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Role of Beer Lipid-Binding Proteins in Preventing Lipid Destabilization of Foam

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The negative effect of fatty acids on the foam stability of beer has been assessed. Long-chain fatty acids are far more damaging than short-chain fatty acids on the foam stability of beer at the concentrations employed. Polypeptides have been isolated from an all malt beer by hydrophobic interaction chromatography. Using this technique five groups of polypeptides were isolated, group 1 being the least hydrophobic and group 5 the most hydrophobic, all of which exhibited similar polypeptide compositions by SDS-PAGE. All five hydrophobic polypeptide groups bound [^{14}C]linoleic acid; however, group 5, the most hydrophobic group, bound the most linoleic acid. Groups 1 and 5 were titrated with *cis*-parinaric acid (CPA) to produce binding curves, which were compared with a binding curve obtained for bovine serum albumin (BSA). Groups 1 and 5 both produced binding curves that saturated at approximately 5.5 μM and 4 μM CPA and had association constants (K_a) of 6.27×10^7 and 1.62×10^7 M^{-1}, respectively. In comparison, BSA produced a binding curve that saturated at 6 μM CPA and had a K_a of 3.95×10^7 M^{-1}. Further investigation has shown that group 1 is pH sensitive and group 5 pH insensitive with respect to lipid binding. The lipid-binding activity of group 5 was also shown to be unaffected by ethanol concentration. Linoleic acid (5 μM) when added to beer resulted in unstable foam. Group 5 was added to the lipid-damaged beer and was shown to restore the foam stability to values that were obtained for the control beer. It has therefore been demonstrated that proteins isolated from beer have a lipid-binding capacity and that they can convey a degree of protection against lipid-induced foam destabilization.

KEYWORDS: Beer; foam; hydrophobic polypeptides; lipid; linoleic acid

INTRODUCTION

Foam and flavor stability are important considerations for a brewer as it is through these that the consumer judges the quality of the beer. However, these foaming and flavor properties are seriously damaged by lipids, which are present in beer, either due to the brewing process or they are introduced to the beer during consumption (*1*).

Food foams may be stabilized by proteins or low molecular weight surfactants, such as lipids, or by mixtures of both classes of molecules. These dispersions are stabilized by two incompatible mechanisms. Proteins form a condensed viscoelastic film of immobile interacting molecules in the interface, whereas surfactants stabilize foams by the Gibbs−Marangoni mechanism involving the minimal interaction of surface-adsorbed molecules and lateral diffusion at the interface (*2*). Destabilization of the foam occurs when there is competition between these two mechanisms. Thus, protein foams can be destabilized when small molecular weight surfactants are competitively adsorbed to the interface, which leads to instability as a result of the disruption of protein−protein interactions (*3*). Lipids, acting like low molecular weight surfactants, displace proteins from the interface, resulting in instability, which causes the foam to collapse. Studies on beer have shown that added lipid decreased foam stability, but following storage for 24 h the foam stability recovers (*4*). One explanation for these observations is that over time the lipid formed complexes with other compounds in the beer, reducing its "foam-negative" effect. The foam-negative effects of lipids can also be counteracted with the addition of an exogenous lipid-binding protein, wheat puroindoline (PIN), to beer (*5*, *6*). It is thought that the PIN acts by binding the residual free lipids in such a way that they can no longer destabilize the foam.

Lipid-binding proteins, such as nonspecific lipid transfer proteins (nsLTPs), have been identified in barley and beer (*7*−*10*) and are known to survive the brewing process (*11*). However, they do undergo marked chemical modifications, including disulfide bond reduction and rearrangement as well as glycation by Maillard reactions (*12*). It has also been shown that LTPs make an important contribution to the foam formation of beer (*10*, *13*, *14*). However, very little is known about whether the protein retains any lipid-binding capacity in the finished beer after the brewing process.

In view of the likely importance of lipid-binding proteins in determining the resistance of beer foam to lipid destabilization, we have characterized the lipid-binding capacity of beer

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polypeptides and investigated the role these proteins might play in the recovery of foam quality in lipid-damaged beer.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise stated, were of analytical grade from Sigma (Dorset, U.K.). An 11 °Plato beer was brewed by Brewing Research International (Surrey, U.K.) in their pilot brewery and was produced from 100% Optic barley malt. The finished beer was filtered, pasteurized, canned, and stored at 2 °C until required.

Fractionation of Beer. The protocol employed to isolate polypeptides from beer was adapted from the method used by Onishi and Proudlove (*15*). Degassed beer (2.5 L) was loaded onto an octyl-Sepharose CL-4B (Amersham Pharmacia Biotech) column (5 × 20 cm) attached to a GradiFrac chromatography system (Amersham Pharmacia Biotech). The column was pre-equilibrated with water. After application of the beer, the column was washed with water until all unbound polypeptides were eluted. The column was subsequently washed with ethylene glycol (500 mL/L) to elute the very strongly hydrophobic polypeptides (group 4) and with 6 M urea to elute the extremely hydrophobic polypeptides (group 5). The group 5 polypeptides were immediately diluted with water to give a urea concentration of 1−1.5 M. The unbound fraction, eluted with water, was adjusted to an ammonium sulfate concentration of 85 g/L with no visible precipitation and reapplied to the column, which had been pre-equilibrated with ammonium sulfate (85 g/L). The column was then washed with ammonium sulfate solution (85 g/L) to elute unbound polypeptides (group 1), with water to elute moderately hydrophobic polypeptides (group 2), and with ethylene glycol (500 mL/L) to elute the strongly hydrophobic polypeptides (group 3). All groups were dialyzed and concentrated on a Vivaflow 200 tangential flow filter (3 kDa molecular weight cutoff) and lyophilized. The groups were stored at −20 °C until required.

SDS-PAGE. Tris/Tricine SDS gel electrophoresis, using the discontinuous buffer system, was performed under reducing conditions according to the method of Schagger and Von Jagow (*16*).

Fatty Acid Binding Assay: Radiolabeled [^{14}C]Linoleic Acid Assay. Labeled linoleic acid (Amersham Pharmacia Bioscience) was dried under nitrogen gas and redissolved in absolute ethanol so that the stock solution had a concentration of 1.8 mM linoleic acid (1-^{14}C specific activity of 2.04 GBq/mmol). The stock solution was further diluted so that an activity of 0.74 kBq was added to isolated beer polypeptides. Linoleic acid (2 μL, 0.74 kBq activity) was added to the isolated beer polypeptides (98 μL), which had been redissolved in 0.1 M citric/citrate buffer, pH 3.5−4.5, mixed, and incubated for 5 min, to give a total volume of 100 μL. An aliquot (10 μL) was taken, 10 mL of scintillant (Zinsser Analytic) was added, and total counts were determined using a Packard Tri-Carb 2700 TR liquid scintillation analyzer, from which the initial linoleic acid concentration was calculated. The remaining sample (90 μL) was applied to an NAP-5 column (Pharmacia Biotech), which had been previously equilibrated with 0.1 M citric/citrate buffer, and protein-bound [^{14}C]linoleic acid was eluted and counted. Samples were assayed in duplicate and the average was calculated; duplicates were within 5−10%.

Fatty Acid Binding Assay: Fluorescent Lipid *cis*-Parinaric Acid (CPA). The CPA method employed was a variation of that used by Di Pietro and Santomé (*17*). CPA was purchased from Molecular Probes, and a stock solution of 30 mM was prepared in absolute ethanol, the concentration of CPA being determined spectrophotometrically using an extinction coefficient of $\epsilon_{308\ nm} = 7.9 \times 10^4\ M^{-1}\ cm^{-1}$. For the production of binding curves 0.2 mg/mL of each of group 5, group 1, and BSA in 0.1 M citric/citrate buffer, pH 4.0 (2.5 mL), was titrated by the addition of 5 μL aliquots of CPA. The excitation and emission wavelengths were 308 and 417 nm, respectively, with excitation and emission slit widths of 5 and 10 nm, respectively. Measurements were carried out in triplicate and the average was taken. Affinity constants (K_a) were calculated using Graphpad Prism 3.0.

Foam Stability. Foam stability was assessed by using the microconductivity technique (*18*). The foaming apparatus consisted of a column with built-in electrodes and an orifice at the base through which nitrogen was sparged. Beer (5 mL) was introduced into the column, so that the electrodes were completely immersed. The conductivity of the beer was then measured using a Radiometer Copenhagen CDM83 conductivity meter connected to the built-in electrodes in the foam column. The sample was removed completely, and 2 mL of beer was reintroduced into the column. Nitrogen gas, presaturated with water, was forced through the gas jet, which possessed a single orifice (15 μm diameter), to create the foam. The gas supply was switched off when the foam reached a predefined height (30 mm). The foam conductivity was measured and recorded for a period of 5 min. Lipid-damaged beer was prepared by adding degassed beer (9.4 mL) slowly to linoleic acid (0−10 μM), which had been dissolved in 0.1 mL of ethanol. This was followed by 0.5 mL of hydrophobic polypeptide groups dissolved in water to give a concentration of 0.4 mg/mL. Control measurements were made using a sample containing degassed beer (9.4 mL), ethanol (0.1 mL), and water (0.5 mL). All samples were analyzed in triplicate and the average was calculated.

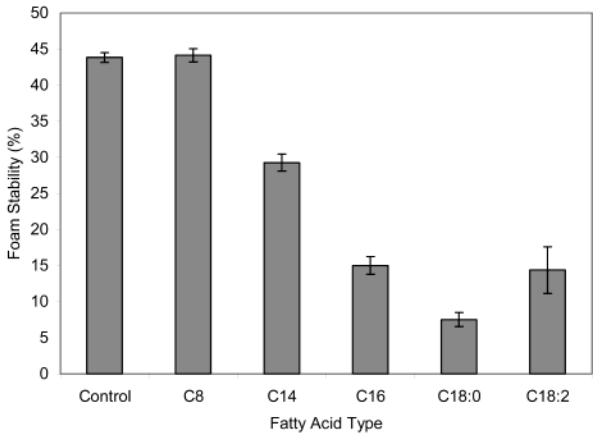

Figure 1. Effect of fatty acid type on the foam stability of beer. Foam stability of beer alone (control) or beer challenged with 5 μM fatty acid was measured for 5 min. The foam stability after 5 min was plotted.

RESULTS

Fatty Acid Chain Length and Its Effect on Foam Stability. Experiments with individual lipids (1 ppm) have shown the order of efficiency in destroying head retention to be dipalmitin, palmitic acid, and monopalmitin (*4*). Furthermore, it has been shown that short-chain fatty acids (C_6−C_{12}), typically produced during fermentation, when added to beer can affect foam stability, but they are much less harmful than the longer chain acids found in wort (*19*).

Initially, studies were performed to determine which was the most appropriate fatty acid to employ to produce a "lipid-damaged" beer. Beer was challenged with 5 μM of various fatty acids (C_8, C_{14}, C_{16}, $C_{18:0}$, and $C_{18:2}$) and the effect on foam stability measured (**Figure 1**). These results show that octanoic acid (C_8) had very little effect on foam stability, whereas an 80% reduction in foam stability was caused by stearic acid ($C_{18:0}$). The addition of linoleic acid ($C_{18:2}$) resulted in 65% reduction in foam stability, a significant foam collapse but not the complete foam collapse that occurred with stearic acid (**Figure 1**). These data suggest that saturated long-chain fatty acids have a more negative effect on foam stability than unsaturated fatty acids. Linoleic acid was therefore selected as the fatty acid that would be employed to simulate lipid-damaged beer and to develop methods to identify relevant lipid-binding proteins in beer.

Lipid-Binding Activity of Beer Polypeptides. SDS-PAGE revealed that the molecular weight distribution among the five isolated hydrophobic groups was very similar and essentially as previously described (*15*). A distinct band of molecular

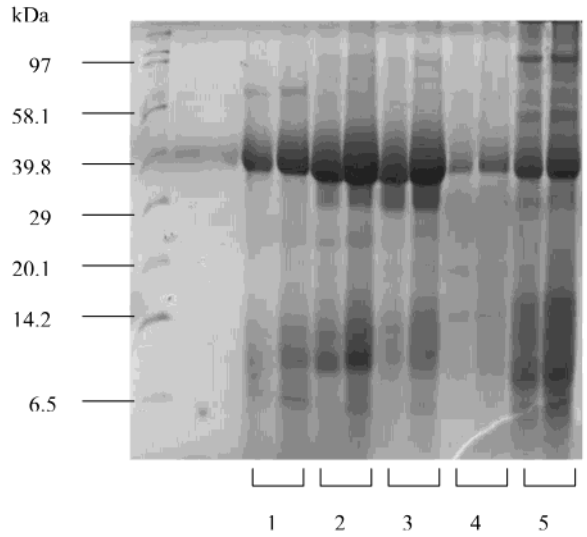

Figure 2. SDS-PAGE of octyl-Sepharose-factionated hydrophobic polypeptides (groups 1–5) isolated from beer: (lanes 1) group 1; (lanes 2) group 2; (lanes 3) group 3; (lanes 4) group 4; (lanes 5) group 5.

Table 1. Lipid-Binding Activity of Octyl-Sepharose-Fractionated Beer[a]

hydrophobic polypeptide group	proportion of beer protein (%)	protein content (%) of hydrophobic groups[a]	linoleic acid binding (nmol/mg of protein)	% of total beer lipid-binding activity
beer	100		0.183	100
1	50	8	0.043	32
2	12	24	0.013	10
3	12	44	0.0097	7
4	10	63	0.0086	6
5	16	61	0.061	45

[a] The protein content of hydrophobic groups isolated from beer and distribution in beer were as previously reported (*15*). Protein content of hydrophobic groups is expressed as percent of dry mass.

weight 40 kDa was apparent with broad smeared bands of molecular weight 8 and 20 kDa. However, group 5 contained a high molecular weight band, >100 kDa, not observed in groups 1−4 (**Figure 2**). Analysis of the protein concentration of the lyophilized hydrophobic groups showed that group 1 contained low levels of protein (~8%) and must therefore be made up of other material such as nonstarch polysaccharides, whereas group 5 contained 61% protein (**Table 1**).

The contribution of each isolated group to the total binding activity of beer was assessed and the distribution of the five hydrophobic groups from beer calculated on the basis of previous studies (*15*). Despite having a low protein concentration group 1 made up 50% of the protein content of beer, whereas group 5 made up only 16% (**Table 1**). The lipid-binding capacity of lyophilized beer was determined using the radiolabeled [^{14}C]linoleic acid assay. The beer used in this study was able to bind 0.18 nmol/mg of protein (**Table 1**). Following fractionation it was found that groups 1 and 5 had the greatest lipid-binding activity, binding 0.043 and 0.061 nmol/mg of protein, respectively, equivalent to 32 and 45% of the lipid-binding activity of the beer. However, the sum of the lipid-binding activities of the octyl-Sepharose fractions was less than that obtained for the lyophilized beer, that is, 0.14 nmol/mg of protein compared to 0.18 nmol/mg of protein. This demonstrates that the fractionation procedure had resulted in a 27% loss of lipid binding activity.

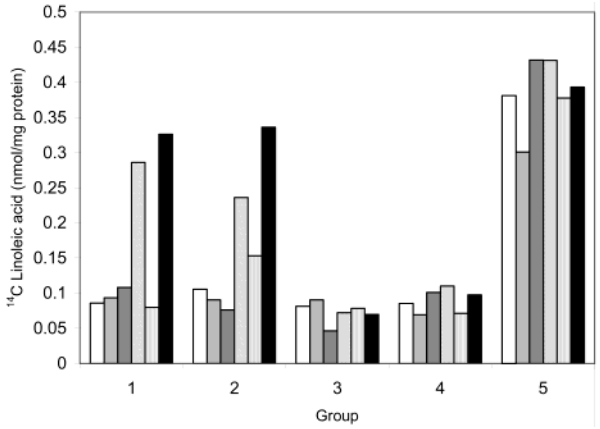

Figure 3. Effect of pH on the lipid-binding activity of hydrophobic polypeptide groups (1–5) isolated from beer. Groups 1–5 were dissolved in 0.1 M citric/citrate buffer. Bars represent, from left to right in each grouping, pH 3.5, 3.7, 3.9, 4.1, 4.3, and 4.5.

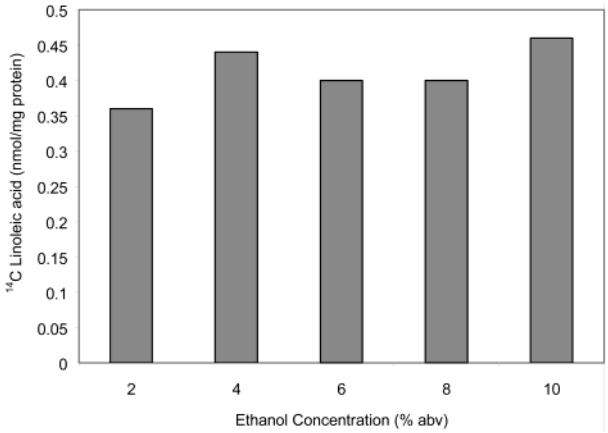

Figure 4. Effect of ethanol concentration on the lipid-binding activity of group 5.

Beer pH and ethanol concentration vary widely and could significantly affect the lipid-binding activity of proteins in beer; therefore, the effect of these parameters was investigated. The lipid-binding capacity of the five groups was assessed at pH 3.5−4.5. Group 5 exhibited greater lipid-binding activity than the other isolated groups but did not respond to the change in pH, binding 0.39 and 0.38 nmol of linoleic/mg of protein at pH 4.5 and 3.5, respectively (**Figure 3**). However, groups 1 and 2 had increased lipid-binding activity at pH 4.5 compared with 3.5; for example, group 1 bound 0.33 nmol/mg of protein at pH 4.5 but only 0.09 nmol/mg of protein at pH 3.5. These results demonstrate that pH does have an effect on the lipid binding of group 1 but not on that of group 5.

As group 5 had a high lipid-binding activity, this fraction was used for all further characterization at pH 4.0. The effect of ethanol (2−10% v/v) on lipid binding was assessed (**Figure 4**), but no clear trend emerged, with the increase in ethanol concentration having only a slight effect on linoleic acid binding. For example, group 5 bound high levels of linoleic acid, ~0.46 and 0.44 nmol/mg of protein, at ethanol concentrations of 10 and 4% (v/v), respectively.

A fluorescent lipid assay, employing CPA, complimentary to the [^{14}C]linoleic acid assay, was used to confirm the lipid-binding activity of the beer fractions as the radiolabeled assay may give anomalous results. For example, a positive result may be obtained by protein binding in a nonspecific manner to lipid aggregates, or a negative result may be found if the lipid

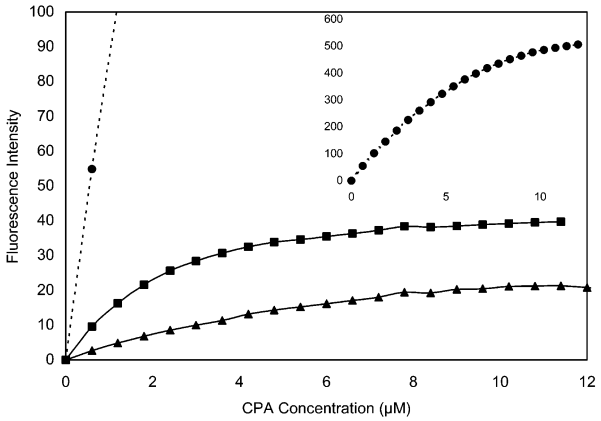

Figure 5. CPA binding activity to group 1 (▲), group 5 (■), and BSA (●). (Inset) CPA binding curve for BSA.

Table 2. Association Constants, K_a ($\times 10^7$ M^{-1}), for CPA Binding to Groups 1 and 5 Isolated from Beer

pH	group 1	group 5	BSA
3.5	2.89	2.02	
3.7	4.21	1.84	
3.9	3.67	1.47	
4.1	6.27	1.62	3.95
4.3	7.29	1.79	
4.5	13.40	2.32	

aggregates and adheres to the glass tubes or the NAP-10 column used in the assay. This is illustrated by the apparently anomalous reduction in binding of [^{14}C]linoleic acid assay at pH 4.3 for groups 2 and 3 (**Figure 3**). CPA is a naturally occurring fatty acid that fluoresces poorly in an aqueous environment, but when bound to a hydrophobic site it is an excellent fluorophore. The CPA assay was used to compare the lipid-binding capacities of groups 5 and 1 with the fatty acid binding capacity of bovine serum albumin (BSA). Group 5 produced a low fluorescent response and a shallow binding curve that saturated at ~4 μM (**Figure 5**). Group 1 also produced a very shallow binding curve and was saturated with CPA at ~5.5 μM. However, BSA produced a far greater fluorescence response and a far steeper initial slope than either group 1 or 5 and became saturated at ~6 μM CPA. **Table 2** shows the affinity constants (K_a) calculated from these data. For example, at pH 4.1 the K_a for group 5 was calculated to be 1.62×10^7 M^{-1} compared with 6.27×10^7 M^{-1} for group 1 and 3.95×10^7 M^{-1} for BSA. The K_a calculated for BSA in this study was found to be comparable with previously published data (*20*, *21*).

Although group 5 showed little sensitivity to pH (**Figure 6A**), the lipid-binding capacity of group 1 increased with increasing pH (**Figure 6B**). Thus, CPA titration curves were superimposable for group 5 between pH 3.7−4.5, increasing slightly at pH 3.5 (**Figure 6A**). However, for group 1 a significant alteration in lipid binding was seen when the pH was raised from 3.5 to 4.5 with the K_a changing from 2.89×10^7 to 13.40×10^7 M^{-1} across this pH range (**Table 2**). This showed a gradual change in lipid binding with pH, unlike the [^{14}C]linoleic acid assay, but clearly confirmed the pH-dependent nature of group 1 lipid-binding activity.

Effect of Beer Lipid-Binding Proteins on Lipid-Damaged Beer Foam. Various concentrations of linoleic acid (2−10 μM) were added to beer, and the effect on foam stability was measured for a period of 5 min. The destabilization of beer foam is shown in **Figure 7**, where the final foam stability, after 5 min, is plotted as a function of linoleic acid concentration. The

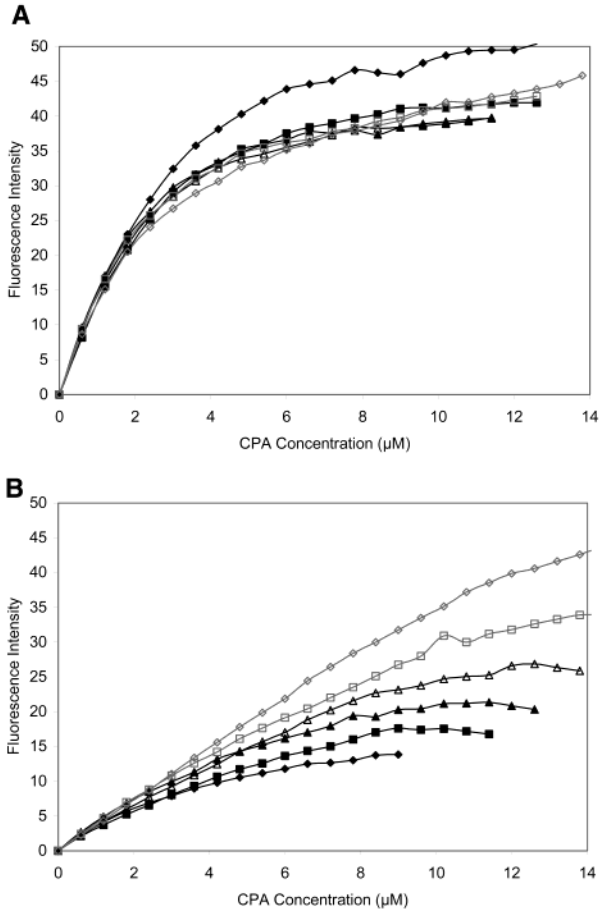

Figure 6. (A) Effect of pH on the lipid-binding activity of group 5: pH 3.5 (◆), pH 3.7 (■), pH 3.9 (▲), pH 4.1 (△), pH 4.3 (□), and pH 4.5 (◇). (B) Effect of pH on the CPA binding activity of group 1: pH 3.5 (◆), pH 3.7 (■), pH 3.9 (▲), pH 4.1 (△), pH 4.3 (□), and pH 4.5 (◇).

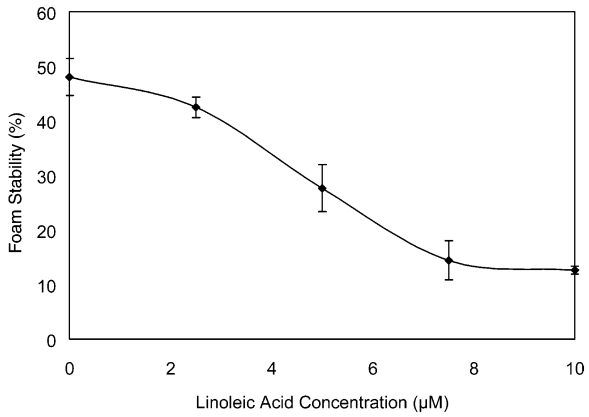

Figure 7. Effect of linoleic acid on the foam stability of beer. The foam collapse was measured for 5 min and the final foam stability plotted.

presence of 10 μM linoleic acid destabilized the beer foam so that it completely collapsed, whereas the addition of 5 μM linoleic acid caused significant, but not complete, collapse of the beer foam and was therefore used to produce lipid-damaged beer for future experiments (**Figure 7**).

Group 5 (0.4 mg/mL mass, 0.24 mg/mL protein) was added to beer, and the effect on foam stability was measured for a period of 5 min. The foam decay curves presented show that the presence of group 5, when added to a control beer, resulted in a small enhancement of the foam stability of the beer (**Figure 8A**). However, when added to lipid-damaged beer, group 5 had

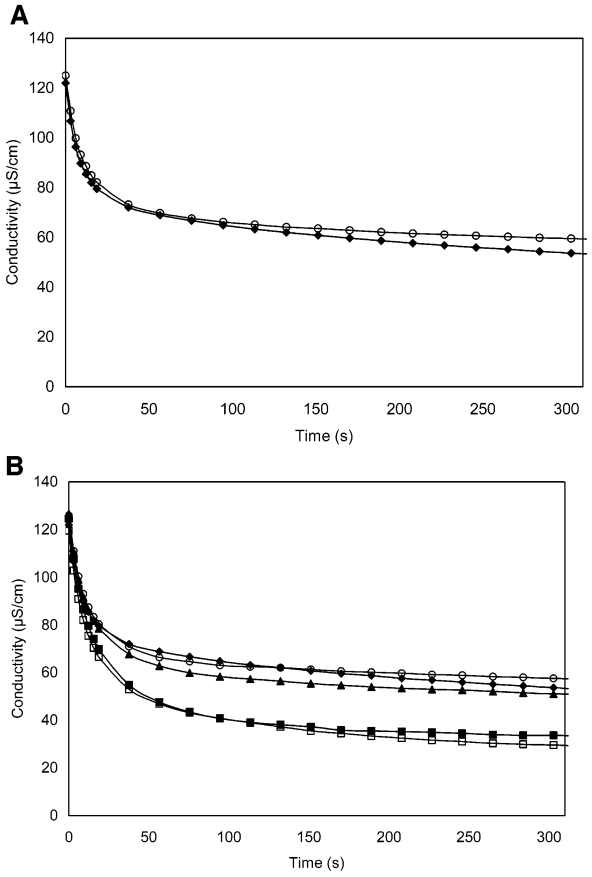

Figure 8. (A) Effect of group 5 on the foam stability of beer. Foam collapse, measured by the fall in conductivity (μS/cm) with time, is shown. Foam conductivity decay curves of degassed control beer (◆) and with the addition of 0.4 mg/mL group 5 (○) are also shown. (B) Effect of group 5 on the foam stability of lipid-damaged beer. Foam decay curves of degassed beer (◆), degassed beer with 5 μM linoleic acid (□), and degassed beer with 5 μM linoleic acid plus 0.4 mg/mL (○), 0.2 mg/mL (▲), and 0.1 mg/mL (■) of group 5 are shown.

a significant effect, restoring the foam stability of the beer to the levels achieved prior to the addition of the linoleic acid (**Figure 8B**). The dose−response effect of adding group 5, at 0.1, 0.2, and 0.4 mg/mL to lipid-damaged beer was investigated (**Figure 8B**). The results show that the addition of increasing amounts of group 5 had an increased effect on restoring the foam stability.

DISCUSSION

This study has confirmed previous observations that fatty acids are damaging to foam, with long-chain fatty acids having a more negative effect than the short-chain fatty acids at the concentration that was employed (*19*). These results indicate that saturated fatty acids have a more negative effect on foam stability than one of the major long-chain unsaturated fatty acids found in beer, linoleic acid. Further work is in progress to elucidate the effect of the various short- and long-chain (including saturated and unsaturated) fatty acids on foam stability.

Using [^{14}C]linoleic acid, we have shown for the first time that polypeptides isolated from beer can bind lipid and have affinity constants of a similar magnitude to BSA, that is, $\sim 10^7$ M^{-1}. When the hydrophobic groups were analyzed for linoleic acid binding potential, group 5, the most hydrophobic group, was found to bind the most linoleic acid with the highest affinity. It has also been shown that groups 1 and 5 contribute the greatest lipid-binding capacity when protein concentration and relative distribution of the hydrophobic groups in beer are taken into account. For example, when the lyophilized samples were analyzed, it was found that group 1 contained only 8% protein but made up 50% of the protein content of beer, whereas group 5 contained 63% protein but made up only 16% of the protein content of beer. It can therefore be seen that group 5 is enriched with lipid-binding proteins compared to the other hydrophobic groups isolated from beer. However, as group 1 contributes 50% of the protein from beer, its impact on lipid-binding activity in beer cannot be discounted.

The pH can affect binding of ligands, such as lipids, to proteins by altering the hydrophobic, electrostatic, and hydrogen bonding as well as the formation of salt bridges via divalent metals such as calcium, involved in the binding reaction (*22*). The pH of beer varies from 3.5 to 4.5 and therefore could play an important role in determining the lipid-binding potential of proteins in beer. Results from the CPA assay have shown that the lipid-binding activity of group 5 was not affected by pH. However, group 1 did respond to the change in pH with an increased lipid-binding response to increasing pH. This suggests that the proteins that bind lipid in group 1 are different from those proteins that bind lipid in group 5. Further work is required to establish why groups 1 and 5 respond differently to changes in pH.

The ability of group 5 to prevent lipid-induced destabilization of foam has also been clearly demonstrated. Results from the CPA binding curves have shown that 0.2 mg/mL of group 5 containing 0.12 mg/mL protein becomes saturated at $\sim$4 μM CPA. Therefore, if sufficient protein was added to the beer, all of the lipid should be "mopped up" by group 5 and unable to damage the foam. The addition of 0.4 and 0.2 mg/mL of group 5 (0.24 and 0.12 mg/mL protein, respectively) had a significant effect in restoring the foam stability of beer that had been damaged by the addition of 5 μM linoleic acid. However, 0.1 mg/mL (0.06 mg/mL protein) had no effect, demonstrating that there was not enough lipid-binding protein present to mop up sufficient lipid to prevent destabilization of the foam.

These observations are similar to that made when purified wheat puroindoline was added to lipid-damaged beer (*5*). However, the concentration of protein added in that study was significantly lower, 0.02 mg/mL puroindoline compared to 0.4 mg/mL of group 5, containing 0.24 mg/mL protein. Results have shown that group 5, although the strongest lipid-binding group isolated from beer, is made up of a number of protein species, and it may be that the lipid-binding protein present in this fraction is present at a very low concentration. Further fractionation of groups 5 and 1 will be required to determine the nature and origin of the lipid-binding proteins in beer and the molecular basis for the differences in their pH sensitivities. This work will also take into account the interactions with other beer components, such as hop acids, which may affect foam stability.

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