CHARACTERIZATION OF STABILIZED AND UNSTABILIZED BEERS

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ABSTRACT

The preparation and properties of polyvinylpolypyrrolidone (PVPP) and silica gel and the mechanisms by which they stabilize beer are discussed. In stabilized beer, chill stability was associated with lower protein sensitivity to precipitation with tannins. Approximately 60% of the proteoses were of low molecular weight (<10,000 daltons). The most acidic of these proteoses were the least stable in solution, being precipitated from beer by cold treatment and adsorbents.

INTRODUCTION

Haze formation is regarded usually as a consequence of the tanning reaction between polyphenols and proteins (Belleau and Dadic 1981; Maza Selas 1987; McMurrough *et al.* 1985; Yu 1987). Simple flavanols (anthocyanogens), owing

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to their tendency to undergo acid-catalyzed and autoxidative polymerization, are generally regarded now as the precursors of protein-polyphenol hazes in unstabilized beers (Gramshaw 1969; Derdelinck and Jerumanis 1986).

On basis of amino acid analyses, it has generally been assumed that the majority of the barley proteins are involved in processes leading to formation of the beer protein found in chill haze (Djurtoft 1965). Beer contains little true proteins, mostly the products of proteolysis, that is, proteoses (Bishop 1975; Dale 1986).

Different adsorbents such as Polyclar AT (polyvinylpolypyrrolidone; PVPP), silica gel and others have been used for beer stabilization (Tarasevich *et al.* 1978; Helin 1980; Gromus 1981; McMurrough and Hennigan 1984; Boschet 1985; Derdelinck and Jerumanis 1986; Jones and Deyber 1986).

Owing to effective stabilization methods in the beer industry, composition of heterogeneous beer proteins and polyphenols have not been studied in detail with emphasis on protein isolation, fractionation and characterization (Meredith and Trachuk 1964; Djurtoft 1965; Woof and Pierce 1968; Asano *et al.* 1984; McMurrough *et al.* 1985).

The aim of the present investigation was to determine and to fractionate the polyphenols, and low and high molecular weight polypeptides after beer treatment with different adsorbents. The techniques of sequence extraction, isoelectric focusing and electrophoresis were used for this purpose.

MATERIALS AND METHODS

Maccabee beer samples were used in this investigation. Control Maccabee beer was prepared using a standard industrial technological process with 70% malt, 17.5% sorghum and 12.5% sugar. Mashing was done by decoction. All cycles were between 21 and 28 days (Control, Table 1).

Two series of experiments were carried out during this investigation.

The first one was conducted under industrial conditions using a single tank process (STP) and included the following preparation. Control A (Table 1) was done exactly under the same industrial conditions as the Control, but only using a single tank process (STP). For stabilization Protesal at 0.05 g/L was used. Test A was conducted under the same conditions as Control A with the use of stabilizing agents such as Polyclar AT (an insoluble, high-molecular weight, cross-linked form of polyvinylpolypyrrolidone) which is reported to remove phenolic impurities (Helin 1980; Siebert *et al.* 1981; McMurrough and Hennigan 1984). PVPP was used at 0.2 g/L and silica hydrogel at 0.5 g/L.

The second experiment involved the following technological process. Maccabee beer was prepared during mashing under the same standard industrial technological process as in the first experiment. After mashing, one part of the

mash was transferred to a vertical fermentation tank (about 8 days fermentation) and the second part to horizontal fermentation tanks using the same time of fermentation. After fermentation, beer samples were transferred to the storage tank separately. Potassium metabisulfide (2 g/hL) and Protesal (5 g/hL) were added to the fermented samples. The samples were kept under such conditions during 14 days. Beer fermented in horizontal tanks after storage was divided into two parts; one of them was filtered (Control B) and the second was treated with PVPP at 0.1 g/L during 24 h at 1 °C (Test B). The same was done with the beer samples fermented vertically; Control C without PVPP and Test C with PVPP at 0.1 g/L. Iron, copper, phosphates, sulfur, organic composition, sugars, chill haze, polyphenols and anthocyanogens were determined by EBC conventional methods of analysis (Analytica-EBC III, 1975–1977).

Nitrogen levels were obtained by micro-Kjeldahl total nitrogen determination and protein nitrogen was assessed by the Lowry method (Lowry *et al.* 1951; Patel and Ingledew 1973). Albumin and globulin concentrations were measured colorimetrically according to Sigma, Technical Bulletins 630 and 560 (1976 and 1976a).

In addition to conventional methods used in analysis of beer, qualitative and quantitative analyses were also conducted, including beer concentration (dialysis and lyophilization), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing (PAGIF) (Nummi *et al.* 1969; Sorensen and Ottesen 1978; Lewis *et al.* 1980; Moonen and Graveland 1987; Dale and Young 1988). Precipitation of beer proteoses by salting out was done according to Nummi *et al.* (1969).

High molecular weight fractions of beer samples were prepared according to Dale and Young (1988). Samples of beer were placed in dialysis tubing and dialyzed against several changes of distilled water for 72 h. The material retained by the dialysis membrane was freeze-dried. In addition, 100 mL of beer sample was placed in a 1 L round bottom flask followed by 500 mL of acetone. The mixture was shaken, then allowed to stand. The precipitate which formed was washed twice with fresh acetone, then freeze-dried. These freeze-dried samples, as well as the precipitated beer proteoses, were applied to SDS-PAGE and PAGIF. The protein content in each of these precipitates was determined by the Bradford assay (1976). Serva Blue G from Serva (Terochem, Laboratories Ltd.) was used instead of Coomassie Brilliant Blue G 250.

PAGIF was done using the Pharmacia Phast System with the PhastGel IEF (isoelectric focusing) media 3–9, containing Pharmolyte carrier ampholytes, pH range 3 to 9. All stages of PAGIF were performed according to the Pharmacia Owners Manual (1987). Broad range pI calibration kit (pI range pH 3–10) was obtained from Pharmacia. Protein concentration of the sample was about 200 μg/mL and 1 μL per lane was applied.

TABLE 1.
RELATIONSHIP BETWEEN HAZE FORMATION IN BEER AND ITS COMPOSITION

No. of a sample	Name of a sample	Haze, FTU	Elemental composition, % by dry substance			Metals, mg/L				Polyphenols, mg/L	Anthocyanogens, mg/L
			C	H	S	Fe	Cu	Ni	P		
1	Control	450	39.67	6.28	0.87	0.072	0.023	0.078	262.5	165	33.4
2	Control A	430	40.48	6.23	0.80	0.063	0.027	0.051	262.5	155	30.1
3	Test A	255	40.77	6.27	traces	0.079	0.017	0.069	321.2	137	20.3
4	Control B	270	39.55	6.50	0.29	0.015	0.020	0.081	169.0	136	25.0
5	Test B	200	39.26	6.29	0.27	0.020	0.010	0.045	173.0	128	16.2
6	Control C	270	39.45	6.20	0.30	0.043	0.023	0.069	177.0	117	11.0
7	Test C	177	39.67	6.47	0.31	0.056	0.030	0.045	173.0	109	10.0

TABLE 1. (cont.)

Tannins, mg/%	Sugars, % Total	Reducing	Nitrogen content, mg/100 mL	Albumin, mg/L	Globulin, mg/L	Total protein content, g/L	Ammonium Sulfate prec. limit, mL	Final clouding after forced test, FTU
84	20.0	15.3	84.3	231.45	3.043	4.98	0.6	518
81	18.0	13.6	81.2	189.30	3.003	4.74	0.6	522
75	17.6	10.6	79.3	150.74	3.003	4.07	0.8	341
79	26.6	13.8	80.9	175.20	2.641	4.21	0.7	392
71	18.0	10.6	77.3	140.13	2.561	3.69	0.8	320
80	24.8	13.0	79.3	170.24	2.385	4.29	0.8	380
69	20.6	10.0	72.5	130.21	2.132	2.24	1.0	295

Control = normal industrial conditions; Control A = Control + STP; Test A = Control A + PVPP treatment; Control B = Control A + horizontal fermentation; Test B = Control B + PVPP; Control C = Control A + vertical fermentation; Test C = Control C + PVPP; STP = single tank process; Forced tests = The beer was cooled at 0°C for 24 h, then kept at 60°C for 8 days, followed by storage for 1 day at 0°C; FTU—formazin turbidity units; Mean average 8 determinations. Average coefficient of variation (CV) = ± 0.68

Preliminary subunit molecular weight determination was done using the same Pharmacia Phast System with preprepared PhastGel gradient (10–15%) and PhastGel buffer strips. All procedures were carried out according to the Owners Manual 110 and 200 (1987). Protein concentration was 2.2 mg/mL. Ten μL of sample was mixed with 10 μL of buffer and 0.5 μL per lane was applied.

For exact molecular weight (MW) determination, a slab gel technique according to Laemmli (1970) was used. The equipment used was: SE 200 Mighty Small I Slab unit, Hoefer Scientific Instruments; voltage, current regulated power supply, Brinkmann Instruments; Gradient Former, Buchler Instruments; Polystaltic pump, Buchler Instruments. Gel dimensions were 83 × 73 × 1.5 mm. Chemicals were purchased from Bio-Rad Laboratories. Mr values for protein subunits were obtained from a calibration graph prepared from the following standards analyzed by SDS-PAGE (subunit Mr in parentheses): I. (MW-SDS-70L Kit) bovine albumin (60,000); egg albumin (45,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); carbonic anhydrase (29,000); bovine pancreas trypsinogen (24,000); soybean Kunitz trypsin inhibitor (20,100) and bovine milk α-lactalbumin (14,200); II. MW-SDS-200 kit; rabbit muscle myosin (205,000); *Escherichia coli* β-galactosidase (116,000); rabbit muscle phosphorylase B (97,400); bovine plasma albumin (66,000); egg albumin (45,000) and carbonic anhydrase (29,000). The standards were obtained from Sigma Chemical Co. The separation gel contained a 10–20% gradient. The running buffer was pH 6.8. The running time was 130 min, using 18 mA per gel. The diluted concentration of lyophilized sample buffer was about 432 μg/mL and 20μL were applied in one well. Mean values of 8 determinations are reported in this study. Analysis of variance was done on the original data. In this study all statistical data were determined by Duncan's (1955) multiple range test. Correlation between the contents of various constituents is made.

RESULTS AND DISCUSSION

From the data of Table 1, it can be seen that the lowest haze formation was found in the sample of Maccabee Test C. Test C also contained the lowest amount of polyphenols, anthocyanogens, total and protein N. Treating beer with adsorbents decreased the N-compounds content from 84.3 to 72.5 mg/100 mL and thus helped to prevent colloid formation in the finished product. Elemental composition of the samples (C; H; S) and the amount of Fe, Cu, Ni, phosphates and sugars were similar in all investigated samples. It was found that treatment of beers with different stabilizing agents, such as Polyclar AT and silica hydrogel, retarded haze formation by restricting protein-polyphenol interactions. These results agreed with the literature (McMurrough and Hennigan 1984; Boschet 1985; Derdelinck and Jerumanis 1986).

During storage of unstabilized beer, the decrease in colloid stability was caused by an increase of polyphenols (Table 1). Stabilization of beer by treatment with either or both Polyclar AT and silica hydrogel increased the stability of the samples. The effect of Polyclar AT was to lower the contents of simple flavanols (anthocyanogens), whereas silica hydrogel affected mainly the more complex substances (Table 1). The highest stability was achieved by PVPP treatment with vertical fermentation.

Anthocyanogens, which are a very specific class of polyphenols and responsible for chill haze in beer, decreased during PVPP treatment. Polyphenols and anthocyanogens, 34.0% and 70.0% respectively, were removed from Test C vs Control. Total tannins and anthocyanogens were determined in fresh and forced (by treating at 60 °C for 72 h) beers (Table 1). Total tannins increased while anthocyanogen values did not reveal any large changes on forcing. The tanninogen values followed the same pattern as the catechin value.

Low molecular weight proteoses, as well as high molecular weight beer polypeptides, were characterized in terms of their isoelectric points. Some material is present at the extreme acid end of the gel (pH 3). All pI values were in the range of 3.75–4.55. The identification and isolation of these proteins, which play an important role in haze formation, may provide information on the effect of certain treatments on beer stability.

Some proteoses were present in experimental beers in concentrations close to their solubility limits. Cold storage lowered the solubility of amphoteric molecules near their isoelectric points and resulted in loss of acidic proteoses. These proteoses, with MW ~ 10,000 daltons, were only precipitable by 15% (w/v) TCA in PAGIF and smaller ones were not detected in our experiments. According to the literature (McMurrough *et al.* 1985; Dale and Young 1988), the acidic proteoses with pI values in the range 4–6 have been cited as a major factor in the formation of chill haze. The ammonium sulfate precipitates obtained from beers following cold storage (1 °C during filtration and PVPP treatment) contained fewer acidic proteoses than did the original untreated samples.

Figure 1 shows that during electrophoresis the proteins concentrated in two bands; the main protein fractions at 44,000 and 14,000–10,000. Comparison of line 4 with 3 and 5 (Fig. 1) shows that fraction 38,000 is lower in sample Test A than in Control A. Sample Test C did not have a minor fraction of about 38,000 as did Control A.

Control A (line 3), Controls B and C (lines 6 and 7, respectively) are very similar to one another and differed from Test A which was treated with PVPP. These data correspond to previous reports that the proteins of beer are a heterogenous group of substances with molecular weights ranging from about 100,000 down to the polypeptide class of material (Bishop 1975; Sorensen and Ottesen 1978; Lewis *et al.* 1980). The data of Fig. 1 correspond exactly with literature

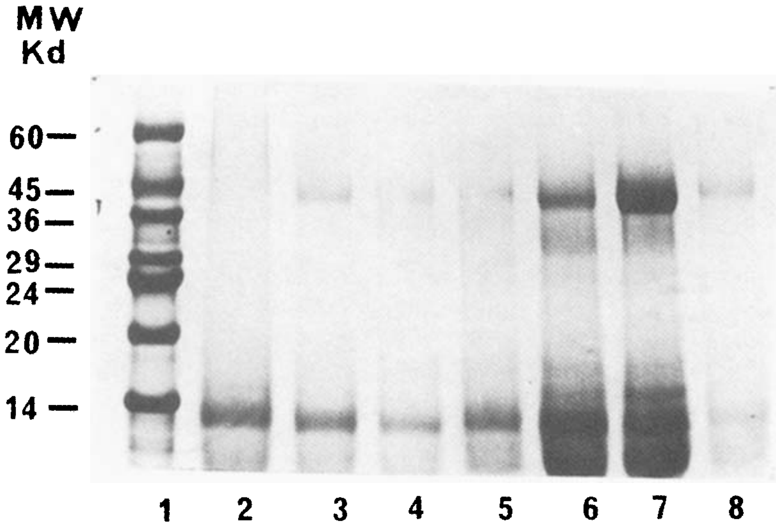

FIG. 1. ELECTROPHORETIC PATTERNS OF BEER SAMPLES
1 - Standard (10 μL); 2, 3, 4, 5, 6, 7, 8- respectively, 20 μL, of lyophilized samples of Test C; Control A; Test A; Control A with acetone precipitation; Control B; Control C; Control C with acetone precipitation.

reports (Sorensen and Ottesen 1978) of a high molecular weight fraction of 44,000, but differed in the low molecular weight range (about 10,000). The difference in low molecular weight proteins, as well as of 32,000, can be explained by different stabilities during treatment of beer samples with adsorbents as well as by use of various techniques for protein precipitation (Fig. 2). Precipitation with ammonium sulfate gave about 70% of the proteins of low molecular weight ($<$15,000, which does not correspond exactly with other authors) (Sorensen and Ottesen 1978; Dale and Young 1988).

Sequential extraction by acetone-water solvents showed that some of the proteins, which are present in beer with polyphenols, may dissociate on treatment with hydrogen bond-breaking solvents like acetone. Acetone precipitation was investigated as a method of avoiding possible interference effects from polyphenolic compounds which have been encountered in the fractionation of plant proteins (Dale and Young 1988).

The main sharp fraction of 44,000 was collected from two SDS-PAGE experiments. The fraction was cut from the gel and electroelution was done using the same running buffer. Then dialysis and lyophilization completed the preparation of a protein sample. SDS-PAGE was performed following the procedure used for separation. The observed protein composition (Fig. 2) indicated that all proteins were in the range of Mr 10,000–16,000 and 30,000–44,000, which have been tentatively identified as proteins previously isolated in association with

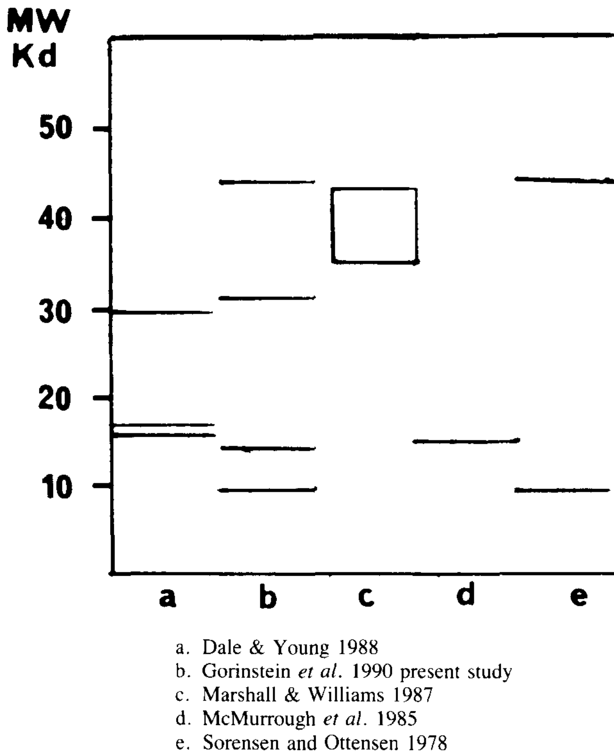

FIG. 2. MAJOR BEER PROTEIN BANDS BY SDS-PAGE AS REPORTED BY SEVERAL AUTHORS

foam and haze formation (Sorensen and Ottesen 1978; Marshall and Williams 1987). The electrophoretic patterns were reproducible both within and between beer samples and were independent of the sample preparation methods or the composition of the sample solubilization mix (Fig. 1).

Both chill and permanent haze involve reversible interactions between high molecular weight (HMW) polypeptides (hordein) and polyphenols (malt and hops). Ionic, hydrophobic and hydrogen bonding leading to chill haze precede the covalent attachment that gives rise to permanent haze. Covalent attachment may occur via the polymerization of polyphenols with phenolic residues of amino acids in the polypeptide chain.

Adjuncts that act as nitrogen diluents e.g., maize grits or carbohydrate syrups, in our case, reduce the level of HMW polypeptides and polyphenols to give improved haze stability.

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