2020, P/N ab275049). The mixture was assayed employing a Nano Drop 3300 fluorescence spectrophotometer (Thermo Fisher Scientific) using excitation at 365 nm and emission at 465 nm.

Purification of hydrophobin

Crude hydrophobin solution was purified by column chromatography using ODS gel (Wakosil 100C18, Cat. # 234-02785, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) (Figure 2). ODS gel was packed in a column (27 mm × 200 mm) and elution was with successive 100 mL additions of 20, 30, 40, 50, 60, 70 and 80% acetonitrile. After chromatography, the eluent was removed by rotary evaporation. Each fraction was dissolved in 100mM acetate buffer (pH 5.5) containing 5% (v/v) ethanol.

Figure 2.

Purification of hydrophobin using reverse phase chromatography. In addition to the assay for hydrophobin, the polypeptide was quantified by its foam stability (Kapp and Bamforth 2002). The error bars show the range of values obtained in triplicate assays.
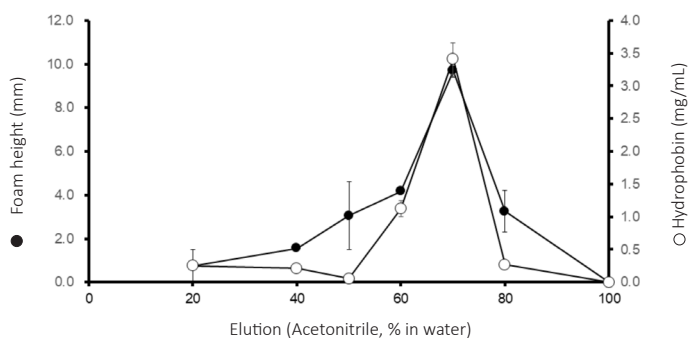
Measurement of foaming of hydrophobin

Evaluation of foaming was carried out according to the Kapp and Bamforth (2002) shake method. Samples (5 mL) were shaken by hand in screwcap culture tubes (15 cm long, internal diameter 1.5 cm) in a 40 cm arc, 10 times, within 3 seconds. The cap was removed immediately after shaking. The depth of the foam remaining after 30 min represents the foam stability. The assay was conducted in triplicate.

Digestion of hydrophobin by enzymes

Hydrophobin (1-5 mg) was dissolved in 1 mL of pH 5.5 acetate buffer (100 mM), and the solution (1 mL each) was poured into 1.5 mL plastic tubes. Enzyme solution (0.01 mL) containing either 1 mg/mL or 5 mg/mL protein in pH 5.5 acetate buffer (100mM) was added to the hydrophobin solution. Four different enzymes were employed: Smizyme LP-G (Shin Nihon Chemical Co., Ltd. Anjyo, Aichi), Thermoase PC (Amano Enzyme; Nagoya, Aichi), Bromelain (Fujifilm Wako Pure Chemical Corporation) and Papain (Fujifilm Wako Pure Chemical Corporation) (Table 1). The mixtures were incubated at 40°C for 2 hr.

Table 1.

Enzymes used in this work.

Enzyme	EC number	Organism	Activity
Smizyme LP-G	3.4.23.1/ 3.4.24.27	Aspergillus	Acidic proteinase plus neutral proteinase
Thermoase PC	3.4.21.62	Geobacillus	Serine endopeptidase
Bromelain	3.4.22.33	Bromelia	Cysteine endopeptidase
Papain	3.4.22.2	Papaya	Cysteine endopeptidase

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

Hydrophobin was analysed by electrophoresis using a modified method of Laemmli (1970). The sample was mixed with sample buffer (EzAppl, AE-1430; Atto Corp.) and heated in boiling water for 5 min. The running gel used e-PAGEL 16.5% (P-T16.5S; Atto Corp.) and Tris-tricine buffer (AE-1415 EzRunT). The protein was run at 20 mA for 75 min and stained using Ez Stain Aqua (#AE-1340; Atto Corp.) Molecular weight markers were Polypeptide SDS-PAGE Molecular Weight Standards (Bio-Rad, #161-0326)

Measurement of gushing

Gushing was assayed according to the method of Deckers et al (2012). Hydrophobin (1-5 mg) after various stages of proteolysis was added to bottles of cooled lager beer (4.7% ABV, 355mL at 2°C) and re-capped. The bottles were shaken horizontally at 150 rpm at 25°C for 3 days on a shaking incubator (BR-300LF, Taitec Co., Saitama, Japan). The weight of the bottle was measured. After standing for 10 min, the bottle was rotated vertically through 180º three times, with a stand of 10 seconds between each turn. After a further 30 seconds, the bottle was opened and when foam loss was complete, the bottles were re-weighed. The weight loss was calculated as the difference between the weight of the bottles before and after gushing.

Preparation of beer

All-malt beer was mashed according to the method of ASBC (1992). Finely ground malt (500 g, Pale ale malt, EBC 5-7, Weyermann, Bamberg, Germany), 500 mg of hydrophobin and 500 mg of papain were mixed with distilled water (2 L) at 45°C. Controls lacked hydrophobin and/or papain. The mixture was stirred at 45°C for 30 min prior to ramping at 1ºC/min to 70°C. A further 1 L of distilled water at 70ºC was added and the mash was held at 60 min. After cooling to room temperature, samples were filtered. Hop pellets (1.3 g Cascade, 6.9% Alpha from Brewland Co.Ltd, Aichi, Japan) were added to the wort, boiled for 70 min and clarified by filtration through sterilised paper. The wort was adjusted to 10° Plato by the addition of sterile water. Yeast (SafAle S-04, Fermentis) was precultured in 10°P wort at 30°C for 48 h and inoculated into the various worts described above. Filtered wort (approx. 2 L) was poured into a 3.2 L stainless pod (*https://www.monotaro.com/p/0673/5714/*) with a headspace of ca. 1 L. Yeast was added at 10 X 10^6 cells/ml wort with incubation at 15°C for 7 days. Yeast was removed by filter paper (No.2 Advantec, Tokyo Japan) in the presence of 20 g diatomaceous earth 045-00875, Fujifilm Wako Pure Chemical Co, Osaka, Japan) which was pre-washed in deionised water.

Bottle conditioning

Safale T-58 yeast (Fermentis) was pre-cultivated in malt extract (10°P) and added to beer at 1 x 10^6 cells/mL. Glucose (2.21 g) was added to the beer in 355 mL bottles targeting a final carbonation of 2.4 (v/v) CO_2. The capped bottles were held at 20°C for 3 days.

Results and discussion

Figure 1 illustrates the growth of *Fusarium graminearum*, together with the development of hydrophobin, which was purified using reverse phase column chromatography (Figure 2).

Hydrophobin was subjected to proteolysis by four different enzymes (Figure 3A). Proteolysis was also measured by the foam stability of the preparation (Figure 3B). Smizyme had the greatest impact on hydrophobin, while bromelain has the least capability to remove/modify hydrophobin. A lower dosage rate of enzyme is sufficient to exert a maximal effect. Furthermore, even when detectable hydrophobin remains, the foaming potential can be substantially removed, especially by Smizyme.

Figure 3A.

Removal of hydrophobin by the action of different proteinases. ● Smizyme LPG, ○Thermoase PC, ■ Bromelain, □ Papain. A1 is with 1 mg hydrophobin (1 mL) treated with 1 mg enzyme (0.1 mL) and A2 is 1 mg hydrophobin (1 mL) treated with 5 mg enzyme (0.1 mL). The error bars are for triplicate assays.

Figure 3B.

Action of four proteinases on the foam stability of hydrophobin solutions. ● Smizyme LPG, ○Thermoase PC, ■ Bromelain, □ Papain. B1 is with 1 mg hydrophobin (1 mL) treated with 1 mg enzyme (0.1 mL) and B2 is 1 mg hydrophobin (1 mL) treated with 5 mg enzyme (0.1 mL). The error bars are for triplicate assays.
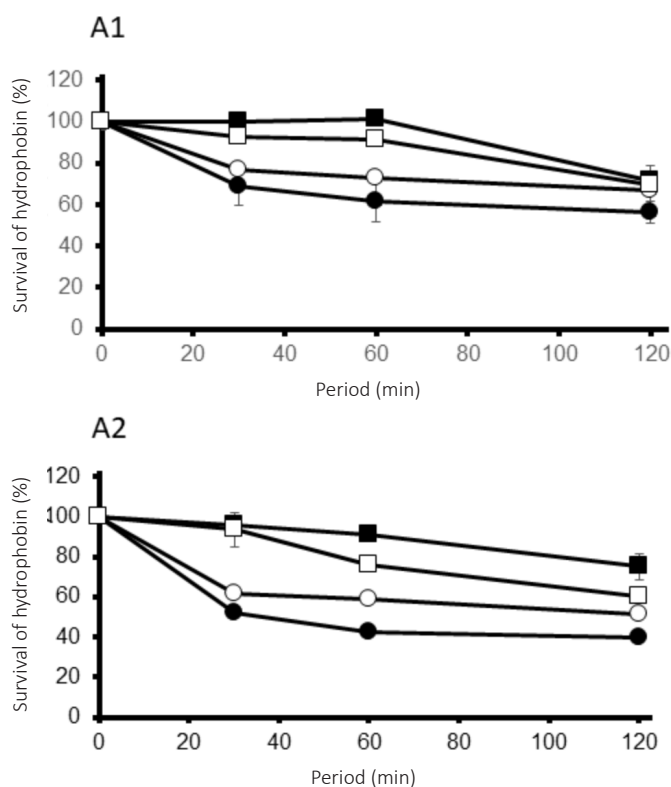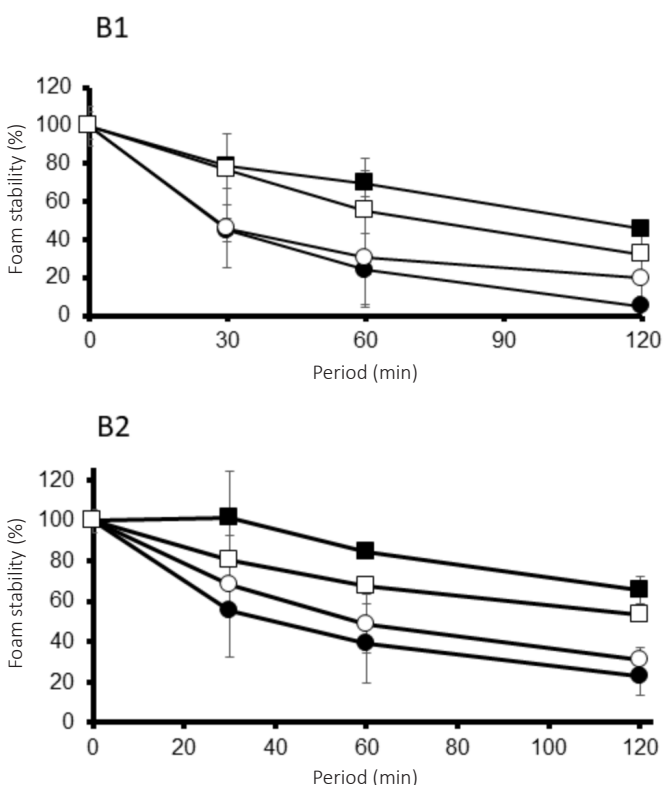

The four enzymes were compared for their ability to lower the ability of hydrophobin to promote the gushing of beer (Tables 2-5). All the enzymes reduced gushing, with Smizyme being the most impactful. Although Thermoase appeared to be more effective than papain in lowering the foam stabilising capabilities of hydrophobin solutions in acetate buffer (Figure 3), papain was superior in lowering the gushing potential of a commercial beer.

In view of its wide availability and familiarity, papain was selected as the enzyme of choice for addition at mashing. Papain has a broad pH range for activity, with 70% of maximal activity at pH 5.0 and 85% at pH 8.0 (Skelton 1968). With its most favourable temperature of 65°C (Lei et al. 2004) it was of interest if the gushing potential of hydrophobin in the finished beer could be overcome and if this could be achieved without deleterious impact on other aspects of beer quality, notably foaming (Table 6).

Table 2.

Gushing of beer containing hydrophobin treated by Smizyme LPG. Gushing as g/bottle.

hydrophobin (mg/mL)	Smizyme LPG (mg/mL)					
	0	1	2	3	4	5
1	43.1 (± 7.1)	45.9 (± 9.7)	43.2 (± 3.8)	36.3 (± 3.1)	31.4 (± 13.5)	5.01 (± 3.2)
2	46.1 (± 3.2)	37.3 (± 0.5)	43.1 (± 4.1)	28.8 (± 4.8)	20.6 (± 12.4)	9.80 (± 4.7)
3	60.5 (± 17.6)	43.9 (± 15.5)	32.1 (± 14.9)	20.4 (± 5.2)	19.7 (± 2.0)	17.6 (± 7.4)
4	67.9 (± 19.5)	45.5 (± 2.3)	30.6 (± 10.0)	17.23 (± 1.5)	11.6 (± 7.7)	17.2 (± 1.5)
5	75.6 (± 4.0)	42.6 (± 3.2)	22.3 (± 13.0)	12.1 (± 7.5)	10.9 (± 1.6)	9.10 (± 6.3)

Table 3.

Gushing of beer containing hydrophobin treated by Bromelain. Gushing as g/bottle.

hydrophobin (mg/mL)	Bromelain (mg/mL)					
	0	1	2	3	4	5
1	43.1 (± 7.1)	40.6 (± 0.4)	39.0 (± 11.3)	38.8 (± 2.4)	39.1 (± 15.6)	36.7 (± 12.3)
2	46.1 (± 3.2)	44.0 (± 14.0)	45.9 (± 5.6)	45.7 (± 13.5)	41.6 (± 15.1)	37.7 (± 11.3)
3	60.5 (± 17.6)	52.5 (± 5.9)	59.2 (± 2.0)	45.0 (± 15.7)	42.6 (± 2.9)	32.1 (± 2.4)
4	67.9 (± 19.5)	64.1 (± 8.6)	62.0 (± 5.2)	52.1 (± 4.9)	46.5 (± 5.0)	38.9 (± 1.8)
5	75.6 (± 4.0)	63.8 (± 9.5)	61.8 (± 10.0)	54.1 (± 0.8)	48.6 (± 3.4)	31.4 (± 2.9)

Table 4.

Gushing of beer containing hydrophobin treated by Thermoase PC. Gushing as g/bottle.

hydrophobin (mg/mL)	Thermoase PC (mg/mL)					
	0	1	2	3	4	5
1	43.1 (± 7.1)	46.5 (± 4.6)	42.5 (± 11.4)	42.1 (± 0.5)	31.4 (± 1.8)	31.4 (± 0.7)
2	46.1 (± 3.2)	48.5 (± 8.2)	40.8 (± 1.8)	50.9 (± 14.4)	30.8 (± 15.5)	32.4 (± 12.1)
3	60.5 (± 17.6)	47.9 (± 10.9)	55.2 (± 1.7)	52.6 (± 0.7)	56.7 (± 2.8)	30.9 (± 7.1)
4	67.9 (± 19.5)	66.1 (± 14.2)	60.7 (± 14.2)	58.9 (± 8.1)	53.6 (± 8.7)	39.5 (± 14.6)
5	75.6 (± 4.0)	66.3 (± 38.6)	69.0 (± 20.5)	57.4 (± 4.0)	58.0 (± 12.4)	39.7 (± 16.2)

Table 5.

Gushing of beer containing hydrophobin treated by Papain. Gushing as g/bottle.

hydrophobin (mg/mL)	Papain (mg/mL					
	0	1	2	3	4	5
1	43.1 (± 7.1)	34.7 (± 7.9)	28.2 (± 3.7)	24.1 (± 9.0)	23.1 (± 1.1)	10.9 (± 1.0)
2	46.1 (± 3.2)	32.7 (± 3.6)	28.0 (± 2.3)	27.9 (± 12.8)	20.1 (± 5.4)	11.3 (± 4.7)
3	60.5 (± 17.6)	34.1 (± 14.1)	30.3 (± 7.1)	28.6 (± 10.0)	20.9 (± 16.8)	14.1 (± 10.6)
4	67.9 (± 19.5)	47.9 (± 8.5)	44.1 (± 2.5)	28.8 (± 10.3)	26.0 (± 12.2)	13.7 (± 0.4)
5	75.6 (± 4.0)	53.2 (± 4.6)	52.9 (± 6.8)	25.6 (± 9.8)	24.6 (± 9.9)	17.3 (± 0.1)

Addition of hydrophobin impacts on the degree of attenuation but this was not consistently eliminated by papain. However, the foam stability of the beers was only marginally decreased by the addition of this enzyme at mashing. By contrast, papain largely eliminates gushing in the bottle conditioned beers. It is evident that the proteolytic enzymes native to the malt are not capable of dealing with the added hydrophobin under the mashing conditions used, although it cannot be ruled out that they were without any impact. It was previously shown by Aldred et al. (2021) that certain exogenous enzymes appear to have a more profound effect on proteolysis in mashing than do the endogenous endo-proteinases of malt. However, papain was the least effective of the proteinases in that study.

A particular concern is that the added proteinase should not damage the foam stability of the beer. Although it has been suggested that the use of papain as a colloidal stabiliser in beer is to the disadvantage of foam stability (Siebert and Lynn 1997), there are significant research reports to suggest that this and other enzymes improve the foaming potential of proteins. Thus, the foaming capacity of gluten (Mimouri et al. 1999; Jasim and Nasser 2020), chickpea protein (Goertzen et al. 2021), pea protein (Tang et al.2023), egg white (Lee and Chen 2002) and soy protein (Zeng et al. 2013) is enhanced by papain treatment and, in some cases, treatment with other proteinases additional to papain is still more efficacious. Kapp and Bamforth (2002) showed that foam stability from barley albumins is not damaged by papain, possibly as Lipid Transfer Protein (LTP1) is an inhibitor of cysteine proteinases such as papain (Jones 1997).

However, it was shown that papain treatment enhanced the foaming properties of hordein. As was demonstrated by Bamforth and Milani (2004), whilst hydrolysed hordein does have inherent foam stabilising ability, it is not as effective as albumins such as LTP1 and Protein Z in this regard. However, the hydrolysed hordein is better able to enter the foam. As a result, the net effect of hordein is to lessen the ability of the albumins to stabilise foam. Inspection of the data in Table 6 would suggest that the impact of the papain addition is limited. It must

	C1	C2	H1	H2	P1	P2	HP1	HP2
Material								
Hydrophobin (mg/100mL)	0	0	20	20	0	0	20	20
Papain (% of malt)	0	0	0	0	0.02	0.02	0.02	0.02
Wort								
Original Gravity (Plato)	10.5	10.6	10.3	10.4	10.3	10.9	10.1	10.0
IBU	25.9	24.8	24.9	23.1	26.1	23.6	23.8	24.8
FAN (mg/L)	133	140	154	175	164	164	170	147
Beer								
Final Gravity (Plato)	1.95	2.04	0.92	1.35	1.60	1.61	1.21	1.81
Alcohol (%)	4.15	4.19	5.10	4.82	4.62	4.13	4.82	4.27
Foam (mm)	33.4	29.9	28.1	36.2	29.0	28.0	29.5	30.1
Gushing (g/330mL)	5.9	4.9	168.8	172.2	2.3	2.3	7.2	14.9

Table 6.

The use of Papain during mashing

C1 and C2; control,
H1 and H2; hydrophobin in wort.

P1 and P2; papain in wort,
HP1 and HP2; hydrophobin and papain in wort.

be stressed that whilst we find no evidence that the addition of papain at the mashing stage is detrimental to foam, this is not to suggest that the traditional use of papain added to the finished beer is not without risk to head retention.

Conclusions

The gushing potential of hydrophobin is reduced by a series of proteolytic enzymes. Of those, papain was selected for further investigation, and it was demonstrated that that addition of papain at the mashing stage could be employed without deleterious impact on the foam stability of the resultant beer.

Author Contributions

Makoto Kanauchi: Conceptualisation, methodology, investigation, writing (review and editing).
Charles Bamforth: Conceptualisation, writing (original draft, editing and review).

Conflict of interest

The authors declare that there are no conflicts of interest.

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