Isolation and Characterization of Foaming Proteins of Beer[1]

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ABSTRACT

"Foaming proteins" that retained the full foaming capacity of the original beer were isolated by ultrafiltration followed by ammonium sulfate precipitation and ion-exchange chromatography on diethylaminoethyl-cellulose. Because the content of foaming proteins correlated well with head formation of many samples of beer, these foaming proteins seem to be responsible for beer foaming. Foaming proteins consisted of three fractions with molecular weights of 90,000–1,000,000, 40,000, and 15,000. These three fractions were all surface-active but differed in the mechanism of their contribution to foaming. The higher and medium molecular weight fractions combined with isohumulones through ε-amino groups to form more surface-active complexes and thereby enhanced foaming. The lower molecular weight fraction did not form these complexes appreciably, probably because of its low content of ε-amino groups. Immunological studies showed that foaming proteins were formed primarily during germination of barley. The level of foaming proteins decreased considerably during brewing, particularly during kettle boiling; 160–620 mg/L of foaming proteins survived in finished beer.

Key words: *Beer, Brewing process, Foam, Isolation, Malting, Protein*

Although some proteinaceous fractions of beer are generally thought to be very important in beer foaming, these proteins have not yet been fully characterized. Since Fürnrohr (8) first suggested the contribution of a proteinaceous fraction to beer foam in 1913, many attempts have been made to isolate and characterize the proteinaceous fractions responsible for head retention of beer. Particularly during the last 10 years, the physical (11,13), chemical (13), and immunological (13,15,18) properties of particular proteinaceous fractions, such as "fraction X" (6), have been elucidated, but the role of these proteinaceous fractions in beer foaming is still unknown. Recently, Sørensen and Ottesen (32) studied the fractionation of beer proteins in detail but were still uncertain which fractions were responsible for foaming.

We succeeded in isolating the "foaming proteins" responsible for foaming of beer. The present article describes the characterization of these foaming proteins and gives clear evidence for their contribution to beer foaming.

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METHODS

Preparation of Samples from Beer

As shown in Fig. 1, 400 ml of unhopped beer was subjected to ultrafiltration, using a Diaflo PM-30 ultrafiltration membrane, and the retentate (Fraction 1) in the ultrafiltration cell was saturated with ammonium sulfate at 0° C for one day. The resultant precipitate (Fraction 3) was collected by filtration through filter paper, redissolved in a minimum volume of deionized water, dialyzed, and applied to a diethylaminoethyl-cellulose column (3.5 × 25 cm) that had been washed with 0.5*M* phosphate buffer, pH 8.0, and deionized water. The column was eluted first with 800 ml of deionized water and then with 500 ml of 0.5*M* phosphate buffer, pH 4.2. The effluent with phosphate buffer (Fraction 6) was dialyzed and lyophilized.

Molecular Weight Estimation

By Gel Chromatography. The sample (2.5–4.5 mg) dissolved in 0.8 ml of 0.05*M* NaCl was applied to a Sephadex G-75 column (2 × 47 cm) equilibrated with 0.05*M* NaCl, and the column was eluted with 0.05*M* NaCl at a flow rate of 20 ml/hr. Effluent was collected in 4-ml fractions, and their protein and carbohydrate contents were measured, respectively, by the methods of Lowry et al (22) and of Molisch, as cited previously (7). The molecular weights of proteins were estimated by comparison of their elution volumes with those of standard proteins such as cytochrom c, myoglobin, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

The fraction eluted in the void volume from the Sephadex G-75 column was rechromatographed on a Sepharose 6B column (1.7 × 44 cm) equilibrated with 0.5*M* NaCl. The column was eluted with 0.5*M* NaCl at a flow rate of 20 ml/hr, and effluent was collected in 3.5-ml fractions and assayed for protein and carbohydrate as described. The molecular weight of protein was estimated using bovine serum albumin, γ-globulin, and apo-ferritin as standard proteins.

By Sodium Dodecyl Sulfate Gel Electrophoresis. The sample (4 mg) was dissolved in 0.5 ml of 0.01*M* phosphate buffer, pH 7.2, containing 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The solution was incubated at room temperature overnight, and then 10 μl was applied to the top of a 10% polyacrylamide gel (0.2 cm^2 × 9 cm) containing 0.1% SDS and subjected to electrophoresis by the procedure of Weber and Osborn

(35). The gel was stained with Amido black and destained by washing with 7% acetic acid. The mobility of the sample was measured by scanning the gel at 477 nm with a Shimadzu CS-910 dual wavelength spectrodensitometer, and the molecular weight of protein was estimated by comparison of its mobility with those of standard proteins such as cytochrome c, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

Affinity Chromatography on Con A-Sepharose

The sample (4.7–25 mg) was dissolved in 1 ml of 0.01 *M* Tris-HCl buffer, pH 8.0, containing 1% NaCl, 1m*M* $MgCl_2$, and 1m*M* $CaCl_2$ and applied to a Con A-Sepharose CL-4B column (2 × 12.5 cm) equilibrated with the same buffer. The column was first washed with 75 ml of Tris-HCl buffer, and then the glycoprotein fraction was eluted with 95 ml of the same buffer containing 0.05 *M* α-methyl-D-mannoside at a flow rate of 40 ml/hr. Effluent was collected in 5-ml fractions, dialyzed, and analyzed for protein (22) and carbohydrate (7).

Electrofocusing

Samples (0.1–0.4 mg) dissolved in 10–40 μl of deionized water were applied to 5% polyacrylamide gel plates containing 2% ampholytes (Ampholine PAG plate from LKB), in a pH range 3.5–9.5, and electrofocusing was performed at 8° C for 3.5 hr at a final voltage of 1,400 V and a final current of 10 mA. Proteins and carbohydrates in the gel were stained with Coomassie Brilliant Blue R-250 and periodic acid-Schiff reagent (12), respectively. The gel was scanned at 560 nm for protein and at 535 nm for carbohydrate.

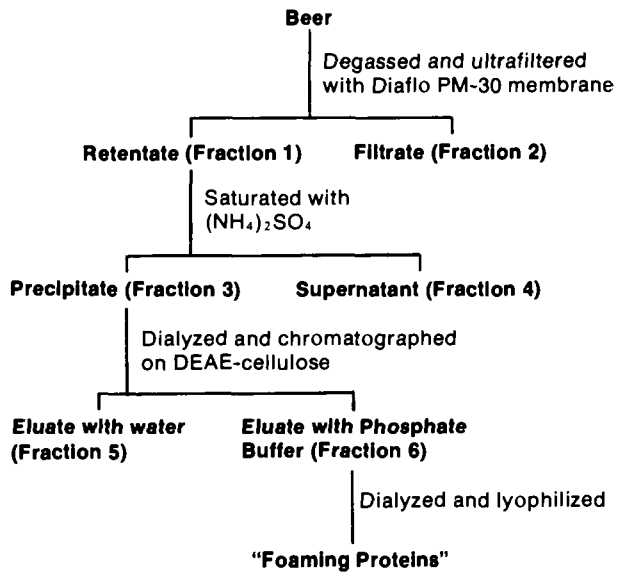

Fig. 1. Procedure for isolation of "foaming proteins" from beer.

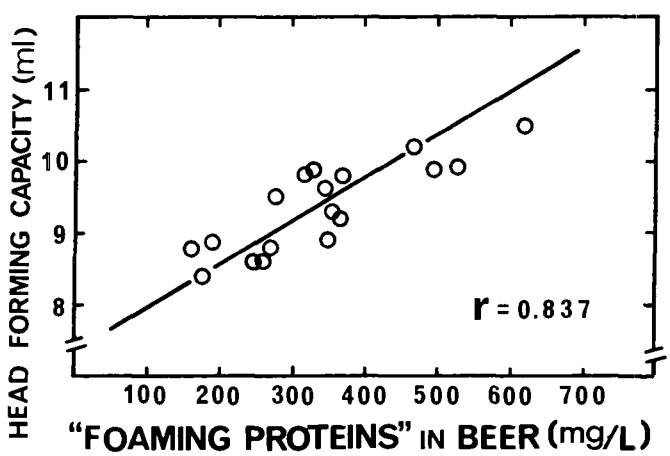

Fig. 2. Correlation between the content of "foaming proteins" and head forming capacity of lager beers. Values for head forming capacity were corrected for variation of isohumulone content (3).

Immunoelectrophoresis

Soluble proteins were extracted from barley, malt, and rice by the method of Grabar et al (10). The rabbit antisera towards these soluble proteins of barley, malt, and rice—intact yeast cells and foaming proteins—were prepared by Japan Immunoresearch Laboratories Co., Ltd.

Immunoelectrophoresis was performed by the method of Grabar and Williams (9) using 1% agarose gel in Tris-barbiturate buffer, pH 8.6, at 8° C for 1 hr at 10 V/cm. After electrophoresis, the trough of the gel was filled with antiserum, and the gel was left at room temperature for 24 hr to allow immunodiffusion. Immunoelectrophoresis at 8° C for 3 hr at 10 V/cm was performed by the method of Laurell (20), using 1% agarose gel in the same buffer containing anti-foaming proteins serum. Protein precipitates in the gel were stained with Coomassie Brilliant Blue R-250.

Chemical Analyses

The protein and carbohydrate contents of samples were determined by the micro-biuret method (17) and the phenol-sulfuric acid method (16), using bovine serum albumin and arabinose, respectively, as standards.

The amino acid composition of samples was analyzed with an amino acid analyzer, JEOL model JLC-6AH, after the samples (5 mg) were hydrolyzed with 3 ml of 6*N* HCl at 110° C for 22 hr in evacuated sealed tubes.

Constituent sugars in samples were analyzed by the methods of Sawardeker et al (29) after the samples (3.4–6.0 mg) were hydrolyzed with 2 ml of 1*N* H_2SO_4 at 100° C for 3 hr.

Measurement of Foam

Samples of 100–300 mg were dissolved in 1 L of 3.6% aqueous ethanol at pH 4.2. Then 20 ml of the solution in a graduated test tube (2.1 × 18 cm) was shaken up and down mechanically at 20° C for 5 sec (400 times per minute, 4 cm amplitude). The volume of the foam was then measured and recorded as the "head forming capacity (ml)." The head forming capacity of degassed beer measured in a similar way correlated well with head formation of carbonated beer determined by the pouring method (3) ($r = 0.91$, $n = 12$).

Preparation of Isohumulone

Isohumulones were extracted from Isolone (isomerized hop extracts of Kalsec Co.) with isooctane and purified by silica gel column chromatography (19).

TABLE I
Head Forming Capacity of "Foaming Proteins"

Beer Fraction	Concentration in Beer (mg/L)	Head Forming Capacity, ml	
		At 100 mg/L	At the Concentration in Beer
1	9,540	1.6	···
2	22,380	0.1	···
3	1,192	3.9	···
4	4,823	0.2	···
5	630	0	···
6 (Foaming proteins)	492	5.7	6.7
Original beer	···	···	6.9

RESULTS

Foaming Proteins from Beer

Table I shows the head foaming capacity of the beer fractions. Fraction 6 contained most of the foam-enhancing substances and had a head forming capacity equivalent to that of the original beer. Because 67% of the material in Fraction 6 was protein, we named this fraction "foaming proteins."

Figure 2 shows that the contents of foaming proteins ranging from 160–620 mg/L in lager beer, correlated well with the head forming capacities of these beers.

Fractionation of Foaming Proteins

A solution of 100 mg of foaming proteins in 10 ml of 0.05*M* ammonium formate was applied to a Sephadex G-75 column (5× 54 cm) equilibrated with 0.05*M* ammonium formate. The column was eluted with 0.05*M* ammonium formate at a flow rate of 55 ml/hr and effluent was collected in 15-ml fractions. Fractions of effluent containing higher, medium, and lower molecular weight materials, respectively, were combined as shown in Fig. 3, and rechromatographed in the same fashion to yield 21.7, 12.9, and 33.4 mg of lyophilized higher, medium, and lower molecular weight materials, respectively.

Figures 4 and 5 show that the medium and lower molecular weight fractions were almost homogeneous with respect to molecular weight. The molecular weights of these two fractions were estimated to be about 40,000 and 15,000 by gel chromatography and 36,000 and 10,000 by SDS gel electrophoresis. Figure 6 shows that the higher molecular weight fraction was composed of at least three subfractions with molecular weights of over 1,000,000, about 400,000, and 90,000, respectively.

Chemical Composition of Foaming Proteins

Table II shows that only 21% of the higher molecular weight fraction was protein, whereas 75 and 65% of the medium and the lower molecular weight fractions, respectively, were proteins. The amino acid compositions of these three fractions were similar to that of barley albumin or globulin (34). The higher and the medium molecular weight fractions contained more lysine than did the lower molecular weight fraction and did not contain cysteine and

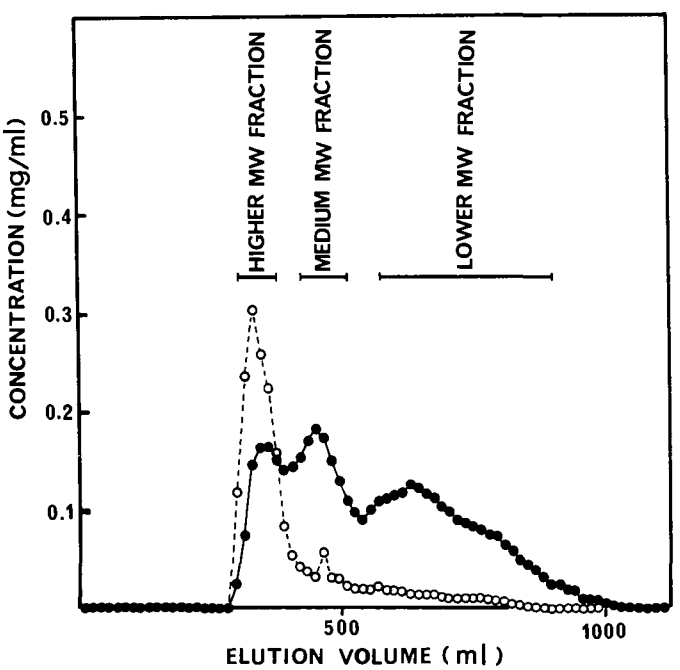

Fig. 3. Fractionation of "foaming proteins" by gel chromatography on Sephadex G-75. • = protein, o = carbohydrate.

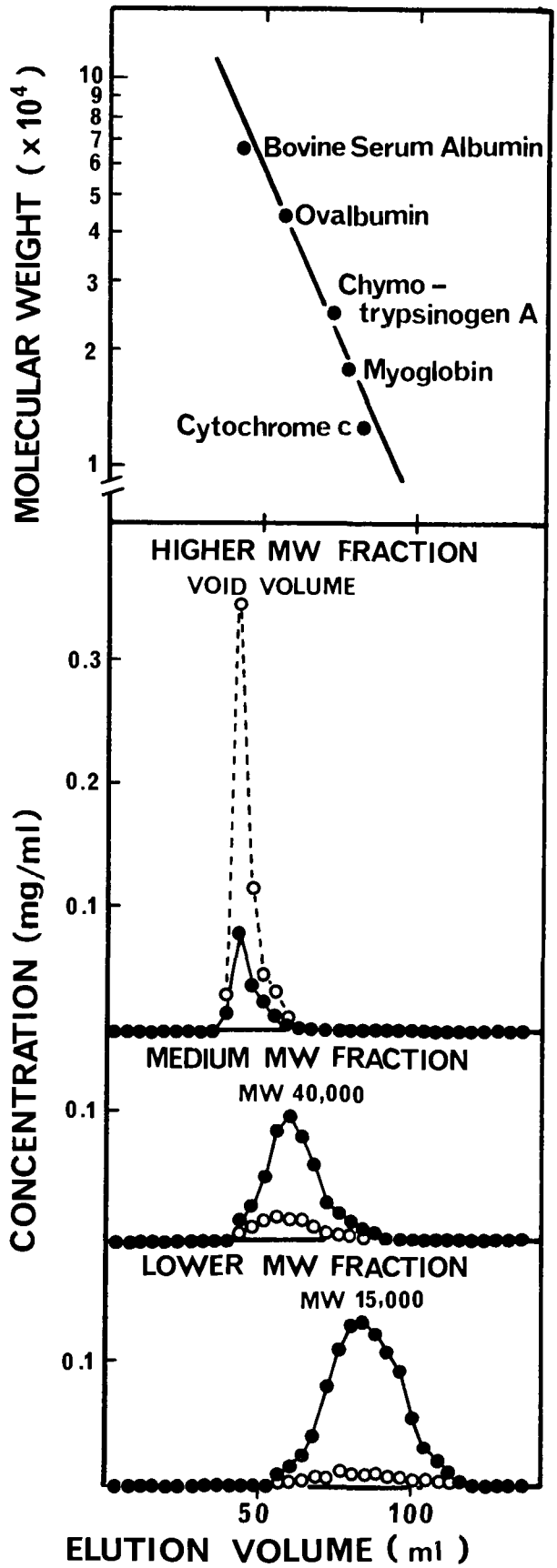

Fig 4. Estimation of molecular weight of higher, medium, and lower molecular weight fractions of "foaming proteins" (3.1, 2.5, and 4.5 mg, respectively), by gel chromatography on Sephadex G-75. • = protein, o = carbohydrate.

methionine.

On the other hand, 64, 17, and 12% of the higher, medium, and lower molecular weight fractions, respectively, consisted of the carbohydrates arabionse, xylose, and glucose with small amounts of mannose and galactose.

Figure 7 shows the isoelectric profiles of foaming proteins. The medium and lower molecular weight fractions contained more than ten protein species with isoelectric points of pH 4–5.5 and pH 3.5–5, respectively. The higher molecular weight fraction also contained at least six protein species with isoelectric points of pH 4–5.5. Because some of the protein species stained with periodic acid-Schiff reagent for carbohydrate, they seemed to be glycoproteins. Con A-Sepharose chromatography (Fig. 8) showed that about 60 55, and 15% of the proteins in the higher, medium, and lower molecular weight fractions, respectively, were glycoproteins.

Mechanism of Foaming of Foaming Proteins

Previously, we (3) found that foaming proteins combined through their ε-amino groups with isohumulones, and the resultant surface-active complexes enhanced the foaming of beer. Figure 9 shows that a solution of the lower molecular weight fraction had the highest surface activity and highest head forming capacity of the three fractions. When isohumulones were added to the solutions, the surface activities and head forming capacities of the

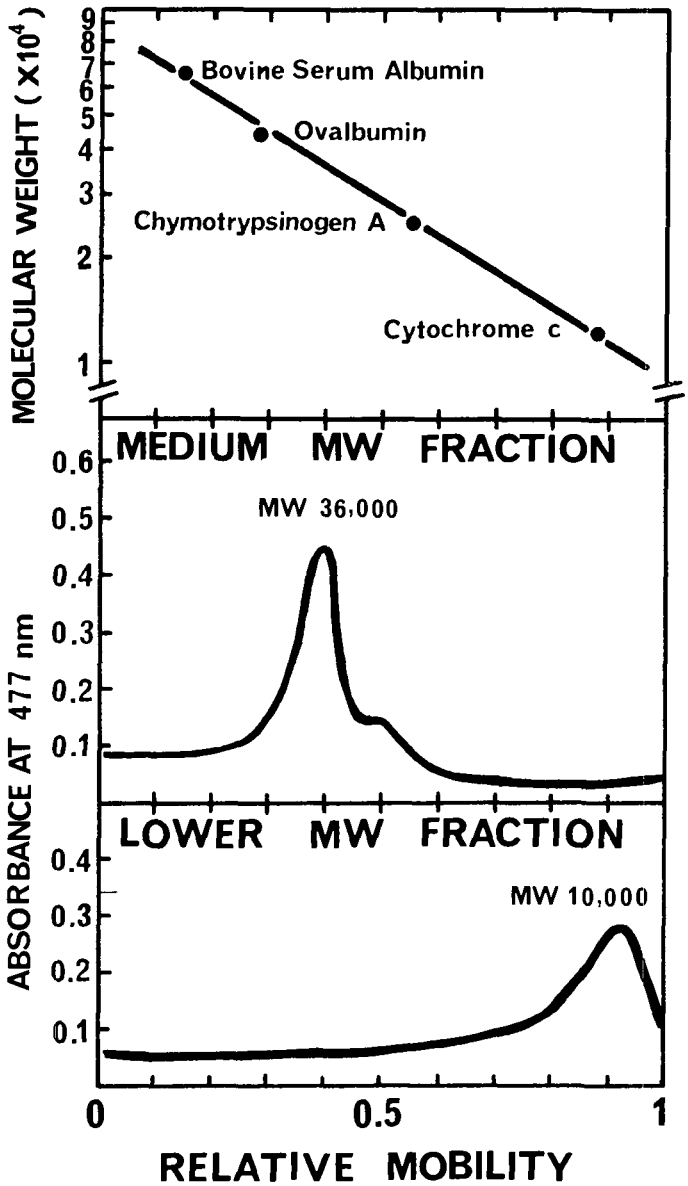

Fig. 5. Estimation of molecular weight of 5 μg of "foaming proteins" by sodium dodecyl sulfate gel electrophoresis. The higher molecular weight fraction did not migrate in 10% polyacrylamide gel.

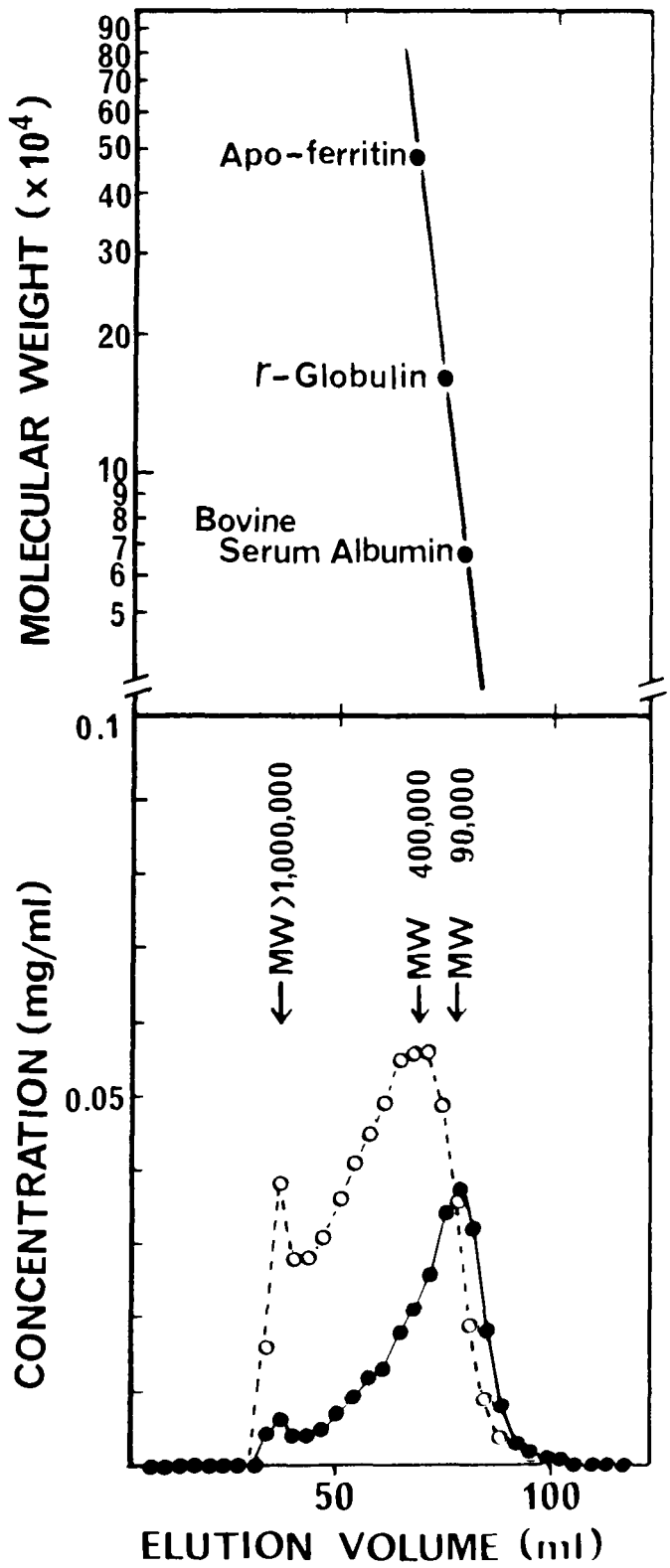

Fig. 6. Estimation of molecular weight of 3 mg of the higher molecular weight fraction of "foaming proteins" by gel chromatography on Sepharose 6B. • = protein, o = carbohydrate.

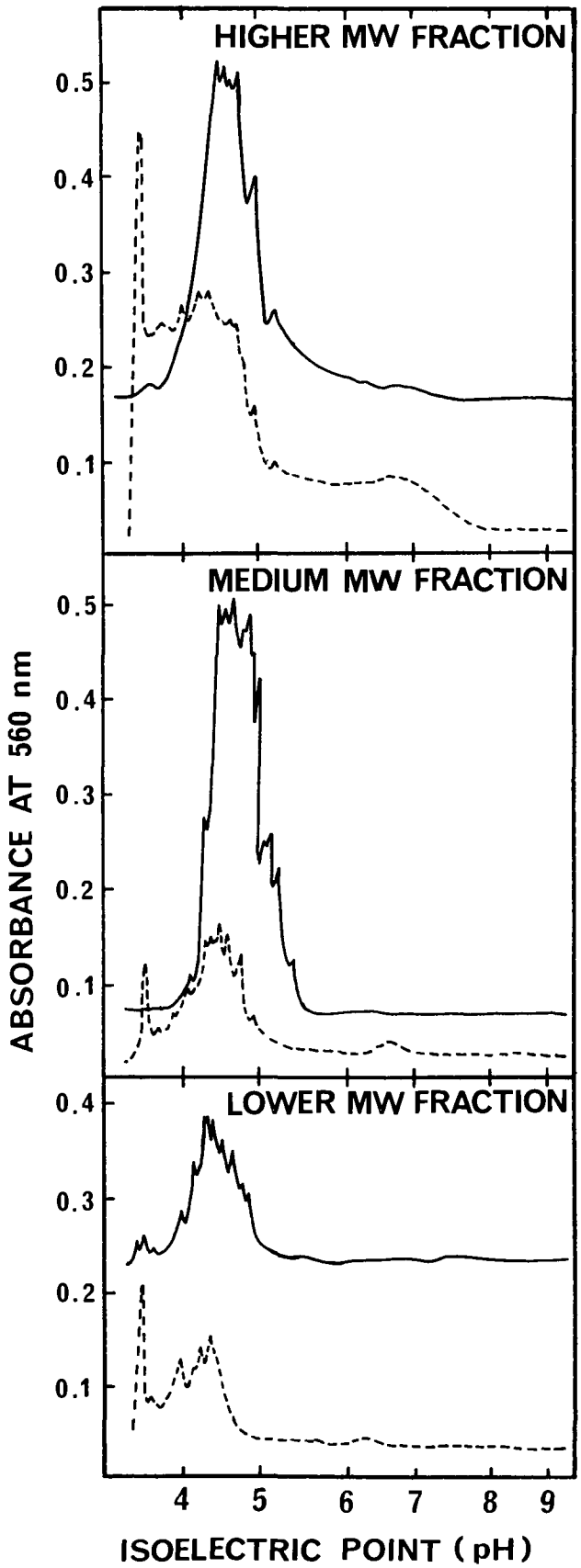

Fig. 7. Isoelectric profiles of higher, medium, and lower molecular weight fractions of "foaming proteins" (0.2, 0.1, and 0.4 mg, respectively). – = protein, --- = carbohydrate.

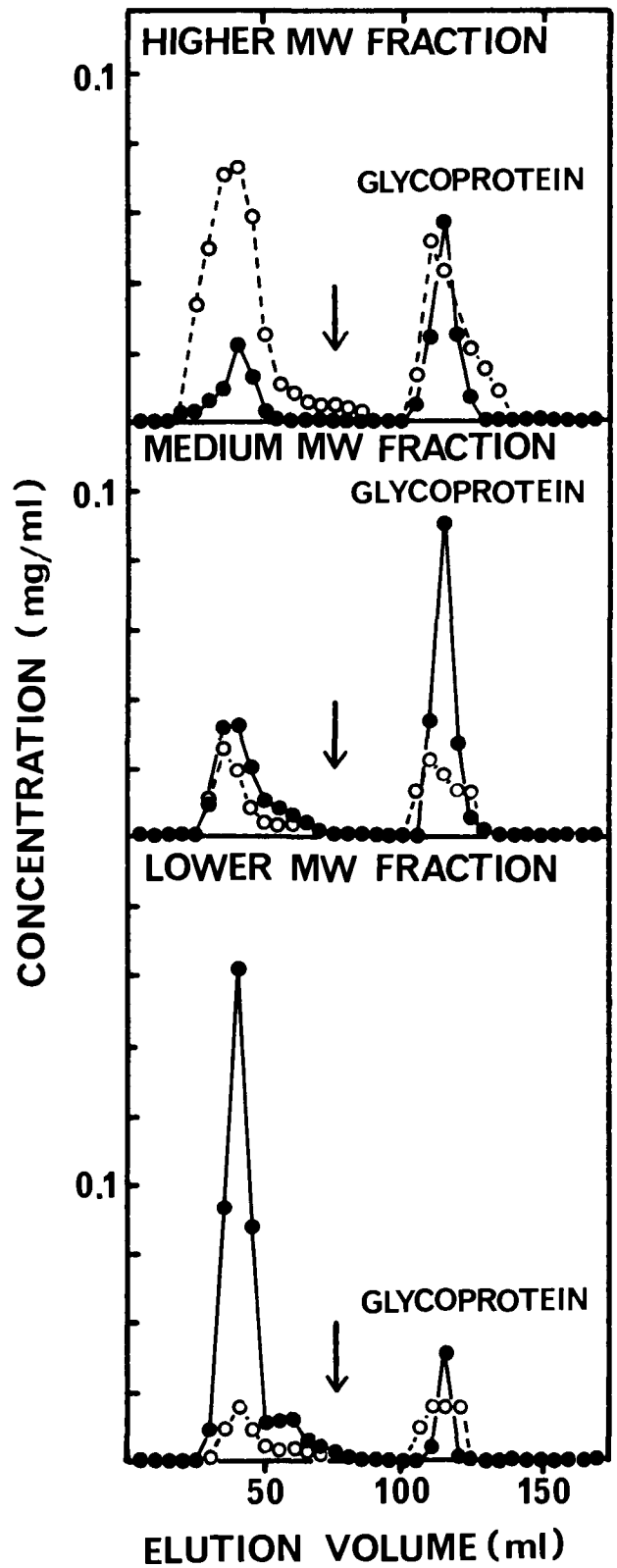

Fig. 8. Affinity chromatography of higher, medium, and lower molecular weight fractions of "foaming proteins" (4.7, 5.0, and 5.0 mg, respectively), on Con A-Sepharose. Arrows indicate the time of additon of 0.05*M* α-methyl-D-mannoside to Tris-HCl buffer. • = protein, o = carbohydrate.

higher and medium molecular weight fractions increased greatly, becoming more than that of the lower molecular weight fraction. (Surface activity was determined as the difference in surface tension of 3.6% aqueous ethanol before and after addition of foaming proteins or of isohumulones.)

Table III shows that the head forming capacities of the higher and medium molecular weight fractions were no longer enhanced by addition of isohumulones when alkaline conditions prevented isohumulones from combining with these fractions by suppressing the dissociation of ϵ-amino groups of the proteins.

The distinct difference in the foaming properties of the higher and medium molecular weight fractions from that of the lower molecular weight fraction led us to examine formation of complexes between these three fractions and isohumulones,using the method of dialysis equilibrium. As described in the previous paper (3), 2 mg of each fraction dissolved in 2 ml of 0.1 *M* phosphate buffer containing 3.6% ethanol, pH 4.2 (inner solution) was put in a cellophane tube and dialyzed against 0.8 mg of isohumulones in 4 ml of the same buffer (outer solution). When isohumulones diffuse from the outer solution into the inner solution to combine with foaming proteins, the concentration of uncombined isohumulones in the inner solution decreases. Then more isohumulones diffuse from the outer solution to the inner solution to restore the equilibrium of isohumulone concentration. Table IV shows that the concentration of isohumulones in the outer solution decreased when the inner solution contained the higher or medium molecular weight fraction but did not decrease appreciably when the inner solution contained the lower molecular weight fraction. This result suggests that isohumulones can combine with the higher and medium molecular weight fractions.

TABLE II
Chemical Composition of "Foaming Proteins"

	Molecular Weight Fraction		
	Higher	Medium	Lower
Protein content, %	21	75	65
Amino acid composition of protein, mol %			
Gly	6.1	7.5	9.2
Ala	6.7	8.6	7.4
Val	4.1	6.4	5.5
Leu	5.7	8.3	5.3
Ile	2.4	3.4	2.8
Ser	6.9	7.5	5.9
Thr	3.6	3.9	4.1
Cys	0	0	1.9
Met	0	0	1.1
Phe	2.2	4.1	1.5
Tyr	0.9	1.1	1.7
Pro	3.9	8.7	10.3
Asp	5.6	6.6	8.5
Glu	9.0	14.2	14.3
Lys	3.6	3.3	2.5
Arg	5.7	3.4	3.6
His	3.4	3.4	2.7
Carbohydrate content, %	64	17	12
Constituent sugars of carbohydrate, mol %			
Ara	48	48	18
Xyl	35	25	19
Glc	7	21	60
Man	5	6	3
Gal	5	trace	trace

TABLE III
Increase in Head Forming Capacity (ml) of "Foaming Proteins" Caused by Isohumulones Under Acidic and Alkaline Conditions[a]

Molecular Weight Fraction	pH	
	4.2	11.0
Higher	+1.9	−0.2
Medium	+3.7	−0.9
Lower	+0.4	−1.3

[a] Average differences in head forming capacities of solutions of various concentration before and after addition of 25 mg/L of isohumulones.

Changes of Foaming Proteins during Malting and Brewing

Because foaming proteins formed immunoprecipitates with antimalt serum, and to a lesser extent with antibarley and antiyeast sera, as shown in Fig. 10, they seem to originate mainly from malt. So the changes of foaming proteins during malting were examined by immunoelectrophoresis. Soluble protein fractions isolated from 5.7 mg of lyophilized germinating barley (Fuji nijo II) were subjected to immunoelectrophoresis by the method of Laurell (20) with 12.8 μl/cm^2 of anti-foaming proteins serum. The content of foaming proteins in the germinating barleys was determined using a calibration curve constructed by immunoelectrophoresis of known amounts of foaming proteins with anti-foaming proteins serum. Protease activities of the germinating barleys were determined by the method of Miller (23). Barley germinated for six days was kilned and the resultant malt was used for preparation of Congress wort.

Figure 11 shows that the levels of foaming proteins in the barley increased rapidly with increase in the protease activity of germinating barley, reaching a maximum on the fourth day of

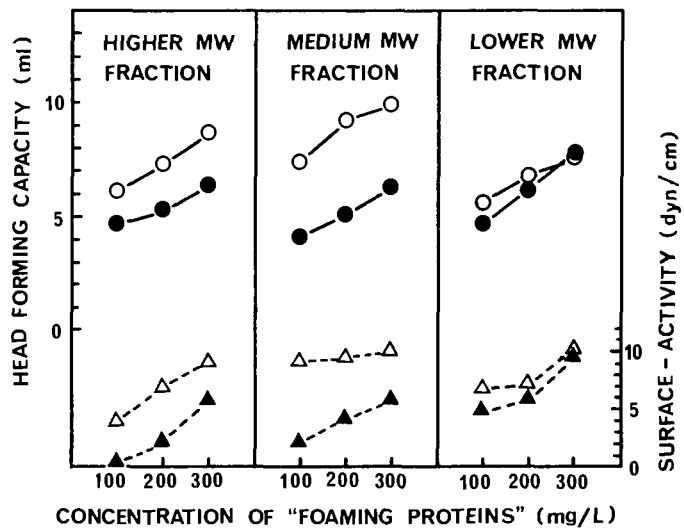

Fig. 9. Head forming capacity (●) and surface activity (▲) of 100–300 mg of "foaming proteins" dissolved in 1L of 3.6% aqueous ethanol, pH 4.2. After 25 mg of isohumulones were added, the solutions were retested (○ and △, respectively).

TABLE IV
Combination of Isohumulones with "Foaming Proteins"

Inner Solution	Isohumulones in Outer Solution	
	Concentration After Dialysis (mg/L)	Decrease in Concentration After Dialysis[a] (mg/L)
Without "foaming proteins"	121.8	···
With "foaming proteins" molecular weight fraction		
Higher	117.2	4.2
Medium	115.2	6.6
Lower	120.8	1.0

[a] Difference between the blank and the sample.

germination and then decreasing gradually in the latter period of germination.

Figure 12 shows the changes of foaming proteins during brewing. Foaming proteins of wort and fermenting wort were isolated by the procedure used for their isolation from beer. The levels of foaming proteins in wort decreased during brewing, particularly during kettle boiling, and only half the total foaming proteins in sweet wort survived in finished beer. Most of the less acidic species of foaming proteins with isoelectric points of pH 4.3–5.5 were lost during kettle boiling (Fig. 13).

Figure 14 shows the effects of process variables on the levels of foaming proteins. Beer brewed from under-modified malt (Kolbach index = 39.9%) retained about 30% more foaming proteins than did beer brewed from control malt (Kolbach index = 43.7%). A short protein-rest in mashing also resulted in an increase of about 10% more foaming proteins in finished beer. When the wort was boiled with hops, the levels of foaming proteins in the resultant beer was 30% less than that in unhopped beer. Similarly, the accelerated decrease of foaming proteins in the wort was caused by boiling with humulones. Therefore, not only polyphenols derived from hops but also humulones and isohumulones seem to accelerate the precipitation of foaming proteins during kettle boiling. Reduction of the boiling time of wort from 90 to 30 min reduced this precipitation or coagulation of foaming proteins. The less acidic species of foaming proteins with isoelectric points of pH 4.3–5.5 were less stable during the brewing process (Fig. 15).

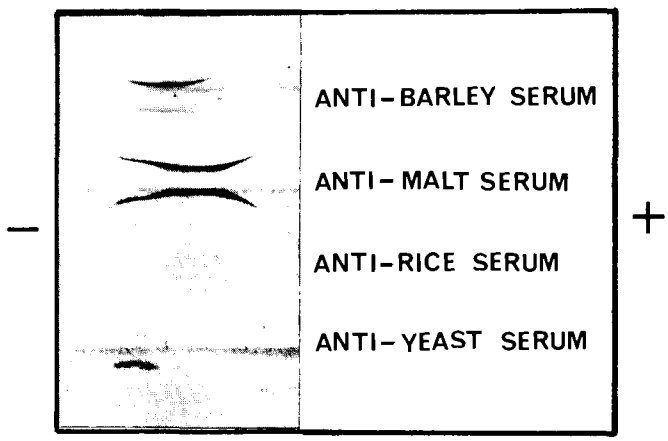

Fig. 10. Immunoelectrophoretic analysis of 100 μg of "foaming proteins" that were allowed to react with 100 μl of antibarley, antimalt, antirice, and antiyeast sera.

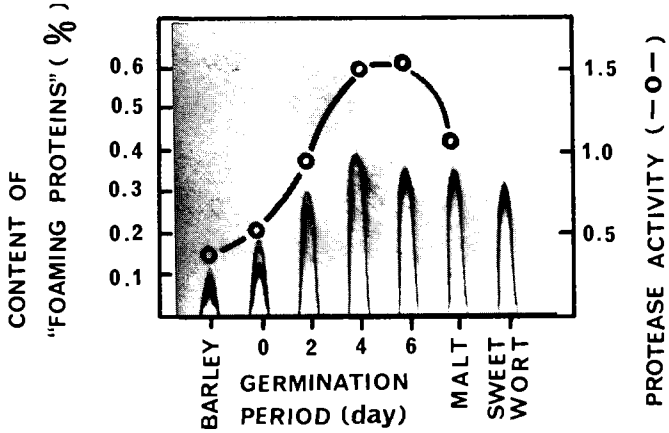

Fig. 11. Changes of "foaming proteins" during malting. The protease activity of the malt is represented as 1 unit.

DISCUSSION

Although many attempts have been made (1,2,4–6,11,21,24–28, 30,33,36), until now foam-enhancing proteins have not been isolated from beer. In the present work, foaming proteins that retained the full foaming capacity of the original beer were isolated and purified. The contents of foaming proteins correlated well with

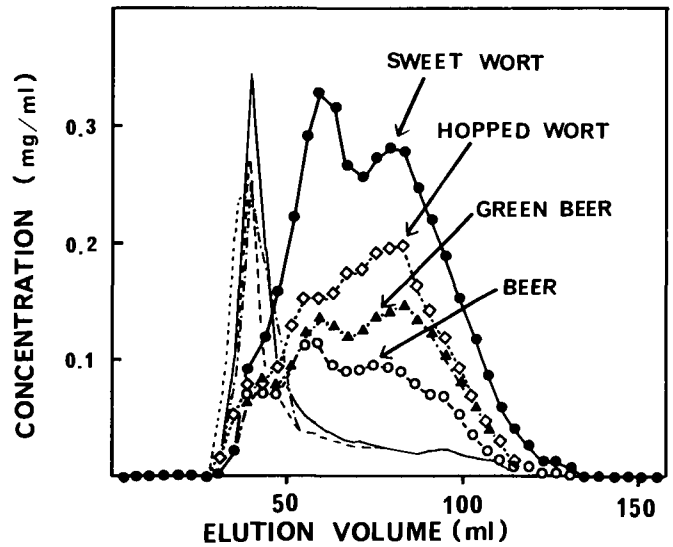

Fig. 12. Changes during brewing of "foaming proteins" isolated from 30 ml of worts and beers, chromatographed on Sephadex G-75. •, ◇, ▲, o = protein; ——, -----, -·-, – – – = carbohydrate.

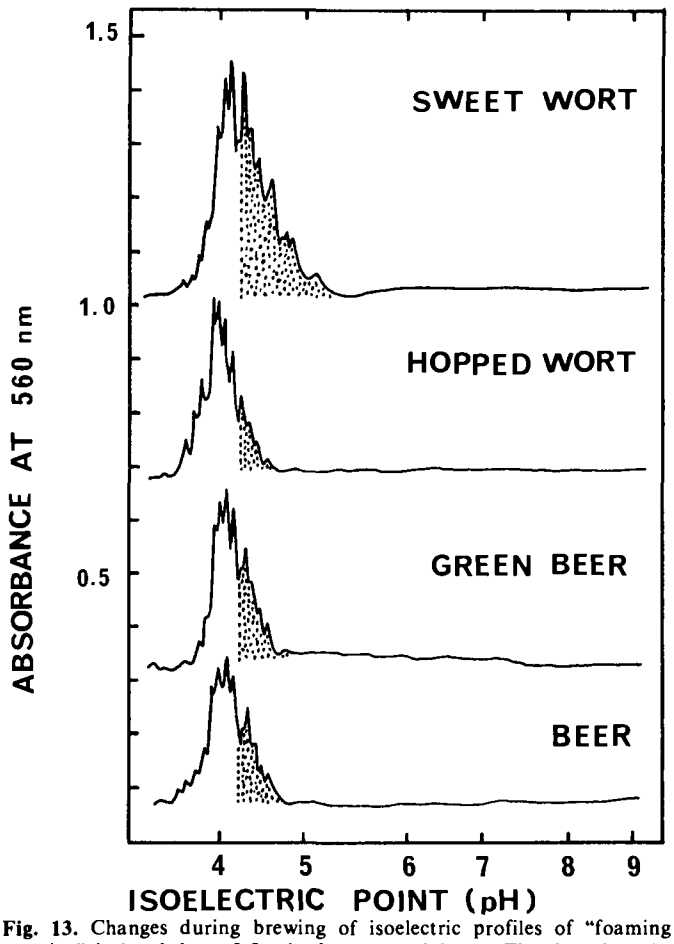

Fig. 13. Changes during brewing of isoelectric profiles of "foaming proteins" isolated from 0.5 ml of worts and beers. The dotted peaks represent less acidic proteins species.

head formation of many samples of beer.

Recently, Schulze et al (31) reported that some proteinaceous fractions concentrating in beer foam were composed of three fractions with molecular weights of 150,000, 90,000, and 10,000, and Hejgaard and Sørensen (13) suggested that barley protein Z (14), with a molecular weight of 40,000, was concentrated in beer foam. Our foaming proteins were also composed of three fractions with molecular weights of 90,000–1,000,000, 40,000, and 15,000. In general, proteins with molecular weights of 10,000–100,000 or more seem to participate in head formation of beer.

The mechanisms by which these three fractions of foaming proteins contribute to foaming of beer differed. The higher and medium molecular weight fractions combined with isohumulones to form more surface-active complexes and thereby enhanced the foaming, whereas the lower molecular weight fraction did not form these complexes appreciably. Because ε-amino groups of lysine residues in foaming proteins combine electrostatically with acidic groups of isohumulone molecules, as reported previously (3), the higher and medium molecular weight fractions, which contain more ε-amino groups than does the lower molecular weight fraction, must combine preferentially with isohumulones. Accordingly, both foaming proteins derived from malt and isohumulones derived from hops are essential for head formation of beer. Foaming proteins, particularly the higher and medium molecular weight fractions, combine with isohumulones, probably in the surface of small bubbles in beer (3), and the resultant surface-active complexes contribute to the foaming of beer.

By immunological studies, we obtained clear evidence that foaming proteins are formed in germinating barley. Precursors of foaming proteins, present in barley in insoluble form, are probably solubilized to foaming proteins by a protease activated in germinating barley. During further germination, the solubilized foaming proteins seem to be degraded enzymatically. Foaming proteins derived from malt are also degraded enzymatically during mashing. Therefore, under-modification of malt and brief mashing are beneficial for head formation of the resultant beer.

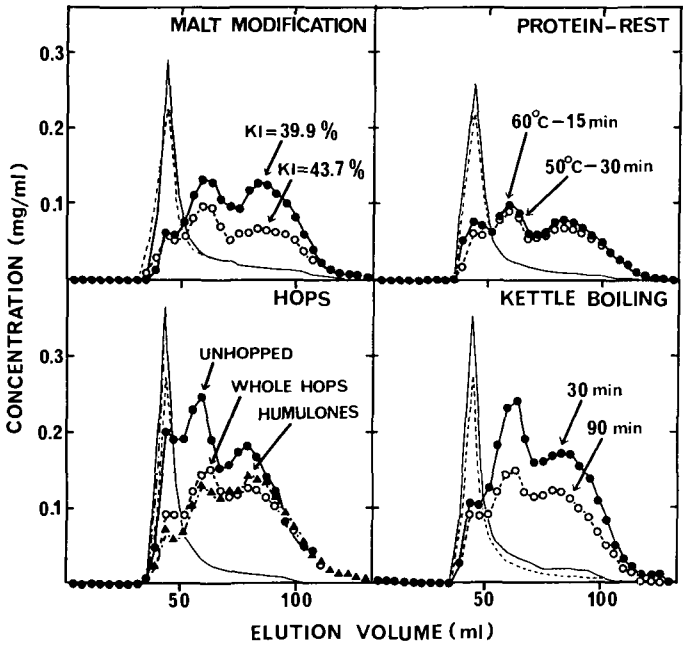

Fig. 14. Effects of process variables on the levels of "foaming proteins" isolated from 30 ml of beers under various conditions, chromatographed on Sephadex G-75. •, o, ▲ = protein; ——, – – –, –·– = carbohydrate.

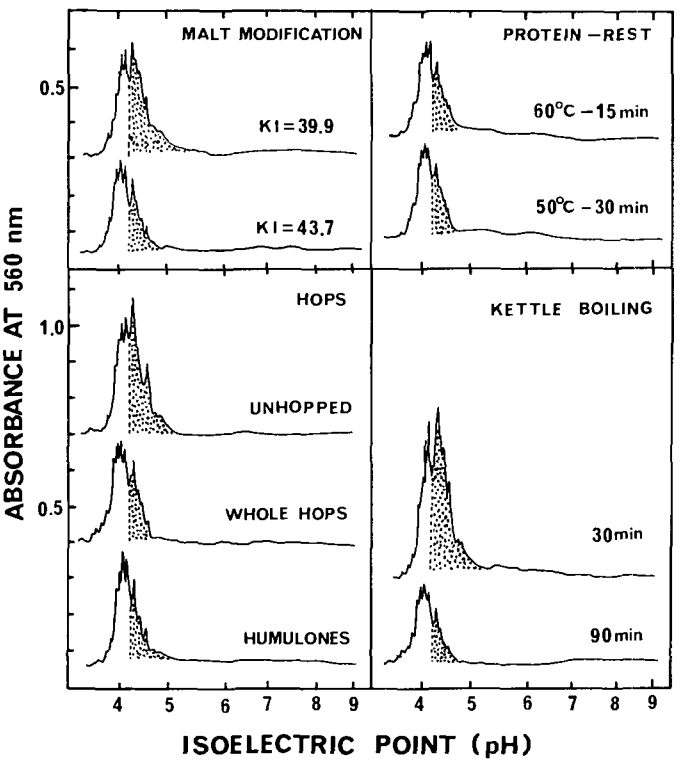

Fig. 15. Effects of process variables on the isoelectric profiles of "foaming proteins" isolated from 0.5 ml of beers brewed under various conditions.

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