The Gel Filtration Chromatographic-Profiles of Proteins and Peptides of Wort and Beer: Effects of Processing – Malting, Mashing, Kettle Boiling, Fermentation and Filtering

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ABSTRACT

J. Inst. Brew. 109(1), 41–50, 2003

Barley and malt proteins, of infusion (IoB) and decoction (EBC) mashing worts as well as commercial wort and beer, obtained from the Castlemaine Perkins brewery, Brisbane, were gel filtered, with or without further treatments. A general, similar pattern of protein and peptide profiles emerged from barley malt and beer. This confirmed the widely assumed fact that beer proteins descend from barley, some transformed and others perhaps mostly unchanged by processing. In the gel-filtrate profiles, a maximum of 8 or 9 fractions were discerned. These fractions were collected and quantified for protein contents and amino acid compositions. The first four fractions contained the proteins and polypeptides of molecular weight higher than 14,000. Consequently, the remaining fractions contain the smaller peptides (<14,000), that were completely removed by dialysis. The effects of processing on proteins and peptides varied contingent upon the type of processing step considered and the pre-chromatographic treatment. Malting was the most effective process remarkably increasing the soluble protein contents, especially the smaller peptide fractions and the colour development. This is the first report, as far as we are aware of, on the gel filtration profiles of wort and beer low molecular weight peptides including those of barley wort. The importance of the smaller peptides in foam formation and retention cannot be overemphasised. The amino acid composition of the fractions revealed much more diversity than was observed in the comparison of the profiles. Proline content of fraction 1 resembled that of barley soluble proteins while fractions F2, F3 and F4 that of glutelin and only fraction 8 that of hordein. The latter, suggests that hordeins or, at least the peptide products rich in proline, are likely to be completely digested to amino acids, during malting.

Key words: Amino acids, barley, beer, protein and peptide-profiles, wort.

INTRODUCTION

Malted barley is the raw material for beer brewing, sometimes complemented with other cereals as adjuncts. Consequently, it is the major source of all beer components. However, most of the beer components have undergone extensive transformation during malting, mashing and fermenting processes. For example, starch, the major component of barley (>60% dry weight)[13] and other cereal grains, is degraded to sugars and fermented to alcohol, the major component of beer.

Similarly, barley proteins are also transformed, but in a different manner and to variable degrees during these processes. This is perhaps because the mature barley grain contains a variety of proteins that differ in function, location, structure and other physical and chemical characteristics. Only about 20% of the total grain proteins are water-soluble. Barley water-soluble proteins are believed to be resistant to proteolysis and heat coagulation and hence pass through the processing steps, intact or somewhat modified, to beer[4,6,26]. The water-soluble protein ratio increases more than 2-fold (>40%) during malting as a result of the degradation of the water insoluble hordein component of the reserve and due to the releasing of bound (latent) proteins[2,5,6,17–19,25].

Protein and its derivatives are major determinants of beer quality. They affect colour, flavour, foam formation and retention, mouth feel and the colloidal stability of beer, which determines the shelf life[1,7–10,20–22]. Researchers' attention has been focussed on beer proteins, especially those involved in foam retention and colloidal stability. Hydrophobicity and molecular weight of these proteins have been identified as the two most important attributes of proteins and peptides involved in foam formation and retention as well as haze formation[7–9,21–23,26,27,29]. Hydrophobic polypeptides of lower molecular weight (5 to 30 × 10^3) were found to enhance foam formation and retention[8,23,26]. In contrast, colloidal instability and haze formation were associated with high molecular weight hydrophobic polypeptides[8,23].

The degree and extent of alteration in barley protein composites during malting, is of paramount importance in transforming barley to malt, as well as shaping malt quality[28]. Appropriate protein modification provides free amino acids for the synthesis of hydrolytic enzymes and provides for yeast nutrition. In addition, it facilitates cell wall breakdown, enhances starch digestibility by freeing the granules maximising fermentable sugars and extract yield and improves processing. Some of these soluble proteins survive through the multiple steps of the brewing process to end up in beer.

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Publication no. G-2003-0306-090
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Proteins are an essential component of beer, contributing to flavour, texture, mouth feel, nutritional value, colour and most importantly, foam formation and retention. On the other hand, beer proteins are also implicated in haze formation, the loss of colloidal stability and the shortening of shelf life. The aim of the present study was to profile and characterise the water-soluble proteins of barley, malt and beer and to identify the barley sources of beer proteins and the effects of malting, mashing, boiling and fermentation.

MATERIALS AND METHODS

Barley and malt

Control Schooner barley samples (designated for all Australian Quality Laboratories) were used in micromalting, malt and barley analysis. Malt was prepared by micromalting on a Phoenix automatic micromalter, kilned and finely ground as described by Osman *et al.*[17,18].

Mashing

Finely ground barley and malt were mashed, in the laboratory under identical conditions using the Institute of Brewing (IoB) and the European Brewing Convention (EBC Analytica 1998) recommended methods. Worts were separated by filtration and divided into two. One of the latter was boiled for an hour (10% evaporation). All samples were centrifuged at 4000 *g* for 10 min to remove any precipitates. Samples of brewery wort, liquor grist ratio of 3.4 L/kg grist and hopping of 62 kg/10^3 hL, (before and after boiling) and beer, (before and after filtration) were obtained from the Castlemaine Perkins Brewery, Brisbane. The barley variety used in the commercial brewery production of 4-X Bitter was Grimmett.

Extraction of barley hordein fraction

Barley hordein fraction was extracted as described by Osman *et al.*[17].

Dialysis

Protein and peptides were dialysed using cellulose membrane dialysis tubing retaining peptides of molecular weight (mol. wt.) 12,000 or greater supplied by Sigma-Aldrich, Sydney, Australia.

Gel filtration chromatography – protein profiling

The water-soluble protein profiling of barley, wort and beer proteins were performed by gel filtration chromatography using Marex® Cellufine™ GCL-300 Medium (Millipore). A column (2.6 × 90 cm) was packed with degassed GCL-300 and equilibrated with 50 mM acetate buffer (pH 5.5) containing 0.02% NaN_3. In order to achieve the best separation of the wort and beer proteins and facilitate a simultaneous inter and intra quantitative and qualitative comparison of the separated protein fractions, the wort and beer samples were prepared, for the column chromatography, as follows. (a) The laboratory and brewery worts (before and after boiling) and beer (before and after filtering) were centrifuged at 4000 *g* and chromatographed (10 mL worts and 15 mL beer). (b) Beer and wort samples after centrifugation were concentrated by ultrafiltration of 20 mL worts and 40 mL beer, using Amicon Bioseparations Centricon Plus 20 with molecular mass cut-off 5,000 or 10,000 Da (Biomax-5, 5×10^3 or 10×10^3 NMWL, Millipore). (c) The centrifuged samples were dialysed, 10 mL each, using dialysis tubing with a molecular mass cut-off of 12,000 Da. These samples (a, b & c) were loaded onto the column, one at a time, and eluted with the equilibration buffer at a flow rate of 50 mL/h, at room temperature (20–22°C). Fractions of 6-mL eluate were collected. The protein contents of the eluted fractions were determined by measuring the absorbance at 280 nm. Fractions under each peak were collected for the protein content measurement with Folin-Ciocalteau reagent according to Lowry *et al.*[12] method and the amino acid composition analysis with Water's PICO-TAG method. The column was washed after each sample with 0.1 N NaOH, distilled water and re-equilibrated before loading the next sample.

Spectral analysis

Fractions of worts and beer, eluted from GCL-300 column of concentrated samples, were concentrated using Centricon Plus 20 (Biomax-5) and analysed using UV-2501 PC, UV-VIS Recording Spectrophotometer (Shimadzu). The spectra were recorded between 250 and 600 nm with medium speed. Similarly, the spectra of wort and beer, without treatment, were recorded.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of barley, wort and beer proteins and those of GCL-300 fractions was carried out using the Hoefer SE 600 vertical slab gel unit according to Laemmli[11]. The SDS-PAGE molecular weight standards were the low range supplied by BIO-RAD. They were phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500) and lysozyme (14,400).

RESULTS AND DISCUSSION

The results presented in Figs. 1 and 2 portray the summary of the laboratory mashing of Schooner barley and malt using the decoction (EBC) and infusion (IoB) mashing methods, respectively. The gel-filtration profiles of the proteins and peptides in Fig. 1, top, show 8 peaks of varying magnitudes and degree of separation. The lower graphs depict the total protein loaded onto the column and the recoveries in the fractions. A similar picture is also seen in Fig. 2 where the separation of barley-wort proteins is better than in Fig. 1. But in totality they are very similar. These profiles were obtained with samples concentrated by ultrafiltration to about 2-fold (20 mL concentrated to 10–13 mL). Furthermore, the same pattern of profiles consistently emerged, again and again, even when the commercial worts and beer samples of a different barley variety (Grimmett) were used, as seen in Fig. 3 or concentrated to 2-fold, as mentioned above (Fig. 4).

However, changes in the profiles began to appear when the samples were further concentrated 8 to 10 times (20 mL to 2.5–2 mL, using a higher molecular mass cut-off [10,000] membrane) or alternatively dialysed (Fig. 5). The

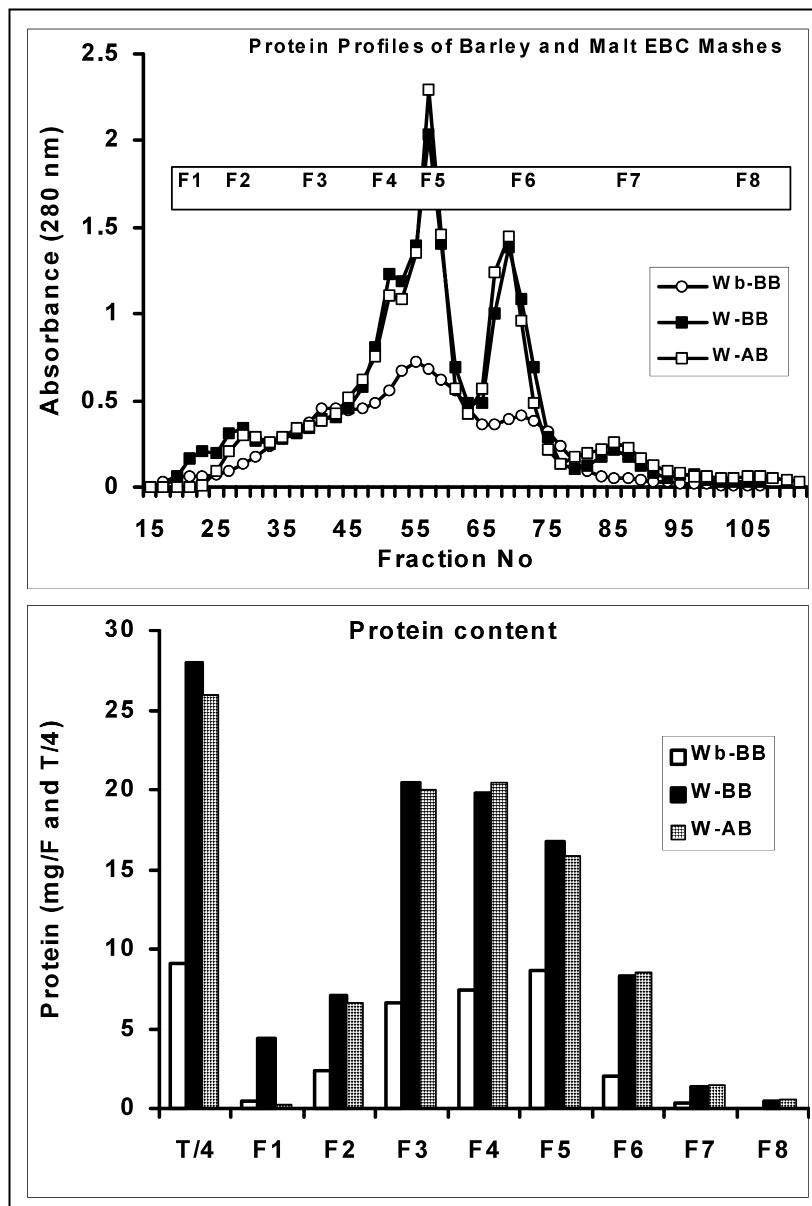

Fig. 1. Gel-filtration protein and peptide profiles of EBC wort of barley before boiling (Wb-BB) and malt wort before (W-BB) and after boiling (W-AB), top and the protein contents of the total (a quarter-T/4) and the fractions (GCL-F1–GCL-F8). Twenty mL wort was concentrated to 11 mL before loading to GCL-300 Column.

highest peaks were diminished to about 20% in the concentrated samples and completely vanished in the dialysed samples (Fig. 5) top and middle, respectively. This means that the peaks and fractions further down from fraction F4, in the profiles, are constituted of proteins and peptides less than 12,000 mol. wt. (dialysis tubing molecular mass cut-off limit). Among the latter group, there are some important proteins, especially for foam formation and retention, such as the lipid transferring protein[6] (LTP1) with molecular weight of about 9,000 Da. It is pertinent to note that other researchers also reported the separation of beer proteins into only four or five fractions using hydrophobic interaction chromatography[9,14,15,26,29]. This means that the hydrophobic techniques exclude the hydrophilic peptides leaving a gap in the comprehensive knowledge and understanding of the role of proteins and peptides in wort and beer.

The quantitative analysis of the proteins and peptides applied to the column and recovered in the fractions, using the Folin Ciocalteau colour reagent[12], revealed some agreement and contrast with the profile pictures. The protein contents in fractions F3 and F4 were higher than that of F5 and F6 and the sum of the contents in fractions F1 to F4 was about 60% of the total recovery. There is no obvious explanation from the amino acid compositions (Table I). However, it is pertinent to add that the first four fractions were all coloured (orange/yellow), while only fraction F5 had a slight yellow colour in this group. Therefore, it is possible that the phenolic and polyphenolic groups might have contributed to the colour.

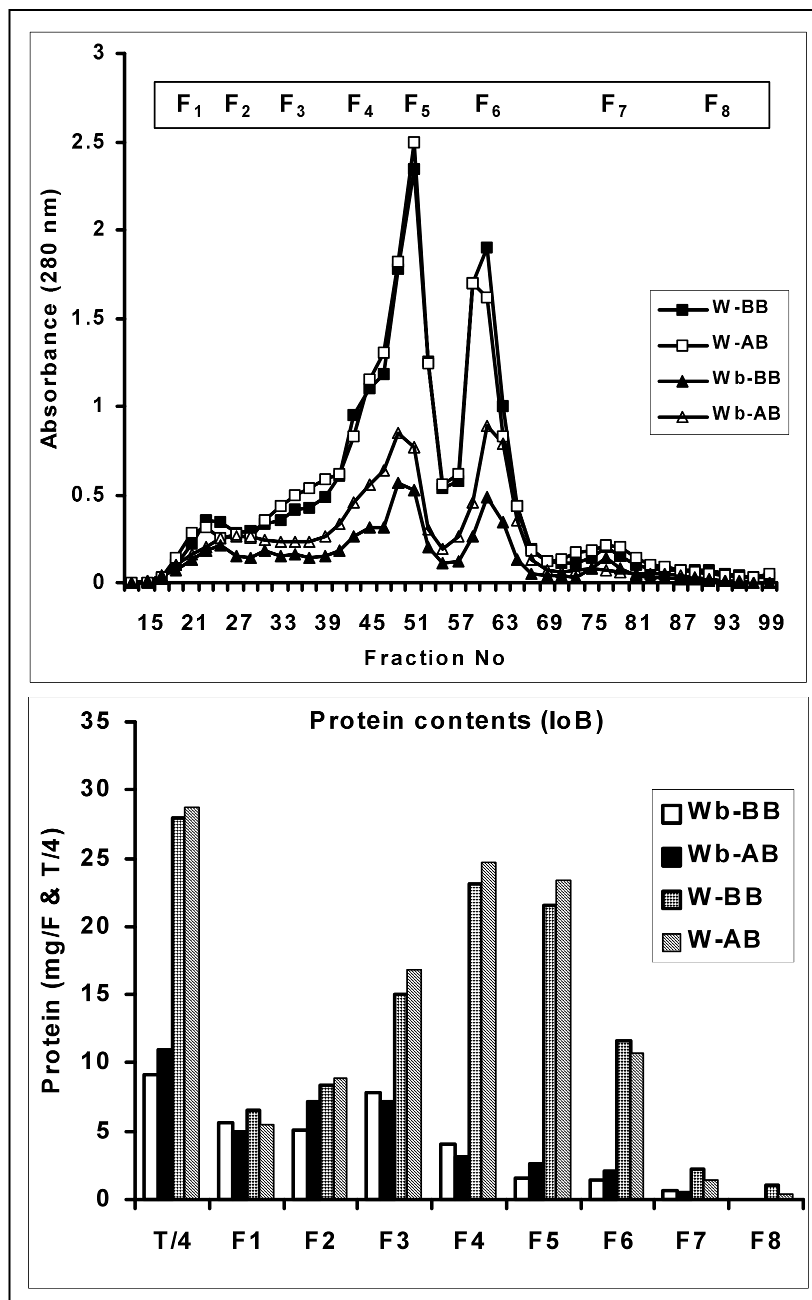

Fig. 2. Protein and peptide profiles of IoB worts of barley (Wb-BB and Wb-AB) and malt (W-BB and W-AB) before and after boiling, respectively, and (bottom) the total and GCL-fractions protein contents, as in Fig. 1.

Further analysis and characterisation of the proteins and peptides of the different fractions using electrophoresis (SDS-PAGE) disclosed more information (Fig. 6). Firstly, the Commassie Blue reagent staining of the gels failed to detect any permanent bands after destaining in the fractions F5 to F8 (results are not shown). No attempts were made to use other reagents at this stage. Secondly, the high molecular weight proteins of GCL-300 fraction F1 showed two spots in wort, three spots in beer and none in barley (Fig. 6). These spots match the molecular weights 40 and high teen thousands in wort and 40, 31 and high twenty thousands in beer. This evidence suggests that fraction F1 contains proteins of combined polypeptides connected, possibly by sulfhydryl bonds. These bonds were reduced by the action of dithiothreitol (DTT) in preparation for SDS-PAGE. Additionally, the results show that beer fraction F1 contains extra proteins compared to wort, which may originate from yeast or proteins (e.g. gelatin) added at the fining stage of processing[22] (about 12.5 mg/L). There is no clear evidence of the presence of high molecular weight single polypeptide proteins in either fraction F2, F3 or F4, which seem to be composed of proteins or peptides of less than 20×10^3. If any high mol. wt. protein is present in these fractions, it must be the result of recombination of smaller proteins and/or peptides by sulfhydryl bonding, as referred to earlier, or by hydrophobic interactions. The

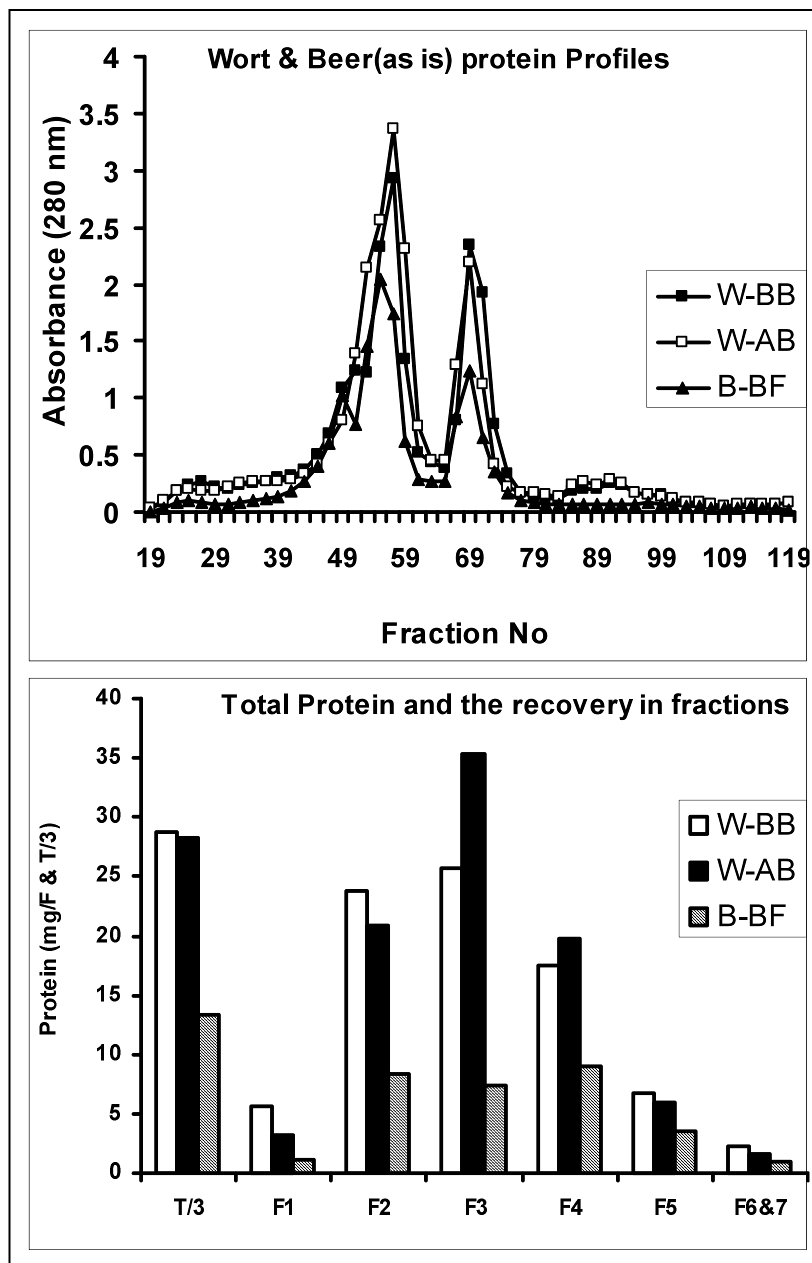

Fig. 3. Protein and peptide profiles of brewery wort before (W-BB) and after (W-AB) boiling and beer before filtering (B-BF) top and bottom the protein contents. Ten mL each, after centrifugation, was loaded to the column without any treatment.

electrophoretic picture presented here is very similar to those reported earlier by other researchers using similar conditions[9,14,15,29]. There is full agreement that beer proteins are mostly constituted by peptides of less than 20×10^3 mol. wt.

All samples were coloured but with different degrees of intensity. The boiled samples of wort from the brewery were the most intensely coloured and the least were the barley worts. This finding confirms the importance of heat treatment in colour development. Colour is developed from the combination of phenols and phenol derivatives with sugars and proteins, through Maillard reactions. The spectral analysis of worts and beer without any treatment (top) in comparison to GCL-300 fraction F1 of the same samples (bottom) are shown in Fig. 7. From both graphs it is clearly evident that these samples absorb light more in the ultraviolet (UV) region than they do in the visible range. Two peaks, one at 330 nm and the other at 290–300 nm, can easily be discerned in the spectra of fraction F1 (Fig. 7, bottom). However, they are fused into a broader peak in constituted whole samples (top). Those two peaks of UV absorption indicate the prevalence of flavonoids and hydroxycinnamic acid derivatives, respectively[3]. Beer, after filtration, has the highest absorption level at 330 nm whereas the boiled wort the lowest level. It is important to note that colour is closely associated with proteins and peptides, especially those of higher molecular weight.

This is an interesting finding as most haze formation is

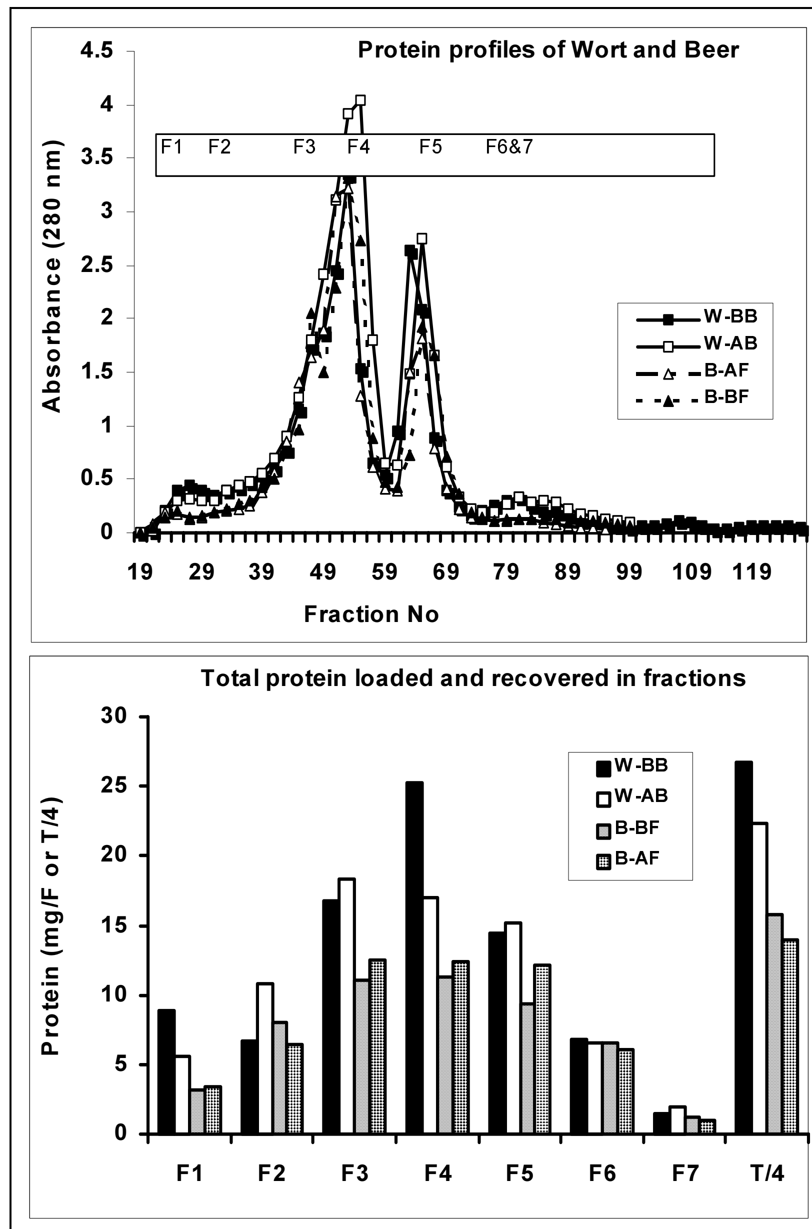

Fig. 4. Protein and peptide profiles of the same wort and beer, as in Fig. 3, except that 20 mL of wort and 40 mL of beer, each, were concentrated to 11–13 mL and loaded to the same column.

considered to be the result of oxidation of beer phenols (presumably free), followed by polymerisation and association with hydrophobic proteins[8,23,26]. In contrast, these results suggest that the phenol association with protein precede further oxidation of either the sulfhydryl groups or phenol-protein or peptide derivatives that lead to polymerisation and beer haze formation. The further oxidation in beer, during storage, might lead to formation of more hydrophobic proteins of even higher molecular weights and hence haze formation. The involvement of smaller peptides in the oxidation and further polymerisation of proteins is a possibility.

There are a number of outstanding features and characteristics revealed by the results presented above. Firstly, the similarities in the profiles of the laboratory and commercial brewery wort samples compounded with the resemblance in barley and beer profiles yield interesting findings. Among the latter, the evidence confirming that laboratory studies, in this case, are a close reflection of what goes on in the brewhouse. Therefore, and as a consequence, the methodology of this study can be applied to barley selection in breeding for proteins of high malting quality attributes such as foam-formation and retention and reducing haze defects. Such studies can be carried out on barley and confirmed with micromalting.

Secondly, the most effective processing step, inflicting quantitative and qualitative changes in the protein contents and profiles, is the malting and to a small degree mashing processes. It is evident that malting (germination) increases the total amount of proteins and peptides by up to three

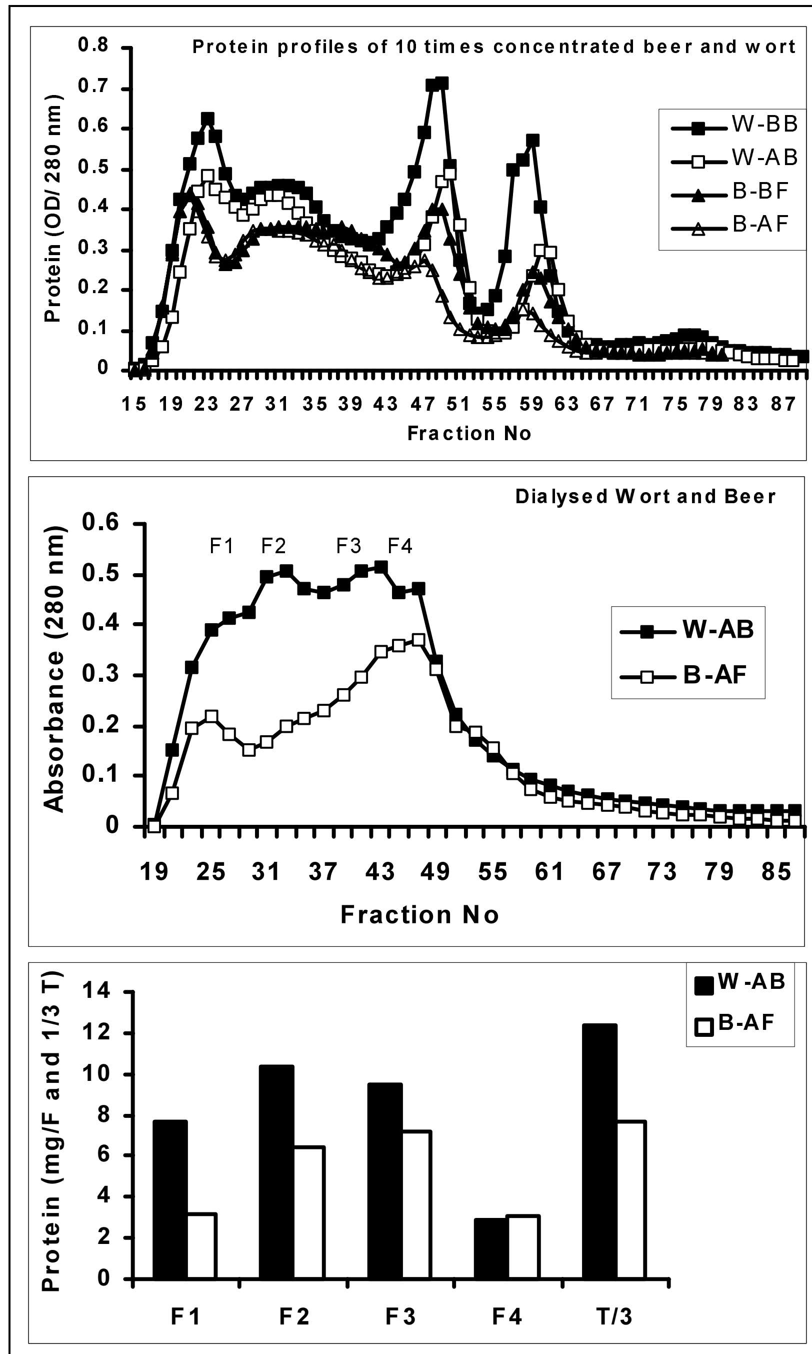

Fig. 5. Protein and peptide profiles of highly concentrated (top) and dialysed (middle) 10 mL and 15 mL each of wort and beer, respectively. The protein contents of the dialysed samples (bottom).

times, adding more proteins and peptides. Mashing seems to increase the soluble proteins, especially in barley worts.

Thirdly, and in contrast to malting and mashing, the remaining processing steps, namely, boiling, fermentation and beer filtering have no significant effects, if any. Boiling of wort, as well as the presence and quantity of hop are generally believed to denature the high molecular proteins and lead to their precipitation and removal, in noticeable quantities. However, there is no evidence here to support such a contention. It appears that only small amounts are removed, in agreement with some recent reports[13,16,30].

As regards the removal of high mol. wt proteins by heat denaturation, which was based mainly on SDS-PAGE results, it has been shown here not to be accurate. There were no bands of water-soluble high molecular weight proteins (>50,000 Da) detected in worts of barley or malt before boiling (see Fig. 6). Furthermore, the protein contents before and after boiling were similar with no drastic differences when all conditions were considered e.g. evaporation, dilution by adding sugars etc. The variations were within the 5 to 10%, which is in our opinion a small fraction. In literature reports[13,16,30], there is often reference to

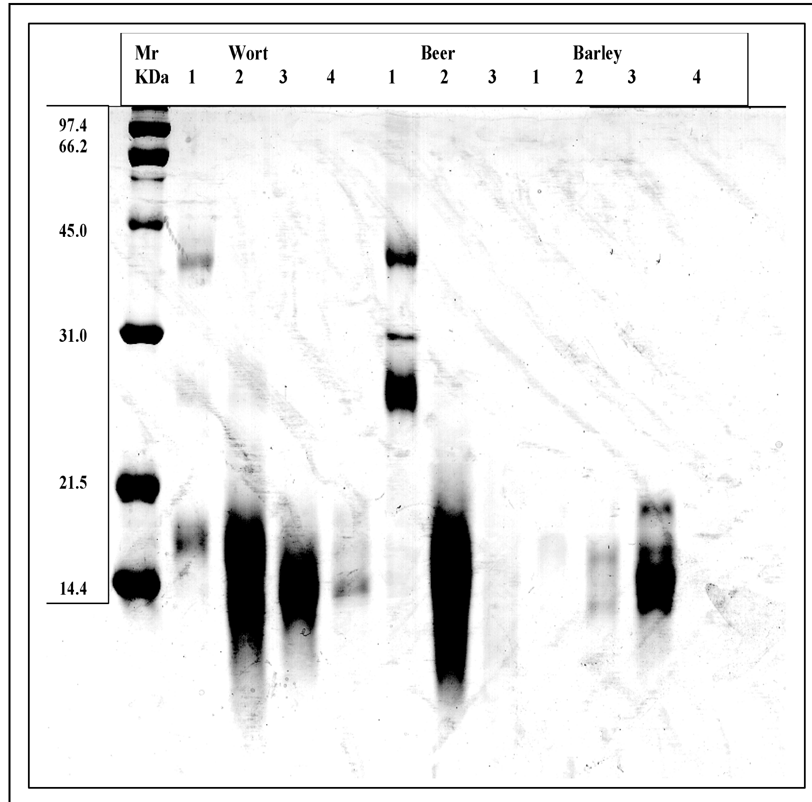

Fig. 6. SDS-PAGE separation of proteins and peptides of GCL-fractions 1 to 4 of wort, beer and barley stained with Coomassie Blue.

the absence of high molecular weight proteins as an indication to their loss by denaturation and precipitation. However, the most likely cause of their absence is either their non-existence or the dissociation of high molecular polypeptides into smaller ones due to reduction, as mentioned above.

Similarly, there is no noticeable removal of proteins during beer filtration. On the contrary the protein level appears to be increased perhaps due to proteins added at the fining stage (12.5 mg/L). Finally, fermentation does not seem to affect the proteins or polypeptides much. The latter suggests that the nutritional needs of yeast seem to be entirely satisfied by free amino acids and, perhaps, the smallest peptides such as di-, tri- and tetra-peptides produced during malting.

Fourthly, the amino acid composition of the proteins and peptides of wort GCL-300 fractions (Table I) indicate that the proteins or polypeptides (fractions F1 to F4) closely match those of barley glutelins. In contrast, the amino acid composition of the smaller peptides of fraction

TABLE I. The amino acid compositions of barley soluble proteins, barley hordein, EBC-wort and EBC-wort fractions separated by gel chromatography on GCL-300 column (mol/100 mL).

Amino acid	Barley soluble proteins	Barley hordein	Barley glutelin	Wort soluble protein	Wort GCL-F1	Wort GCL-F2	Wort GCL-F3	Wort GCL-F4	Wort GCL-F5	Wort GCL-F6	Wort GCL-F7	Wort GCL-F8
Asp D/N	7.32	0.74	6.88	6.9	6.97	5.7	8.32	9.77	7.45	3	2.6	5.28
Glu E/Q	10.97	10.52	17.14	17.62	13.58	16.36	17	16.06	8.1	4.2	8.72	3.75
Ser	6.17	4.03	6.31	6.45	7.16	5.68	6.9	6.97	6.87	3.39	2.47	1.89
Gly	9.38	3.18	8.96	8.96	7.87	8.18	10.43	10.93	7.6	7.37	7	.062
His	2.38	1.4	2.57	1.92	2	1.53	1.84	1.72	1.91	1.29	2.8	0.35
Arg	5.59	1.53	4.57	3.14	3.6	2.13	2.44	2.96	6.95	2.71	1.74	1.38
Thr	6.08	3	5.27	5.67	6.27	6.9	5.23	5.11	5.25	3.1	6.36	5.97
Ala	11	1.44	7.91	9.2	10.72	17.8	9.28	9.31	7.56	4.56	3.73	0.8
Pro	9.11	52.58	12.9	14.92	8.15	17.85	18.1	15.26	12.83	6.53	4.1	55.77
Tyr	3.41	9.12	2.99	0.92	3.12	0.92	1.11	1.98	4.12	0	0.78	9.25
Val	7.92	3.51	6.39	7.09	8.15	5.71	6.38	6.09	5.75	1.87	1.23	0.34
Met	2.13	0.69	1.68	1.43	2.5	1.25	1.19	2.04	2.75	1.48	1.5	5.38
Leu	2.95	3.35	2.35	2.83	3.06	1.93	2.47	3.22	13.11	0	0	6.88
Ile	7.19	1.54	6.83	6.76	9.19	4.77	5.35	4.32	0.44	59.24	55.63	0
Phe	3.29	3.19	3.89	3.14	4.63	2.34	2.11	2.02	7.08	0.82	0.46	2.34
Lys	5.1	0.13	3.65	3.28	3.03	0.95	1.86	2.23	2.22	0.39	0.86	0

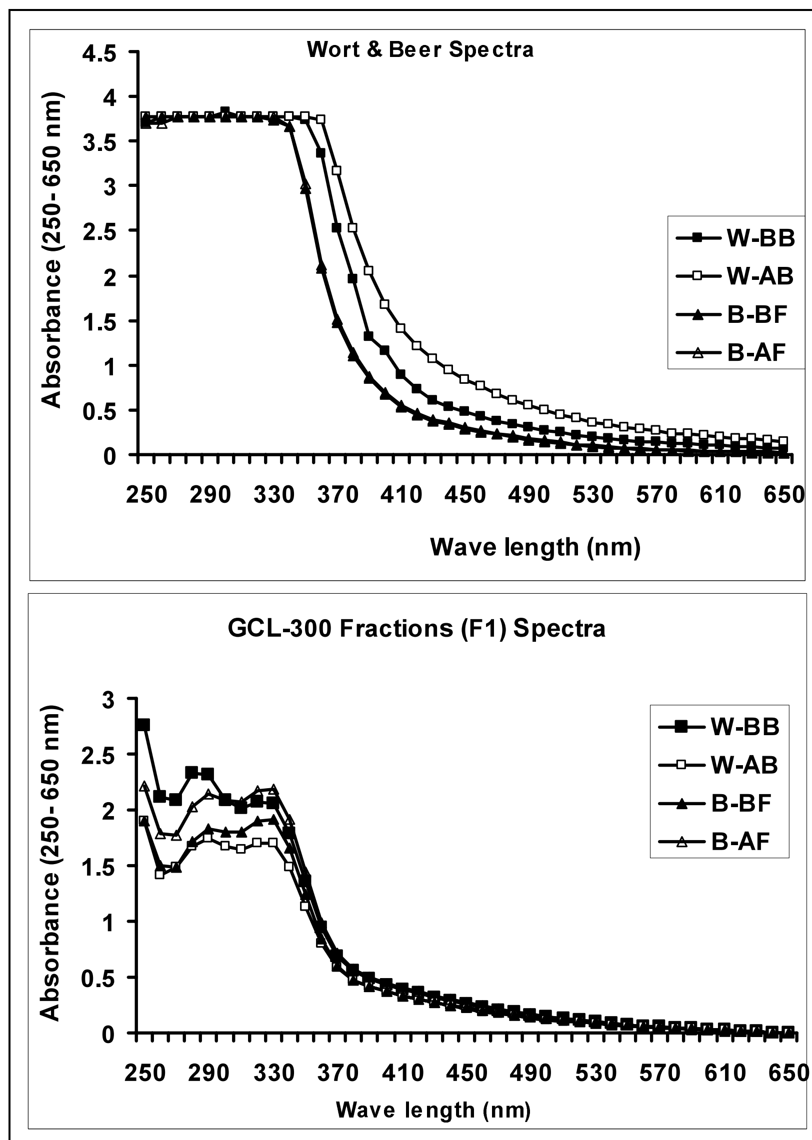

Fig. 7. Spectrograms of wort and beer without any treatment (top), and the spectrograms of fractions F1 from Fig. 5 top, concentrated before measuring their spectra, bottom.

F5 to F8, which were mainly produced during malting, appear as a mixture of hordein and glutelin. This suggests that the proteins degraded during malting are most likely a mixture of hordein and glutelin[20,25,28]. It is also possible that hordeins are more completely degraded to amino acids and smaller peptides compared to glutelins which appear to be the source of most peptides, confirming our earlier reports[17,18]. This is further supported by the view that some hordein and glutelin groups may structurally overlap and are hard to separate[24]. From our amino acid composition data, it is more likely that true hordeins are high in proline and true glutelins are high in glutamines. A mixture of some of the two groups may have similar characteristics due to common homologous sequences. For instance, 8 monoclonal antibodies from a library developed for wheat prolamins were found to interact with barley and beer polypeptides[9]. There is a strong need to further investigate and characterise the cereal prolamins, including classification. That will lead to better understanding of these proteins, especially, their technological attributes and the nutritional values.

CONCLUSIONS

The results of this study confirm that soluble protein more than doubled during malting due to increases in proteins of 40×10^3 mol. wt and lower and especially smaller proteins and peptides of 14×10^3 mol. wt and lower. The combined and integrated results of our chromatographic and SDS-PAGE data strongly suggest that most of the wort and beer high mol. wt (HMW) proteins are formed by combination of peptides. This is most likely, resulting from the oxidation of both or either sulfhydryl groups and/or phenol derivatives. There was no evidence to support the widely perceived view that considerable amounts of HMW proteins are coagulated and removed during kettle boiling.

Only small changes were observed. In contrast, boiling increased the protein contents due to evaporation and raised stability of the soluble proteins as was clearly evident in the case of barley wort. The results also indicate that wort and beer colour are closely associated with their protein component of common origin, barley and malt.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Grains Research and Development Corporation (GRDC). They also express their appreciation and gratitude to the staff of Barley Quality Laboratory for their support and Mr C. Wood of UQ for amino acid analysis. The contributions of Professor J. de Jersey, Professor S.E. Hamilton and Dr P.A. Inkerman are highly appreciated.

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(Manuscript accepted for publication November 2002)