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Haze-active protein and turbidity in commercial barley and wheat beers at different storage temperatures

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Abstract

The objective of the present work was to investigate the haze-active (HA) protein and its relationship with the turbidity in commercial clear barley beer (BB) and cloudy wheat beer (WB) stored at 0 - 20°C for seven days. It was found that the maximum turbidity occurred at 0 or 5°C in samples, and the turbidity had a significantly negative correlation with temperature. Hence, it was recommended to store BB at 10 or 15°C to avoid the haze formation while WB at 0 or 5°C to promote a stable and high turbidity value. Furthermore, HA protein was extracted by silica, whose relative molecular weight (Mr) was determined by HPSEC and divided into four fractions. Mr of HA protein in BB was higher than that in WB in each fraction. For the correlation of turbidity and different fractions of HA protein, there were similarities between BB and WB. The content of low Mr fractions consisting of fraction II (8.34 - 13.92 kDa) in BB or fraction I&II (< 7 kDa) in WB had positive influences on turbidity, while high Mr portion including fraction III (38.08 - 45.91 kDa) in BB or fraction IV (59.28 kDa) in WB showed negative effects on turbidity.

Introduction

Beer is one of the most popular drinks all over the world, and the colloidal stability of beer has become a critical issue for transportation and warehousing. Generally, the haze formation may be promoted when beer undergoes unavoidable long distance transportation or is exposed to the complex temperature environment (Speers *et al.,* 2003; He *et al.,* 2012). Consequently, it is necessary to identify the correlation between temperature and turbidity of beer, and the influence of different fractions in HA protein to avoid the stability problems during storage.

Haze is an important appearance characteristic reflecting the quality of beer. Therefore, turbidity is very important for brewers, because it is the first visual quality indication of beer to consumers (Iimure *et al.,* 2012). In the market, most BBs are crystal clear with turbidities less than 2 EBC (European Brewery Convention), since haze is unacceptable by consumers (Steiner *et al.,* 2010). However, most of WBs are turbid and their turbidities are usually over 2 EBC. In addition, the quality characteristics of the cloudy wheat beer include both the observed intensity and stability of the haze, which are accepted by the consumers. Cloudy WB has a homogeneous, intense, and stable haze, which the consumers desire (Delvaux *et al.,* 2000). There are many factors affecting beer haze. For beer itself, many substances in beer can cause turbidity, such as dextrin (Cai *et al.,* 2016), β-glucan (Speers *et al.,* 2003), protein, polyphenols, hop resin, and yeast (Steiner *et al.,* 2010). Previous studies established that the major compounds of haze was from the specific combination of protein and polyphenols (Asano *et al.,* 1982; Siebert *et al.,* 1996). Some beer proteins were considered to have a critical impact on the haze; Barley dimer alpha-amylase inhibitor-1, barley trypsin inhibitor, hordeins (Colgrave *et al.,* 2012; Iimure et al., 2012; Konečná *et al.,* 2012; Picariello *et al.,* 2012), and protein Z (Curioni *et al.,* 1995; Evans *et al.,* 2003) were all considered to cause the haze in BB. Schulte *et al.* (2016) demonstrated that in the haze proteome, relative abundance of protein Z4 and lipid transfer protein 1 was more than the other detectable protein. However, a protein-polyphenol haze formation model system was built by gliadins (alcohol-soluble wheat gluten) and the haze-active polyphenols in WB (Siebert *et al.,* 1996). Delvaux *et al.* (2003) found that wheat gluten proteins and polyphenols formed the haze or precipitate, which was dependent on the gluten concentration.

For external reasons, temperature (He *et al.,* 2012) could also affect the haze of WB in multiple ways (Delvaux *et al.,* 2000). On the one hand, lowering the temperature could reduce solubility of the marginal soluble materials and produce a higher level of particles. This led to the phenomenon known as "chill haze" or "reversible haze" (Steiner *et al.,* 2010) presented at around 0°C. Normally, heating the sample would eliminate

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most of the turbidity caused by chilling. On the other hand, elevated temperatures could accelerate the interaction of substances forming the insoluble particles, resulting in a faster haze development (Siebert, 2009). Haze existing in both warm and cold temperatures was known as 'permanent haze' (Speers *et al.,* 2003).

The main objective of the present work was to invetsigate the haze-active protein and its influence on the turbidity of beers at different storage temperatures. Here, we analysed the correlation of the turbidities and physiochemical indicators of beers; and the correlation of different fractions of HA protein with the turbidities were also analysed to reveal the effect of HA protein on the turbidity.

Materials and methods

Materials and reagents

Ten BBs and ten WBs were purchased from e-commerce platforms (JD and Tmall, China). These beers were placed in 0, 5, 10, 15, and 20°C incubators for seven days except for the controls. Standard protein molecular weight marker was from 6.5 to 158 kDa (GE Healthcare Gel Filtration Cal Kit, UK). Silica gel powder with micro-aperture of average 14.0 nm and diameter of 10 μm were purchased from Stabifix (Germany). All other chemicals were of analytical grade.

Analysis of basic indicators

Ethanol content, real concentration, and original concentration were analysed by a Beer Analysing System (Alcolyzer Plus+DMA4500 Density Meter, Anton Paar, Austria). Protein content was determined by Kjeldahl method (Speed Digester K-425 and Distillation Unit K-350, Buchi, Swiss). The nitrogen protein conversion factor was 6.25.

Turbidity measurement

The turbidity measurement was referred to ASBC and EBC analysis methods (ASBC Beer 26 Formazin Turbidity Standards, EBC 9.29 Haze in Beer: Calibration of Haze Meters). To determine the initial turbidity of each beer sample, 200 mL of degassed beer was added to a 250 mL beaker and incubated at 20°C for 30 min. The beer samples stored for seven days were degassed on the eve of the test and taken from 0, 5, 10, 15, and 20°C incubators before testing. Unfiltered beer sample was poured into a test bottle, and a calibrated turbidity meter was used to monitor the turbidity (WGZ-4000, Xinrui, China). The measurements were performed in triplicate.

HA protein extraction

The extraction of HA protein was performed following the methods of Apperson *et al.* (2002) and Siebert and Lynn (2007). Briefly, 0.2 g of dry silica gel was added into 500 mL of degassed beer to mix well by stirring at 4°C for 1 h. Then it was centrifuged at 2,740 *g* for 10 min at 4°C (TGL-20Br centrifuge, Anting, China). The precipitate was collected after decanting the supernatant, and then 5 mL of 0.1 mol/L NaOH was added to release the HA protein from the silica. The mixture was stirred and then centrifuged at 7,012 *g* for 10 min. This step was repeated, and the supernatant was recovered and made up to 50 mL with ultra-pure water. Subsequently, 22.5 g of ammonium sulphate was added to the recovered solution and stirred for 1 h, and the solution was then centrifuged at 7,012 *g* for 7 min to decant the supernatant. Next, 20 mL of ultra-pure water was added to the remaining precipitate and shaken until the protein was reconstituted in the solution. The protein was purified by dialysis, then recovered by freeze-drying, and frozen at -20°C for further analysis.

HA protein molecular weight determination by HPSEC

Analytical method was carried out according to Xie *et al.* (2014). HPSEC was calibrated by markers. A LC-20AT system with a SPD-20AT detector (Shimadzu Kyoto, Japan) was connected to the column of TSK gel Super SW2000. The composition of mobile phase was 80% (v/v) phosphate buffer solution (0.2 M, pH 7.0) containing 0.15 mol/L NaCl and 20% (v/v) acetonitrile. The flow rate was 0.2 mL/min, the analytical time was 50 min, the column oven temperature was kept at 25°C, and the detection wavelength was set at 214 nm (Silva *et al.,* 2008). Markers and HA protein samples were dissolved in the mobile phase and filtered by 0.45 μm filter membrane (Pall, USA). Filtrate was collected in the sample bottle and the injection volume was 20 μL. GPC software was used for the chromatographic analysis.

Statistical analysis

Data were processed by the statistical software SPSS (IBM, USA). The difference at $p < 0.05$ was considered significant. Correlation analysis was performed by Pearson's correlation double-tailed test, and the *p*-values under 0.05 and 0.01 were considered significantly correlative.

Result and discussion

Basic indicators of BBs and WBs

The basic indicators of beers are shown in Table 1. The range of ethanol content, real concentration, and original concentration in BBs were 4.59 - 5.62,

Table 1. Basic indicators of ten barley beers and ten wheat beers.

	Barley beer						Wheat beer				
No.	Ethanol content (v/v, %)	Real concentration (m/m, %)	Original concentration (Plato, °P)	Protein content (g/L)	Turbidity (EBC)	No.	Ethanol content (v/v, %)	Real concentration (m/m, %)	Original concentration (Plato, °P)	Protein content (g/L)	Turbidity (EBC)
1	4.64 ± 0.01^{g}	3.58 ± 0.00^{e}	10.64 ± 0.02^{i}	2.41 ± 0.12^{bc}	0.09 ± 0.00^{h}	1	4.58 ± 0.00^{g}	3.77 ± 0.00^{g}	10.76 ± 0.01^{g}	3.75 ± 0.29^{bcd}	3.4 ± 0.10^{j}
2	4.98 ± 0.01^{d}	3.58 ± 0.00^{e}	11.13 ± 0.01^{g}	1.70 ± 0.16^{d}	0.33 ± 0.04^{e}	2	4.24 ± 0.00^{i}	3.97 ± 0.01^{f}	12.17 ± 0.00^{b}	2.97 ± 0.03^{d}	16.0 ± 0.20^{e}
3	5.02 ± 0.01^{d}	3.19 ± 0.02^{g}	10.80 ± 0.00^{h}	0.99 ± 0.07^{e}	0.02 ± 0.00^{j}	3	5.08 ± 0.00^{b}	3.81 ± 0.02^{g}	11.51 ± 0.01^{e}	4.56 ± 0.46^{bc}	14.5 ± 0.00^{f}
4	5.33 ± 0.01^{b}	3.69 ± 0.02^{d}	11.81 ± 0.00^{b}	2.58 ± 0.27^{bc}	0.05 ± 0.00^{i}	4	5.05 ± 0.01^{c}	4.02 ± 0.01^{e}	11.70 ± 0.00^{c}	4.12 ± 0.06^{bcd}	32.5 ± 0.00^{a}
5	5.08 ± 0.01^{c}	3.71 ± 0.01^{d}	11.44 ± 0.00^{c}	2.77 ± 0.27^{b}	1.30 ± 0.00^{b}	5	5.48 ± 0.01^{a}	4.08 ± 0.01^{d}	12.37 ± 0.01^{a}	5.03 ± 0.52^{b}	3.9 ± 0.00^{i}
6	4.59 ± 0.01^{h}	3.51 ± 0.00^{f}	10.54 ± 0.00^{j}	3.79 ± 0.18^{a}	0.28 ± 0.03^{f}	6	4.72 ± 0.00^{f}	4.13 ± 0.01^{c}	11.32 ± 0.00^{f}	8.92 ± 0.53^{a}	7.0 ± 0.00^{h}
7	4.92 ± 0.02^{e}	3.84 ± 0.01^{c}	11.31 ± 0.00^{e}	2.64 ± 0.04^{bc}	0.47 ± 0.06^{c}	7	4.11 ± 0.01^{j}	4.48 ± 0.02^{a}	12.38 ± 0.01^{a}	4.15 ± 0.40^{bcd}	7.2 ± 0.10^{g}
8	4.99 ± 0.02^{d}	3.80 ± 0.01^{c}	11.39 ± 0.01^{d}	2.58 ± 0.04^{bc}	0.24 ± 0.00^{g}	8	4.84 ± 0.01^{e}	4.31 ± 0.00^{b}	11.64 ± 0.02^{d}	3.57 ± 0.12^{cd}	24.2 ± 0.10^{b}
9	5.62 ± 0.00^{a}	3.87 ± 0.01^{b}	12.36 ± 0.01^{a}	3.14 ± 0.13^{b}	0.45 ± 0.06^{d}	9	4.41 ± 0.00^{h}	3.50 ± 0.00^{h}	10.22 ± 0.02^{h}	3.80 ± 0.04^{bcd}	17.9 ± 0.00^{d}
10	4.79 ± 0.01^{f}	3.96 ± 0.02^{a}	11.23 ± 0.02^{f}	2.14 ± 0.09^{cd}	1.54 ± 0.09^{a}	10	4.99 ± 0.00^{d}	3.98 ± 0.01^{f}	11.50 ± 0.03^{e}	4.07 ± 0.35^{bcd}	21.6 ± 0.10^{c}

Different lowercase letters within columns indicate significant differences ($p < 0.05$).

3.19 - 3.96, and 10.54 - 12.36%, respectively. The protein content was 0.99 - 3.79 g/L which was mainly distributed in the range of 2 - 3 g/L. The turbidity in all samples was below 2 EBC, which ranged from 0.02 to 1.54 EBC. Similarly, the ethanol content, real concentration, and original concentration in WBs (Table 1) were 4.11 - 5.48, 3.50 - 4.48, and 10.22 - 12.37%, respectively. The protein content was 2.97 - 8.92 g/L, which was higher than that of BBs. Meanwhile, the difference analysis showed that the protein content was more concentrated (3 - 5 g/L). The turbidities of all WBs were above 2 EBC. The maximum turbidity was 32.5 EBC in sample 4, while the minimum value was 3.4 EBC in sample 1.

Turbidity changes of 10 BBs and 10 WBs at 0 - 20°C storage

The turbidities of 10 BBs following seven-day storage at 0 - 20°C are shown in Figure 1. The turbidities of 10 BBs at 15 and 20°C were below 2 EBC. Especially, the turbidities of samples 1, 3, and 4 at 0 - 20°C were all below 0.2 EBC, and the highest value appeared at 5°C. The maximum value over 2 EBC occurred at 0°C in samples 2, 6, 7, 8, 9, and 10. In sample 5, the turbidity increased from 2.47 EBC to the maximum 7.51 EBC when the storage temperature decreased from 10 to 5°C. However, this maximum value dropped to 2.68 EBC when the temperature decreased to 0°C. The maximum change was found in sample 10, and the initial turbidity was 1.54 EBC (Table 1). Following storage at 20 and 15°C, the value was 1.55 and 1.66 EBC ($p > 0.05$), respectively, no big change was found. However, the maximum change appeared after the storage temperature decreased from 5 to 0°C when the turbidity rose dramatically to 21.68 from 7.71 EBC.

As presented in Figure 1, all turbidities of WB were far greater than 2 EBC, and the turbidities in all samples stored at 20°C did not change much in comparison to the initial turbidities. Seven WBs, including samples 1, 2, 3, 5, 7, 8, and 9, showed maximum turbidities at 5°C. But their turbidities at 5 and 10°C were significantly different ($p < 0.05$), except sample 5, whose turbidities at 5 and 10°C were 11.91 and 11.36 EBC ($p > 0.05$), respectively. The highest turbidity occurred in samples 4, 6, and 10 at 0°C. The maximum change appeared in sample 10 whose turbidity rose from 21.86 EBC to the maximum 49.49 EBC when the temperature decreased from 20 to 10°C. Meanwhile, samples 9, whose turbidity rose slightly from 15.19 to 20.11 EBC, had the minimal change in turbidity after the storage temperature decreased from 15 to 5°C. In comparison to the initial turbidity of this sample, the turbidities at 0 - 20°C only showed small fluctuations.

In accordance with Figure 1, WBs showed that the maximum turbidity of beer also appeared at 0 or 5°C, and the higher the initial turbidity, the higher the turbidity after storage. In addition, it was reasonable to accept that the haze of WB was larger than that of BB because of different materials and brewing methods, as well as temperatures and filtration that might have a far-reaching influence on the turbidity (Delvaux *et al.,* 2000). Besides, the turbidities of most samples stored at 10, 15, and 20°C were lower than those at 0 and 5°C, and the difference between the above two temperature intervals was significant ($p < 0.05$). It was found that storage temperature was an important factor leading to this phenomenon. Formation and precipitation of insoluble complexes were promoted by cold storage. At the same time, the lower temperature reduced the solubility of some potential haze materials (Siebert, 2009). Therefore, the present work recommends that BB should be stored and transported at 10 or 15°C to avoid the risk of forming chill haze and rapid haze formation at high temperatures. For safety, energy conservation and avoiding the potential freezing, WB should be stored at 0 or 5°C to obtain a stable and high turbidity value.

Correlation between turbidity and storage temperature of beers

As shown in Figure 1, it could be inferred that the turbidities were related to the storage temperature. Therefore, the correlation analysis of storage temperatures (0 - 20°C) and their corresponding turbidities in beers was analysed by Pearson's r correlation double-tailed test. The turbidity of beer had a negative correlation ($p < 0.05$) with storage temperature, and the absolute value of Pearson's r (-0.419, $p < 0.01$) of BB was higher than that (Pearson's r = -0.355, $p < 0.05$) of WB. It could be concluded that the turbidity of BB showed more correlation with storage temperature than that of WB.

Correlation of turbidities and the basic indicators

As shown in Table 2, the real concentration significantly correlated with the turbidities of the beers stored at 5, 10, and 15°C ($p < 0.05$), and showed extremely significant correlation ($p < 0.01$) with the turbidities of beers stored at 0 and 20°C. While other indicators had no correlation with the turbidities of BBs stored at 0 - 20°C. In WB, all indicators in Table 2 had no correlation with the turbidities at 0 - 20°C.

By correlation analysis, the basic indicators of beers were not simple factors affecting beer turbidity directly. Actually, beer is a colloidal solution with complex composition and low stability; thus, proper storage temperature would promote the haze or precipitation in beers during storage.

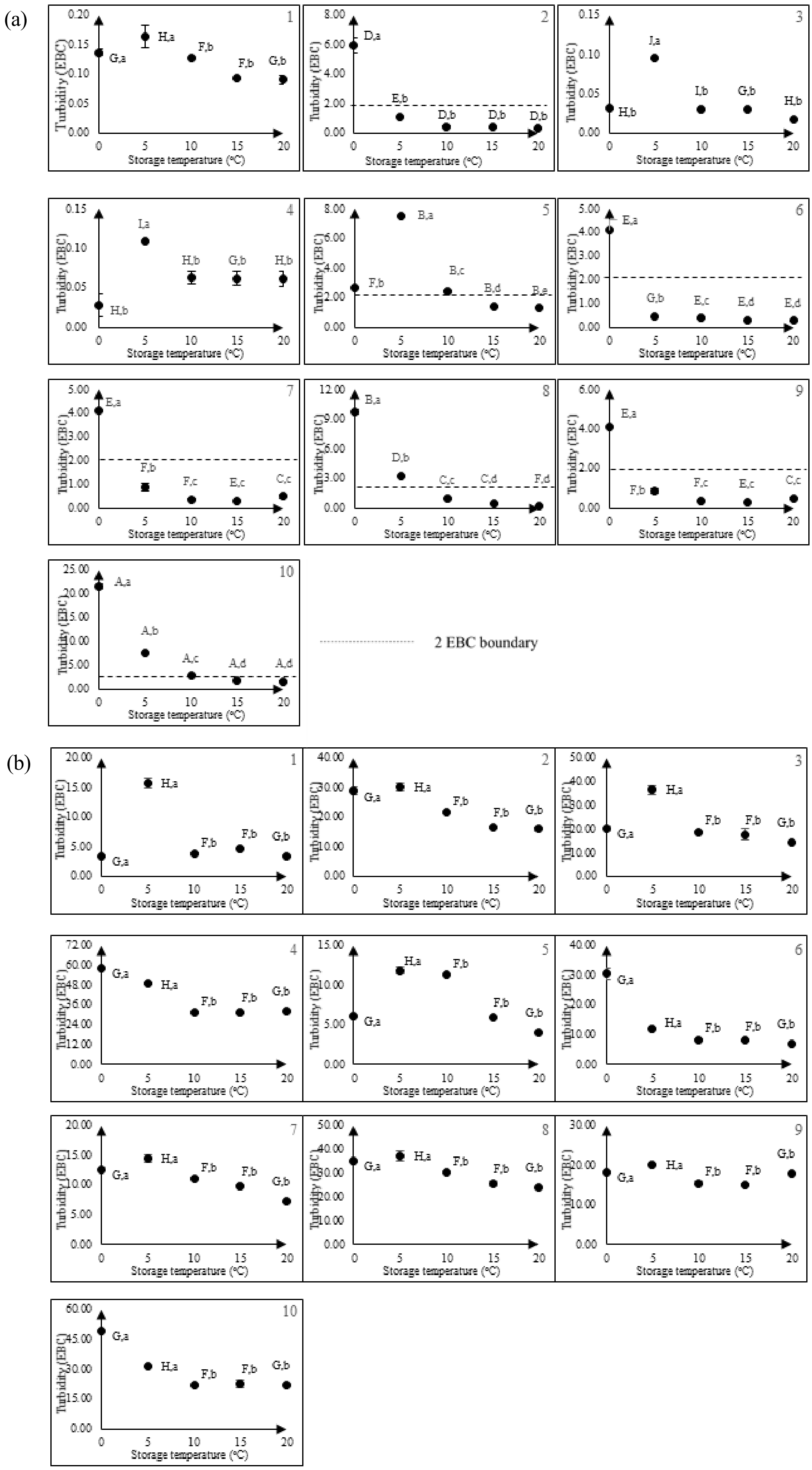

Figure 1. Turbidities of barley beers (a) and wheat beers (b) at 0 - 20°C following seven days storage. Different lowercase letters indicate significant differences ($p < 0.05$) in the same sample; different uppercase letters indicate significant differences ($p < 0.05$) at the same temperature.

Table 2. Correlation analysis of the basic indicators and turbidities of 20 beers stored at different temperatures for seven days.

Turbidity	Temperature	Pearson's correlation (*r*)			
		Ethanol content	Original concentration	Real concentration	Protein content
BB	0°C	-0.216	0.053	0.596**	-0.028
	5°C	-0.092	0.123	0.492*	0.014
	10°C	-0.133	0.092	0.499*	0.047
	15°C	-0.130	0.100	0.516*	0.057
	20°C	-0.059	0.179	0.571**	0.105
WB	0°C	0.193	0.107	0.051	0.005
	5°C	0.244	-0.057	0.058	-0.424
	10°C	0.220	0.124	0.189	-0.405
	15°C	0.186	0.031	0.030	-0.360
	20°C	0.140	-0.072	-0.080	-0.363

* = Correlation is significant at the 0.05 level (two-tailed); and ** = Correlation is significant at the 0.01 level (two-tailed).

Relative molecular weight of HA protein in beers stored at different temperatures

As shown in Table 3, the Mr of HA protein in BB were divided into four fractions according to HPSEC chromatographic profile, and named as I, II, III, and IV, respectively (Figure 2). Among them, fraction I (4.03 - 4.80 kDa) and II (8.34 - 13.92 kDa) were low molecular weight (LMW, < 15 kDa); III was 38.08 - 45.91 kDa, mostly belonging to high molecular weight (HMW, > 40 kDa); and IV (86.46 - 119.22 kDa) was HMW. The relative content of component I, II, III, and IV in all samples was 5.62 - 15.18, 40.31 - 56.39, 18.48 - 33.63, and 7.99 - 29.31%, respectively. The mean content of fraction

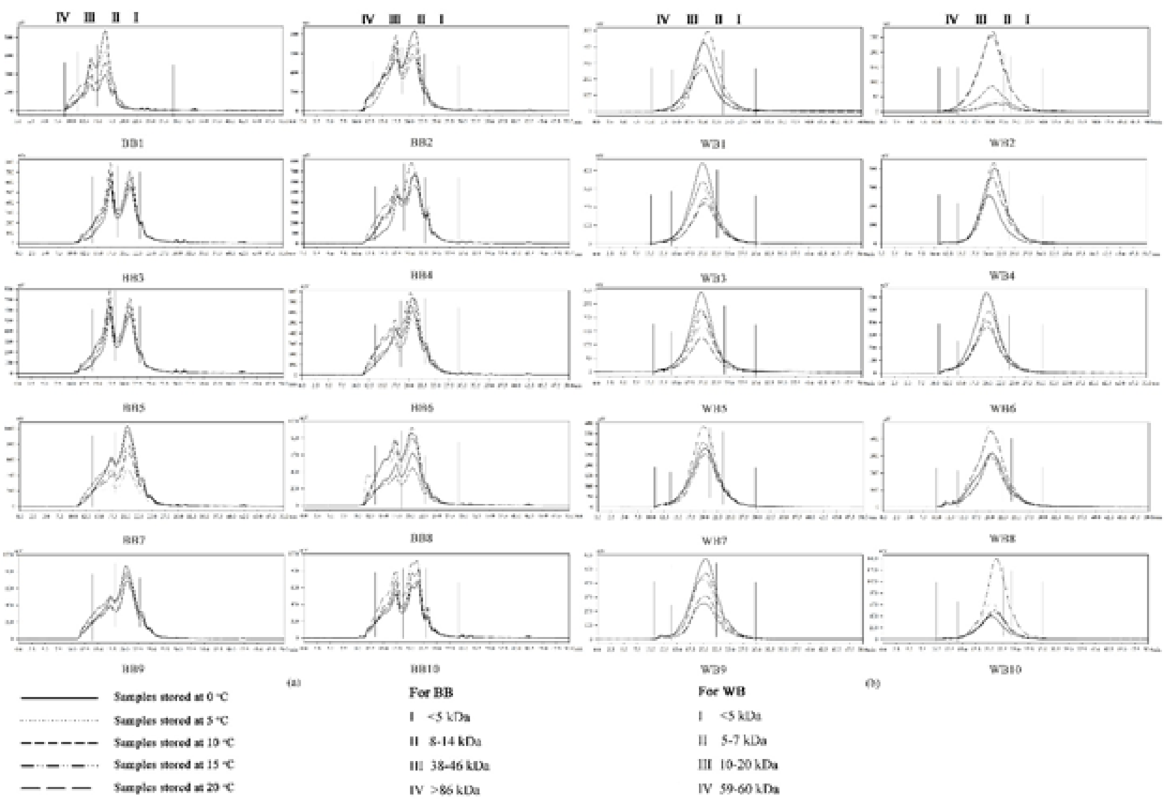

Figure 2. Molecular weight distribution of HA protein in barley beers and wheat beers. Each picture represents the elution profile of a beer stored at 0 - 20°C, which is plotted by elution time on the horizontal axis and signal intensity at 214 nm on the vertical axis. Elution profiles of HA protein in barley beers (a), and elution profiles of HA protein in wheat beers (b). The numbers after letters represent the sample number, for example, BB1 = sample 1 of barley beer.

Table 3. Mr and relative content of HA protein in barley beers and wheat beers stored at different temperatures.

No.[1]	Barley beer I < 5 kDa Mr	Barley beer I < 5 kDa %	Barley beer II 8 - 14 kDa Mr	Barley beer II 8 - 14 kDa %	Barley beer III 38 - 46 kDa Mr	Barley beer III 38 - 46 kDa %	Barley beer IV > 86 kDa Mr	Barley beer IV > 86 kDa %	No.[1]	Wheat beer I < 5 kDa Mr	Wheat beer I < 5 kDa %	Wheat beer II 5 - 7 kDa Mr	Wheat beer II 5 - 7 kDa %	Wheat beer III 10 - 20 kDa Mr	Wheat beer III 10 - 20 kDa %	Wheat beer IV 59 - 60 kDa Mr	Wheat beer IV 59 - 60 kDa %
1-0[a]	4.80	9.67^{Dd}	10.00	53.55^{Da}	39.81	28.69^{Bd}	86.46	8.09^{Ie}	1-0	4.07	8.41^{Db}	6.59	8.47^{Cb}	15.82	74.40^{Ec}	59.28	8.72^{Hc}
1-5	4.80	10.48^{Bc}	9.94	48.52^{Eb}	41.32	26.61^{De}	86.46	14.39^{Id}	1-5	4.07	8.30^{Gc}	6.59	7.51^{Ec}	16.78	75.19^{Eb}	59.28	9.00^{Eb}
1-10	4.09	12.77^{Aa}	10.04	44.45^{Ic}	41.16	26.87^{Dc}	86.46	15.90^{Hb}	1-10	4.07	7.71^{Ge}	6.59	8.46^{Db}	16.52	77.52^{Aa}	59.28	6.31^{Id}
1-15	4.17	11.38^{Bb}	10.12	42.93^{Id}	41.98	30.40^{Ba}	86.46	15.29^{Ic}	1-15	4.07	11.90^{Ca}	6.59	12.69^{Ba}	12.08	73.74^{Ed}	59.28	1.68^{Ie}
1-20	4.20	9.04^{Ee}	10.12	40.31^{Je}	42.48	30.14^{Db}	110.06	20.51^{Ca}	1-20	4.07	8.05^{Hd}	6.59	6.61^{Hd}	18.57	72.82^{Fe}	59.28	12.53^{Ba}
2-0	4.80	7.67^{Ia}	10.54	49.11^{Ga}	41.81	23.41^{Ge}	86.46	19.80^{Ab}	2-0	4.07	13.59^{Bb}	5.47	19.92^{Aa}	11.43	60.75^{Jd}	59.28	5.74^{Ie}
2-5	4.24	6.31^{Ic}	11.32	45.31^{Gc}	41.96	29.45^{Cd}	86.46	18.93^{Ed}	2-5	4.07	14.30^{Aa}	5.69	17.82^{Ab}	10.66	60.59^{Je}	59.28	7.30^{Gd}
2-10	4.21	7.39^{Fb}	11.96	46.36^{Fb}	41.78	30.91^{Aa}	86.46	15.33^{Ie}	2-10	4.07	10.05^{Dd}	6.96	11.26^{Bc}	14.68	68.98^{Hc}	59.28	9.70^{Eb}
2-15	4.31	5.62^{Je}	10.74	40.59^{Je}	42.94	30.16^{Cc}	86.46	23.63^{Aa}	2-15	4.07	10.02^{Fe}	6.96	10.79^{De}	14.55	69.43^{Ib}	59.28	9.77^{Ca}
2-20	4.80	6.08^{Id}	11.27	43.53^{Id}	42.15	30.69^{Bb}	86.46	19.70^{Ec}	2-20	4.07	10.26^{Dc}	6.96	11.14^{Ad}	15.00	69.49^{Ja}	59.28	9.10^{Fc}
3-0	4.33	8.50^{Fa}	9.84	48.20^{Ja}	44.25	30.94^{Ad}	86.46	12.37^{Ge}	3-0	4.07	6.07^{Ge}	6.25	6.55^{Gc}	17.76	77.70^{Ba}	59.28	9.68^{Ec}
3-5	4.47	6.48^{He}	10.27	45.29^{Hc}	45.51	31.91^{Ac}	86.46	16.32^{Gc}	3-5	4.07	6.84^{Jd}	6.25	5.89^{Je}	18.55	75.33^{Dc}	59.28	11.95^{Aa}
3-10	4.46	6.71^{Jc}	10.02	44.41^{Jd}	45.37	29.96^{Ce}	86.46	18.92^{Ea}	3-10	4.07	7.61^{Hc}	6.25	6.33^{Hd}	17.57	75.64^{Cb}	59.28	10.42^{Db}
3-15	4.46	6.68^{Gd}	9.97	44.29^{He}	45.01	31.95^{Ab}	86.46	17.08^{Gb}	3-15	4.07	9.69^{Ha}	6.25	8.21^{Ea}	14.59	74.15^{Dd}	59.28	7.95^{Fe}

3-20	4.37	7.26^{Hb}	10.30	46.00^{Eb}	44.20	33.63^{Aa}	86.46	13.10^{Jd}	3-20	4.07	9.13^{Gb}	6.25	7.84^{Eb}	15.10	73.67^{Ee}	59.28	9.36^{Ed}
4-0	4.10	11.48^{Bb}	10.18	52.40^{Fa}	38.08	28.14^{Cd}	86.46	7.99^{Je}	4-0	4.07	14.29^{Ab}	6.25	12.57^{Bb}	10.61	70.37^{Id}	59.28	2.77^{Jc}
4-5	4.04	10.38^{Cc}	11.65	44.93^{Ic}	42.77	30.73^{Ba}	86.46	13.96^{Jc}	4-5	4.07	8.80^{De}	6.25	7.72^{Ce}	15.71	79.19^{Aa}	59.28	4.29^{Ia}
4-10	4.09	9.73^{Ce}	11.63	44.87^{Gd}	42.30	30.55^{Bc}	86.46	14.85^{Jb}	4-10	4.07	16.25^{Ac}	6.25	12.60^{Aa}	10.54	68.57^{Ie}	59.28	2.58^{Je}
4-15	4.03	15.18^{Aa}	10.20	48.94^{Cb}	41.30	27.76^{De}	86.46	8.13^{Jd}	4-15	4.07	14.63^{Aa}	6.25	11.38^{Cc}	11.32	71.29^{Gc}	59.28	2.69^{Hd}
4-20	4.12	9.85^{Ad}	11.43	44.06^{He}	42.75	30.58^{Cb}	86.46	15.51^{Ia}	4-20	4.07	13.34^{Ad}	6.25	10.52^{Cd}	13.23	72.27^{Gb}	59.28	3.88^{Jb}
5-0	4.22	12.08^{Ab}	12.03	54.84^{Ca}	41.57	21.69^{Hc}	86.46	11.39^{He}	5-0	4.07	5.81^{He}	6.25	5.27^{Id}	19.52	78.92^{Aa}	59.28	10.00^{Dd}
5-5	4.21	12.10^{Aa}	12.33	51.71^{Ab}	42.95	21.06^{He}	86.46	15.14^{Hd}	5-5	4.07	9.61^{Ba}	6.25	6.44^{Ga}	17.70	73.43^{Ie}	59.28	10.52^{Dc}
5-10	4.25	11.02^{Bc}	12.38	50.18^{Bc}	43.54	21.62^{Hd}	86.46	17.18^{Gc}	5-10	4.07	6.52^{Jd}	6.25	5.16^{Je}	19.62	76.09^{Bb}	59.28	12.24^{Ba}
5-15	4.30	8.10^{Fe}	13.61	48.92^{Dd}	42.55	23.71^{Hb}	86.46	19.27^{Db}	5-15	4.07	8.46^{Ib}	6.25	6.34^{Jb}	17.53	75.92^{Bc}	59.28	9.28^{De}
5-20	4.25	8.26^{Fd}	13.50	47.30^{Ce}	42.60	24.07^{Ha}	86.46	20.37^{Da}	5-20	4.07	7.25^{Jc}	6.25	5.90^{Jc}	19.06	75.78^{Ad}	59.28	11.06^{Db}
6-0	4.33	7.67^{Ib}	13.01	53.41^{Ea}	43.07	24.09^{Ec}	86.46	14.83^{Ee}	6-0	4.07	5.68^{Id}	6.25	5.50^{Hd}	18.90	77.31^{Ca}	59.28	11.51^{Bd}
6-5	4.27	7.54^{Gc}	12.94	48.49^{Fb}	43.13	23.40^{Fd}	86.46	20.57^{Cc}	6-5	4.07	7.25^{Hc}	6.25	6.16^{Ic}	17.49	74.73^{Fb}	59.28	11.87^{Bb}
6-10	4.30	6.92^{He}	13.16	46.81^{Ed}	43.50	24.86^{Fa}	86.46	21.42^{Bb}	6-10	4.07	7.55^{Ib}	6.25	6.15^{Ic}	18.02	73.48^{Fd}	59.28	12.83^{Aa}
6-15	4.19	9.47^{Da}	12.50	47.13^{Fc}	43.35	24.63^{Gb}	86.46	18.77^{Fd}	6-15	4.07	10.03^{Ea}	6.25	6.95^{Ha}	15.71	72.30^{Fe}	59.28	10.73^{Ae}
6-20	4.26	7.28^{Gd}	12.85	45.53^{Fe}	43.57	25.33^{Ee}	86.46	21.86^{Ba}	6-20	4.07	7.54^{Ib}	6.25	6.23^{Ib}	18.20	74.68^{Cc}	59.28	11.55^{Cc}
7-0	4.21	9.15^{Eb}	13.40	56.39^{Aa}	43.62	20.85^{Ia}	86.46	13.60^{Fe}	7-0	4.07	7.24^{Ed}	6.25	7.63^{Fa}	16.26	73.78^{Gc}	59.28	11.35^{Ca}
7-5	4.16	9.11^{Dc}	13.01	49.52^{Ce}	42.51	18.48^{Je}	86.46	22.89^{Ba}	7-5	4.07	7.24^{Id}	6.25	7.63^{Da}	16.26	73.78^{Gc}	59.28	11.35^{Ca}
7-10	4.22	8.28^{Ee}	13.30	51.92^{Ad}	42.97	20.51^{Ib}	86.46	19.29^{Db}	7-10	4.07	8.46^{Fb}	6.25	6.80^{Gc}	18.22	75.29^{Db}	59.28	9.45^{Fb}

7-15	4.80	9.06^{Ed}	12.89	53.79^{Ab}	44.16	20.21^{Ic}	86.46	16.94^{Hd}	7-15	4.07	8.12^{Jc}	6.25	6.63^{Id}	18.05	75.97^{Aa}	59.28	9.28^{Dc}
7-20	4.23	9.83^{Ba}	12.73	52.74^{Bc}	42.92	19.27^{Jd}	86.46	18.16^{Fc}	7-20	4.07	9.25^{Fa}	6.25	7.49^{Fb}	16.34	74.73^{Bd}	59.28	8.54^{Gd}
8-0	4.21	7.94^{Ha}	12.87	49.05^{Ha}	43.55	24.02^{Fc}	86.46	18.99^{Ce}	8-0	4.07	4.27^{Jd}	6.27	8.47^{Ca}	14.82	73.22^{Hb}	59.28	14.04^{Aa}
8-5	4.16	6.14^{Jd}	13.24	42.45^{Je}	44.36	22.11^{Ge}	119.22	29.31^{Aa}	8-5	4.07	8.72^{Ec}	6.25	6.21^{He}	17.89	73.72^{Ha}	59.28	11.35^{Cc}
8-10	4.22	7.27^{Gb}	13.14	44.64^{Hd}	44.32	22.96^{Gd}	86.46	25.12^{Ab}	8-10	4.07	11.01^{Ca}	6.27	7.32^{Fd}	15.82	70.57^{Gd}	59.28	11.10^{Cd}
8-15	4.80	6.57^{Hc}	13.44	46.13^{Gb}	45.91	25.12^{Fa}	86.46	22.18^{Bd}	8-15	4.07	11.02^{Da}	6.25	7.77^{Fb}	15.41	70.92^{Hc}	59.28	10.29^{Be}
8-20	4.23	6.04^{Je}	13.69	45.13^{Gc}	45.33	24.98^{Fb}	86.46	23.85^{Ac}	8-20	4.07	9.55^{Eb}	6.25	7.36^{Gc}	15.80	70.43^{He}	59.28	12.67^{Ab}
9-0	4.31	9.82^{Cb}	13.89	55.59^{Ba}	43.07	19.28^{Jd}	86.46	15.31^{De}	9-0	4.07	7.08^{Fe}	6.25	7.75^{Db}	14.89	76.06^{Da}	59.28	9.11^{Fa}
9-5	4.35	8.36^{Fe}	13.92	51.61^{Bd}	44.11	19.62^{Ib}	86.46	20.41^{Db}	9-5	4.07	8.70^{Fd}	6.25	7.02^{Fe}	17.46	75.55^{Cb}	59.28	8.72^{Fb}
9-10	4.38	8.64^{Dd}	13.69	50.02^{Ce}	44.94	20.25^{Ja}	86.46	21.09^{Ca}	9-10	4.07	9.69^{Ec}	6.25	7.43^{Ed}	16.63	75.07^{Ec}	59.28	7.82^{Gd}
9-15	4.35	10.13^{Ca}	13.25	52.33^{Bc}	45.21	18.59^{Je}	86.46	18.94^{Ec}	9-15	4.07	9.80^{Gb}	6.25	7.73^{Gc}	14.71	74.53^{Cd}	59.28	7.95^{Fc}
9-20	4.80	9.62^{Dc}	13.36	53.67^{Ab}	45.84	19.34^{Ic}	86.46	17.37^{Hd}	9-20	4.07	10.86^{Ca}	6.25	8.11^{Da}	15.01	74.34^{De}	59.28	6.69^{He}
10-0	4.24	8.17^{Gc}	8.35	48.22^{Ic}	44.71	24.40^{De}	86.46	19.21^{Bb}	10-0	4.07	9.60^{Cd}	6.25	7.64^{Ee}	15.41	73.81^{Fb}	59.28	8.95^{Ga}
10-5	4.20	8.41^{Eb}	8.34	49.38^{Da}	44.96	24.49^{Ed}	86.46	17.72^{Fe}	10-5	4.07	9.14^{Ce}	6.25	7.99^{Bd}	14.51	76.91^{Ba}	59.28	5.96^{Hd}
10-10	4.31	6.87^{Id}	8.34	48.80^{Db}	45.03	25.96^{Eb}	86.46	18.37^{Fc}	10-10	4.07	12.22^{Bc}	6.25	10.53^{Cc}	11.84	70.57^{Gc}	59.28	6.68^{Hb}
10-15	4.31	6.33^{Ie}	9.35	47.30^{Ed}	45.35	26.02^{Ea}	86.46	20.35^{Ca}	10-15	4.07	14.48^{Ba}	6.25	12.83^{Aa}	10.43	68.83^{Je}	59.28	3.86^{Ge}
10-20	4.15	9.77^{Ca}	8.55	47.28^{De}	44.46	24.88^{Gc}	86.46	18.07^{Gd}	10-20	4.07	12.94^{Bb}	6.27	10.90^{Bb}	11.35	70.17^{Id}	59.28	6.00^{Ic}
Mean	4.35	8.69	11.42	48.17	43.32	25.39	87.59	17.76	**Mean**	4.07	9.49	6.30	8.51	15.60	73.24	59.28	8.76

Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) at the same temperature and in the same sample, respectively. [1] = sample no. - storage temperature, for example, 1-0 means sample 1 stored at 0°C.

I and IV were 8.69 and 17.76%, respectively. Fraction II possessed the highest mean level (48.17%), followed by component III (25.39%). From the overall trend, the content of fraction II decreased but fraction III increased in each sample along with the increasing storage temperature.

The Mr of HA protein in BBs ranged from LMW to HMW were divided into four fractions. These results corroborated the findings of numerous previous works in the Mr of HA protein. Iimure *et al.* (2009) found that HA protein in BB was identified as protein Z4, protein Z7, and trypsin/amylase inhibitor pUP13 whose Mr was 35 - 45 kDa. Asano *et al.* (1982) elucidated that polypeptides (10 - 30 kDa) originated from barley primarily was responsible for the haze formation. However, no research of fraction IV was found in the previous studies. Therefore, a further study with more focus on fraction IV was suggested.

HA protein in WB was also divided into four fractions named I (< 5 kDa), II (5 - 7 kDa), III (10 - 20 kDa), and IV (59 - 60 kDa), respectively (Table 3). Among them, fractions I and II fell into LMW range, fraction III ranged from the low to medium molecular weight, and fraction IV fell into HMW range. The content of fractions I, II, III, and IV were 4.27 - 16.25, 5.27 - 19.92, 60.59 - 79.19, and 1.68 - 14.04%, respectively. The mean content of fractions I, II, and IV were 9.49, 8.51, and 8.76%, respectively; but the most abundant was fraction III, which accounted for 73.24%. Overall, the components content of fractions II and III accounted for 80%, but fractions I and IV only accounted for 20%.

Depraetere *et al.* (2004) developed barley malt with proteolytic activity that promoted the breakdown of wheat protein into smaller proteins which led to smaller particles being suspended in beer and better stability of the haze. However, some literatures about the influence of wheat proteins on the haze formation of beers were contradictory. Delvaux *et al.* (2000) stated that HMW proteins from wheat increased the haze. On the other hand, wheat had a strongly negative effect on the permanent haze intensity because of the water-soluble or solubilised gluten proteins (Delvaux *et al.,* 2001).

After comparing Mr of HA protein components, the Mr of fractions I, II, III, and IV in WB was smaller than that of BB, correspondingly. Especially, most HA protein in WB were in low and medium molecular weight, the mean level (Table 3) was more than 90%. In the bright BB, the range of LMW HA protein components (I and II) (Table 3) was 46.21 - 66.92%, while the range of HMW HA protein components (III and IV) was 33.08 - 53.79%. However, no medium molecular weight HA protein fraction was found in BB samples. Brijs *et al.* (2002) found that HMW proteins were likely to form the haze and speculated that precipitates were formed when the molecular weight of proteins was too high.

In the normal brewed beer, the potentially turbid HMW proteins or peptides could be inevitable. And some sensitive proteins would also undergo polymerisation and oxidation during the long-term storage, thereby increasing the size of molecules. Thus, the turbidity would eventually occur (Stewart, 2004).

Correlation between turbidity and four HA protein fractions content in beers

As shown in Table 4, fraction II was significantly positively correlated with the turbidity of barley

Table 4. Correlation of turbidity and contents of four HA protein fractions in barley beers and wheat beers.

Barley beer			**Wheat beer**		
Fraction	**Turbidity**		**Fraction**	**Turbidity**	
	Pearson correlation	**Sig. (2-tailed)**		**Pearson correlation**	**Sig. (2-tailed)**
I	0.011	0.9738	I	0.355*	0.011
II	0.322*	0.024	II	0.309*	0.029
III	-0.347*	0.015	III	-0.251	0.079
IV	0.046	0.752	IV	-0.312*	0.028
I&II	0.273	0.060	I&II	0.351*	0.012
II&III	-0.073	0.616	II&III	0.250	0.080
III&IV	-0.273	0.060	III&IV	-0.351*	0.012
I&II&III	-0.048	0.744	I&II&III	0.312*	0.027

* = Correlation is significant at the 0.05 level (two-tailed).

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beer (Pearson's r = 0.322). While fraction III was significantly negatively correlated with the turbidity (Pearson's r = -0.347), but the latter was more correlative with the turbidity than the former. Fraction II (8.34 - 13.92 kDa) indicated a positive influence on the turbidity but fraction III (38.08 - 45.91 kDa) showed a negative influence on the turbidity (p < 0.05).

In WB, the correlation of the turbidity and contents of four fractions as well as their combination is shown in Table 4. Except for fraction III, the remaining components were all significantly correlated to the turbidity. Fractions I, II, and their combination I&II were positively correlated with the turbidity (p < 0.05); however, the correlation of I&II (p = 0.012) and I&II&III (p = 0.027) were lower than that of I (p = 0.011). It was suggested that the promotion effect of the combination of fractions I and II on the turbidity was not a simply additive relationship, which was actually related to inside and outside environments of WB and the composition of the amino acids in these components. Meanwhile, the content of fractions IV (Pearson's r = -0.312) and III&IV (Pearson's r = -0.351) had a negative correlation with the turbidity in WB. Therefore, it could be concluded that LMW fraction (< 7 kDa) in WB including I and II significantly (p < 0.05) positively influenced the turbidity; instead HMW fraction IV significantly negatively influenced the turbidity; but fraction III did not show any influence on the turbidity although it possessed the highest level in HA protein.

Siebert (1999) found that the haze-forming activity of HA protein was mainly in connection with the mole percent of proline in protein. And Leiper *et al.* (2003) observed that silica adsorbed a wide range of polypeptides, including a 46 kDa protein found in all beer types. Jin *et al.* (2009) found bands in the range of 40 - 66 and 10 - 30 kDa, indicating that proteins of approximately 40, 25 - 29, and 6.5 - 17 kDa might be significant for the haze formation. Previous research had found either HMW component or LMW component was inseparable from the formation of HA protein. LMW fraction, which led to the small aggregate particles that tended to remain in the suspension, could result in more stable turbidity; on the contrary, HMW fraction was prone to forming sediment (Depraetere *et al.,* 2004). This could explain why fraction II in BB and fraction I and II (LMW) in WB had a positively significant (p < 0.05) correlation with the turbidity; and fraction III in BB and fraction IV in WB (HMW) was negatively significantly correlative with the turbidity (p < 0.05).

Hence, for BB, reducing the protein level of low and medium molecules (< 40 kDa) in the finished beer was in favour of decreasing the turbidity. Instead, brewing WB might be suggested to increase the degradation of HMW protein that would be the content of LMW protein, which could be beneficial for the haze formation and stabilisation.

Conclusion

By storing the commercial BBs and WBs for a 7-day period at 0 - 20°C, the beer turbidity was determined; and HA protein in beers was extracted, fractionated based on HPSEC elution peaks, and its relationship with beer turbidity was analysed. The maximum turbidity occurred at 0 or 5°C. The higher the turbidities of the initial samples, the higher the turbidities after storage. Therefore, BB is recommended to be stored and transported at 10 or 15°C to avoid the haze formation, while the cloudy WB should be stored and transported at 0 or 5°C to promote a stable and high turbidity value. Mr of each fraction in BBs was higher than that in WBs correspondingly. In BBs, fraction II (8.34 - 13.92 kDa) possessed the highest level of 48.17% and positively affected the turbidity, whose amount was suggested to be reduced in brewing to make BB clearer. In WBs, fractions I and II (Mr < 20 kDa) positively influenced the turbidity. The cloudy WB would be more fullness and have better stability of haze by promoting the amounts of fractions I and II during WB wort making.

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