The Formation, Nature, and Prevention of Precipitates in Frozen and Thawed Beers

GERHARD J. HAAS[1] and ALAN I. FLEISCHMAN[2]
Liebmann Breweries, Brooklyn, N. Y.

A nonproteinaceous precipitate, which forms in beer by repetitive freezing and thawing, was isolated. The precipitating material was found to be a carbohydrate polymer containing glucose, glucuronic acid, and *N*-acetylglucosamine in a molar ratio of 5:2:3. The polymer comes from the malt and is solubilized in the wort during the mashing procedure. Addition of the enzyme beta-glucosaminidase to the beer during storage in the cellars will prevent the formation of the precipitate. The enzyme can be isolated from gastropods and from filtrates of *Aspergillus oryzae* fermentations.

Upon freezing and thawing of beer, precipitation can occur. Formation of these precipitates cannot always be predicted; sometimes a shipment which accidentally has frozen will remain clear upon thawing; at other times an unsightly formation of flakes will result. These flakes are distinct both in appearance and composition from what is understood as chill haze (chill haze is predominantly proteinaceous and is formed by holding beer at cold temperatures, but not in the frozen state; it is a fine cloud or haze like precipitate). The precipitate formed on freezing and thawing—hereafter called frozen beer precipitate—is predominantly of carbohydrate nature and was first described by Nissen and Estes (*19*). These authors reported that it did not contain protein, and was a retrograded dextrin of indeterminate polymer size.

In the study reported here, more information was sought on the mechanism of formation, composition, and prevention of this precipitate.

Formation of the Precipitate

Reproducible quantities of precipitate could be obtained by freezing beer in 12-ounce metal cans at −18° C. for 24 hours, followed by thawing at 24° C. for 24 hours, for a total of 10 cycles. Additional freezing and thawing beyond 10 cycles failed to increase the yield of precipitate. The rate of precipitate formation is given in Table I.

For these determinations, the contents of duplicate cans, after alternate cycles, were filtered, washed free of beer solids with water, dried, and weighed. The composition of the beer used in these experiments is given in Table II.

Table I. Rate of Frozen Beer Precipitate Formation

Cycle	Yield, Gram per 12 Ounces
Original beer	0.000
1	0.010
3	0.014
5	0.026
7	0.034
9	0.049
10	0.056

Table II. The Composition of Beer Used in Freezing Experiments

Composition	
Apparent extract, %	2.99
Real extract, %	4.73
Alcohol (weight), %	3.78
Original extract, %	12.02
Degree of attenuation, %	60.8
Reducing sugars, %	1.21
Protein, %	0.31
Acidity (as lactic acid), %	0.14
pH	4.25
Carbon dioxide, volumes	2.67
Air content, ml. per 12 ounces	1.1

Factors which might cause precipitation were studied. Precipitation was found to be independent of carbonation, alcohol content, or anions. For example, recanned decarbonated beer, when subjected to a freeze-thaw cycle, formed a precipitate. Similarly, vacuum-distilled beer, reconstituted to its original volume with water after removal of alcohol and other volatiles, also formed a precipitate. Anions were removed by passing decarbonated beer through a 2-foot bed of Amberlite IRA-45 (Rohm and Haas Co.) in the hydroxyl cycle. The pH of the effluent was 8.0. After readjustment with phosphoric acid to pH 4.2, the effluent was canned, frozen, and thawed. The precipitate formed.

The source of the precipitate was sought from among the raw materials used in the brewing process. Upon alternate freezing and thawing, precipitates with the same chemical composition were found in beer brewed with adjuncts, all-malt beers, malt liquors, beer brewed with dehusked malt, hopped wort, and unhopped wort. The precipitate occurs in the absence of hop, cereal adjuncts, and husk, but also occurs in unfermented wort, thus excluding yeast fermentation as a causative agent. Thus the carbohydrate polymer apparently originates in the wort and hence the malt kernel. In all cases, frozen beer precipitate could be distinguished from chill haze by its chemical composition, its inability to redissolve upon warming, and the failure of papain to prevent its formation.

Composition of the Precipitate

For analytical purposes, frozen beer precipitate was prepared from normal beer, isolated by filtration, and washed free of beer with distilled water. The precipitate was insoluble in both cold and boiling water, cold or boiling 10% sodium hydroxide, and cold dilute sulfuric or hydrochloric acids. It was soluble in hot concentrated sulfuric acid. Ignition of the precipitate produced an ash which was identified as calcium oxide. Calcium was determined chelometrically (*20*) and titrimetrically after oxalate precipitation (*4*). Oxalate was determined manometrically (*11*). The precipitate contained 25% calcium oxalate and 75% organic matter. This ratio will undoubtedly vary from beer to beer depending upon the oxalate content. No calcium oxalate was detected in frozen beer precipitate prepared from anion-free beer, indicating that the calcium oxalate was a coprecipitated contaminant, and not necessary for precipitate formation.

[1] Present address: General Foods, Technical Center, White Plains, N. Y.
[2] Present address: Atherosclerosis Research Group, St. Vincent's Hospital, Montclair, N. J.

Frozen beer precipitate from anion-free beer was washed several times with distilled water and then used in the analysis of the organic matter. Elementary analysis by sodium fusion indicated the presence of nitrogen and phosphorus, and the absence of sulfur and halogen (*18*). Tests for amino acids and lipids were negative. Positive Molisch (*7*) and anthrone tests for carbohydrates were obtained. The material reduced alkaline copper tartrate only after hydrolysis with dilute hydrochloric acid, indicating that it was a nonreducing polymeric carbohydrate.

Bial's orcin test for pentoses (*2*) and the phloroglucinol test for pentoses or galactose (*13*) were negative. After a 2.5-hour hydrolysis in 4*N* hydrochloric acid, Seliwanoff's test for ketoses (*7*) was negative. Reaction of the partially hydrolyzed material with phenylhydrazine and sodium acetate yielded an osazone of indeterminate nature (*5*). Descending chromatography of the partially hydrolyzed material on Whatman No. 1 paper in a water-saturated phenol system, and subsequent spraying with *p*-anisidine phosphate (*3*), tentatively indicated the presence of D-glucuronic acid, and glucose. Spraying with ninhydrin tentatively indicated glucosamine.

A 2.5-hour hydrolysis with 4*N* hydrochloric acid under a nitrogen atmosphere in which the effluent gases were trapped in barium hydroxide showed that carbon dioxide was given off. A furfural derivate was detected in the pot by the phloroglucinol reaction, further indication of a uronic acid. The uronic acid was confirmed by reaction of partially hydrolyzed precipitate with alcoholic carbazole and sulfuric acid to give a characteristic purple color (*7*). D-Glucuronic acid was confirmed by reacting partially hydrolyzed material with thioglycolic acid and mannose in the presence of sulfuric acid (*7*). A red solution was formed, the absorbance of which was greater at 410 mμ than at 480 mμ. This difference in absorbances is characteristic of D-glucuronic acid and differentiates it from other uronic acids. Glucuronic acid was quantitatively determined by titrating the carbon dioxide evolved after destructive distillation with hydrochloric acid in a nitrogen atmosphere (*14*).

Glucosamine was confirmed by reacting partially hydrolyzed material with acetyl acetone under alkaline conditions to give 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole: this in turn was reacted with *p*-dimethylaminobenzaldehyde to yield a product whose absorption maximum was at 540 mμ (Elson-Morgan Reaction) (*8*, *14*). Although this test is quantitative for monomeric hexosamines, it was used only qualitatively since partial destruction of the hexosamine occurs under the conditions of hydrolysis. Milder conditions failed to hydrolyze or even appreciably solubilize the precipitate. Glucosamine was quantitatively determined on the unhydrolyzed material by the method of Gardell (*10*). Since glucosamine is often found in the acetylated form, a quantitative acetyl determination was made (*18*).

Reaction of partially hydrolyzed material with ferric chloride and phloroglucinol according to the method of Lindh (*15*) tentatively confirmed glucose. Final confirmation of glucose was obtained with the unsulfonated resorcinol reaction of Devor *et al.* (*6*), and quantitatively determined with their presulfonated resorcinol method.

Quantitative analysis of the polymer is given in Table III. The precipitate is thus found to be a polymer of glucose, glucuronic acid, and *N*-acetylglucosamine in a molar monomer ratio of 5:2:3. The function of the trace amount of phosphate was not followed up further.

Table III. Quantitative Analysis of Frozen Beer Precipitate

Component	Per Cent
Gross	
Calcium oxalate	25.0
Organic matter	75.0
Organic Matter	
Elemental	
Nitrogen (amino)	2.66
Phosphate ion	1.02
Constituents	
Glucose, polymerized	48.4
Glucosamine, polymerized	27.0
Glucuronic acid, polymerized	19.2
N-Acetyl groups	5.0

Table IV. Enzymatic Analysis of Lyophilized Snail Enzyme

Enzyme	Units Per Gram	Method Reference
Beta-glucuronidase	22,800	(*9*)
Amylase	0	(*1*)
Cellulase	1,550	(*17*)
Beta-glucosaminidase	6,000	(*16*)

Prevention of the Precipitate

To find a method for the prevention of precipitation during freezing and thawing of beer, addition of various enzymes was studied. Since the precipitate contained both glucose and glucuronic acid monomers, enzymes which have been reported to cleave polymers of this type were examined. The procedure used was to add graded amounts of the enzyme under study to beer and to incubate the mixture for 5 to 10 days at room temperature, and at 8° C. After this incubation period, the beer was frozen and thawed 10 times and examined for precipitate formation. In all cases, the beer was treated with papain preparations to prevent chill haze formation.

Graded amounts of bovine liver beta-glucuronidase (Worthington Biochemical Corp.), three units to 600 units per liter of beer, did not prevent precipitate formation. Also ineffective were β-glucosidase (Worthington Biochemical Corp.), an enzyme which attacks the D-glucopyranosyl-beta-D-glucopyranose linkage; amylase (Worthington Biochemical Corp.); cellulase (Takamine Division of Miles Laboratories); and hemicellulase (Takamine Division of Miles Laboratories).

As already reported in the patent literature (*12*), frozen beer precipitate formation could be prevented by incubating with the beer 0.39 mg. per liter of a partially purified lyophilized aqueous extract of the snail, *Otala lactea*. The method for preparing the extract, and partially purifying it, is given below.

Fifty snails were distended by submerging in water for several minutes. After deshelling, the fleshy portions were extracted with ice water by grinding in a Waring Blendor for 2.5 minutes. The active extract, 190 ml., was decanted. Solid ammonium sulfate was added at 0° C. to 40% saturation. The inactive precipitate which formed was removed by centrifugation. After adjusting the active supernatant to pH 6.5 with ammonium hydroxide, solid ammonium sulfate was added to raise the concentration to 50% saturation. The active precipitate was dissolved in 0.1*M* sodium acetate buffer at pH 4.5 and dialyzed against 0.1*M* phosphate buffer, pH 7.0, for 20 hours at 4° C. The active nondialyzable fraction contained 1.3 mg. protein per ml.

An enzymatic analysis of the extract is shown in Table IV. As beta-glucuronidase and cellulase were ineffective, beta-glucosaminidase apparently was the active enzyme.

Confirmation that the active enzyme was beta-glucosaminidase was found in a series of experiments in which 100 mg. of lyophilized filtrate from an *Aspergillus oryzae* fermentation, sold under the trademark "Mylase P" (Wallerstein Co., Division of Baxter Laboratories), was incubated with 100 ml. of beer at 35° C. for 24 hours. No precipitate formed after the usual freezing and thawing test. "Mylase P" contained 24,200 units of beta-glucosaminidase but had no detectable beta-glucuronidase and cellulase activity. The preparation did contain amylase which had previously been shown to be absent in the snail preparation and ineffective when employed alone with beer.

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PROTEIN QUALITY IMPROVEMENT

Improvement in the Protein Efficiency of Soybean Concentrates and Isolates by Heat Treatment

JOHN B. LONGENECKER,[1] WILBUR H. MARTIN, and HERBERT P. SARETT

Department of Nutritional Research, Mead Johnson Research Center, Evansville, Ind.

Protein efficiency values were determined for three soybean concentrates (approximately 60% protein) and four soybean isolates (approximately 90% protein) before and after mild heat treatment. Values for the unheated samples were 0.34 to 1.91, as compared to 2.44 (81% of the casein control) for a processed soybean flour. Mild heat treatment improved the protein efficiency values to 2.02 to 2.29, except for one, which remained low (1.46). The latter isolate contained appreciable cysteic acid apparently from destruction of cystine. Addition of methionine hydroxy analog to this isolate markedly improved its protein value. The findings indicate that soy isolates and concentrates may contain inherently good protein, but often require mild heat treatment to bring out maximal protein value. This must be considered in assessment of nutritive value and in use of these materials in nutritional products.

SOYBEANS are a very valuable protein source for many nutritional products designed for human and animal use. Soybean flour must be properly heat treated to achieve maximal nutritional value, although excess heat can be detrimental (*1–3, 9, 15*). The improvement in the nutritional quality of soybean protein resulting from mild heat treatment is due partly to the destruction of trypsin inhibitor(s) (*8, 10, 15*) and partly to modification of the protein permitting more complete digestibility and utilization of the growth-limiting sulfur amino acids (*3, 10*).

There is increasing interest in the use of soybean concentrates and isolates in place of soybean flour in nutritional products because of the many technological advantages relating to taste, texture, and other characteristics (*6, 13*). Successful use of such special preparations also depends to a great extent on their nutritional qualities. Three soybean concentrates (approximately 60% protein) and four isolates (approximately 90% protein) were studied to evaluate their protein qualities and to determine whether they had received adequate heat treatment in manufacture to promote maximal protein quality, and whether processing had reduced their potential protein quality. The results of these experiments are presented.

[1] Present address: Department of Home Economics, University of Texas, Austin, Texas.

Experimental

The soybean protein preparations were obtained from Archer Daniels Midland Co., Central Soya Co., Hercules Powder Co., and Ralston-Purina Co. The conditions of manufacture were not divulged by the companies. Methionine, cystine, and tryptophan content of several of the soybean preparations were determined by microbiological assay with *Leuconostoc mesenteroides* (*5*), and the other amino acids (and cysteic acid) by ion exchange chromatography (*11, 14*) using a Beckman-Spinco Model X120 amino acid analyzer. Values for methionine by the latter method were similar to those found microbiologically.

Each sample was tested in rat studies as received, and again after heat treatment, in which the soybean samples were spread in a thin layer (approximately 1/2 inch) in flat pans and exposed to live steam at 105° C. in the autoclave for 30 minutes. The materials were then dried in an oven at 100° C. for 1 hour. In all experiments, groups of 10 male weanling rats each (McCollum-Wisconsin strain) with an average initial weight of approximately 50 grams were selected on the basis of body weight and litter. The animals were individually housed in metal, screen-bottomed cages in an air conditioned animal room.

The general composition of the diets is shown in Table I. In all experiments, food and water were offered *ad libitum* and their intakes recorded; the animals were weighed each week. After 4 weeks, the protein efficiency value was calculated for each animal, and an average value and standard deviations were determined.

Results and Discussion

The protein efficiencies for the unheated and heated soybean concentrates