Functional (Breadmaking) and Biochemical Properties of Wheat Flour Components. VII. Petroleum Ether-Soluble Lipoproteins of Wheat Flour[1]

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ABSTRACT

Two protein fractions were isolated by a relatively simple solvent system from the petroleum ether-soluble oil of wheat flour. One protein fraction (about 0.7% of the total free lipids) contained 60% protein and 35% carbohydrate. The second protein fraction (22% of total free lipids) contained 30% protein, 7.5% carbohydrate, and about 62.5% lipid. Neither protein fraction had a significant role in breadmaking. The lipoproteins apparently were soluble in salt solution as well as in petroleum ether. Extracting flour with 6% salt (NaCl) yielded a globulin protein fraction containing 32.5% lipid. Proteins were detected in petroleum ether extracts of the globulin fraction.

The presence of protein in petroleum ether extracts of flour was first reported by Balls and Hale (1). They isolated the protein called purothionin as a crystalline hydrochloride, and found that it had an unusual amino acid composition (2), containing 20% arginine and 16% cystine.

Recently Fisher et al. (3) further characterized purothionin. Purothionin hydrochloride, prepared essentially as described by Balls and Hale (1), was fractionated on Sephadex into two fractions. Electrophoretically, fraction B corresponded to the "fast-moving globulin doublet" of wheat flour. The molecular weight of that fraction was estimated at about 10,000 by gel filtration. Fraction A had a molecular weight of about 45,000 by the same technique.

Fraction A contained significantly more glutamic acid and aspartic acid but less lysine and cysteine than fraction B. The amino acid composition of fraction B was similar to a "fast-moving globulin" preparation from wheat flour. The similarity of the "fast-moving globulin" and purothionin was stressed in a similar and independent study by Nimmo et al. (4). Their globulin preparation resembled purothionin in amino acid composition, electrophoretic mobility at pH 3.1 and pH 8.5, ultraviolet spectra, and gel-filtration properties. Additional evidence of the close similarity between purothionin and globulin was recently reported by Redman and Fisher (5). They found purothionin and the globulin "fast-moving doublet" to have the same C-terminal end-group, the same peptide "fingerprint" after trypsin digestion, and similar, but not identical, amino acid composition.

Our purpose was to isolate the petroleum ether-soluble proteins without using

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the potentially disruptive hydrochloric acid, to determine the contribution of those unique proteins to breadmaking, and to identify lipids associated with the proteins.

MATERIALS AND METHODS

The oil was extracted in a large Soxhlet, with petroleum ether (b.p. 35° to 60°C.), from a pilot-milled, composite HRW wheat flour. The flour contained 0.36% ash and 12.1% protein on 14% moisture basis. The extracts were evaporated under vacuum and stored at 4°C. until used.

Starch-gel electrophoresis was performed in a vertical position with pH 3.2 aluminum lactate and lactic acid buffer containing 3.0M urea. Electrophoresis was performed at 30 ma. and approximately 325 v. for 4 hr. at room temperature. The gel was sliced and stained with 0.1% Amido Black 10B. Details of the procedure have been given previously (6).

Thin-layer chromatography (TLC) was performed on 100 γ of lipid. After they were developed with chloroform:methanol:water (65:25:4), the plates were sprayed with a 70% (by volume) aqueous sulfuric acid saturated with $K_2Cr_2O_7$ and charred at 150°C. for 30 min. The plates were photographed under ultraviolet light.

Baking tests were made from 10 g. of flour (14% moisture basis). The formula included (on flour basis), in percent: sucrose, 6; salt, 1.5; yeast, 2; 60°L. malt syrup, 0.5; nonfat dry milk, 4; commercial vegetable shortening, 3; water as needed; and optimum potassium bromate. Details of the procedure were given by Shogren et al. (7).

Paper chromatography was used to identify sugar residues following hydrolysis. The samples were hydrolyzed with 2N sulfuric acid in an evacuated, sealed tube at 110°C. for 5 hr. The hydrolysate was neutralized with sodium hydroxide, adjusted to volume, and spotted on Whatman No. 4 paper. The chromatogram was developed for 18 hr. with the top layer of a mixture of butanol, acetic acid, and water (63:10:27). Spots were visualized by dipping the chromatogram in a solution of silver nitrate in moist acetone, followed by dipping in dilute potassium hydroxide in methanol. The spots were fixed by dipping in an aqueous photographic fixer. Additional details of the procedure have been given by Linko et al. (8).

Protein in lipid fractions was detected by a spot test. The lipid in a suitable solvent (petroleum ether or 2:1 chloroform-methanol) was spotted on filter paper and the solvent was allowed to evaporate. The paper was dipped in a 0.1% aqueous solution of Amido Black 10B for 5 min. The paper was removed from the dye and washed repeatedly with distilled water. The presence of protein was indicated by a dark spot on a white background.

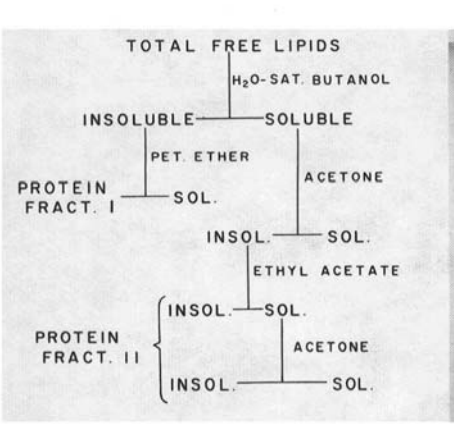
Amino acids were determined with a Beckman Model 120C amino acid analyzer. Twenty-five milligrams of sample was hydrolyzed with 6 ml. of 6.0N HCl in an evacuated, sealed tube for 20 hr. at 110°C. The hydrolysate was filtered (Whatman No. 4), evaporated to dryness over sodium hydroxide, and diluted to volume.

Protein was determined by the Kjeldahl procedure (9), and total carbohydrate by the phenol-sulfuric acid method (10).

RESULTS AND DISCUSSION

Isolation of Protein Fractions

The petroleum ether-extracted oil from a hard winter wheat flour forms a clear



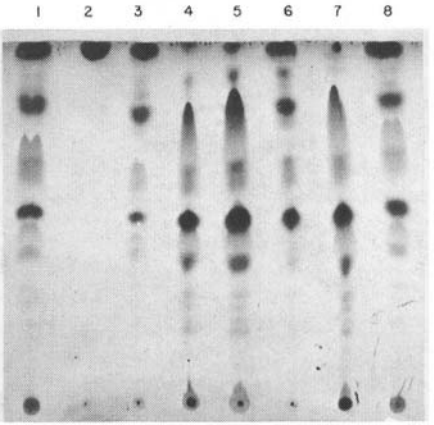


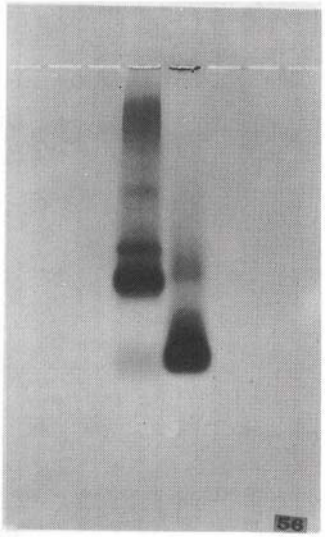
Fig. 1 (left). Fractionation scheme for isolating protein fractions from total free flour lipids.

Fig. 2 (right). Thin-layer chromatogram of: total free lipids (patterns 1 and 8), water-saturated butanol-insoluble, petroleum ether-soluble (pattern 2) acetone-soluble (patterns 3 and 6), protein fraction II (patterns 4 and 7), and ethyl acetate-soluble (pattern 5). Spots are tentatively identified as: A, unresolved nonpolar lipids; B, monogalactosyl diglycerides; C, phosphatidyl ethanolamine; D, digalactosyl diglycerides; E, phosphatidyl choline; and F, lipoprotein.

solution in petroleum ether. The presence of protein in the oil was confirmed by a spot test with aqueous Amido Black 10B on filter paper. Attempts to characterize the protein by starch-gel electrophoresis were unsuccessful because the oil was insoluble in the aluminum lactate-lactic acid buffer.

The protein was assumed to be soluble in petroleum ether because of its association with lipids. If the binding of protein to lipid was not covalent, then polar solvents, such as those used to extract bound lipids, would break the association and thus free the protein. Therefore, the petroleum ether-soluble oil was dissolved in water-saturated butanol (1 part oil to 10 parts butanol). The spontaneous precipitate (Fig. 1), a white solid, was removed by filtration, washed with water-saturated butanol, and then extracted in a Goldfisch extractor with petroleum ether. The petroleum ether solubles contained no protein by the spot test, and appeared to be sterols by TLC (pattern 2, Fig. 2). The insoluble fraction was designated as protein fraction I.

The oil soluble in water-saturated butanol gave a position protein reaction. Therefore, the water-saturated butanol was evaporated to recover the oil. The oil was then washed several times with acetone. The oil soluble in acetone contained little or no protein. The acetone-insoluble fraction (Fig. 1) was then treated with several washings of ethyl acetate. Both the ethyl acetate-insoluble and -soluble fractions contained protein. The ethyl acetate was evaporated from the soluble fraction and the residue was washed with acetone. The acetone-soluble fraction contained little or no protein. The second acetone-insoluble fraction appeared to be identical with the ethyl acetate-insoluble fraction by nitrogen analysis and starch-gel electrophoresis. Therefore the two fractions were combined to form protein fraction II.



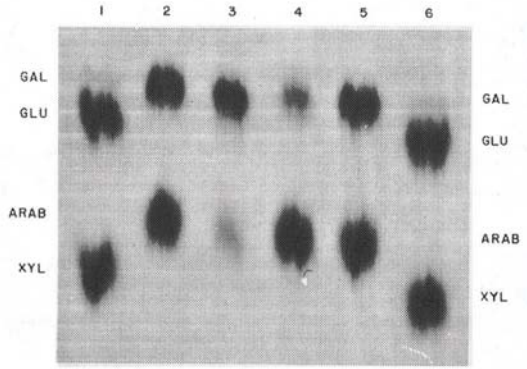


Fig. 3 (left). Starch-gel electrophoretic patterns of protein fraction I (left) and protein fraction II (right).

Fig. 4 (right). Paper chromatogram of sugars: standards (patterns 1, 2, 5, and 6), hydrolysate of protein fraction I (pattern 3), and hydrolysate of protein fraction II (pattern 4). Gal, glu, arab, and xyl are abbreviations for galactose, glucose, arabinose, and xylose, respectively.

Characterization of Protein Fractions

Protein fraction I (about 0.7% of the total free lipids) contained 60% protein (N X 5.7) and about 35% carbohydrate. That fraction should contain little or no lipid. Starch-gel electrophoretic pattern of protein fraction I (Fig. 3) has one predominant band and one extremely light band. Paper chromatography (Fig. 4) identified the carbohydrates as mainly galactose with a smaller quantity of arabinose. Amino acid analysis of fraction I (Table I) was characterized by

TABLE I. AMINO ACID COMPOSITION (g. AMINO ACID PER 100 g. PROTEIN), PROTEIN FRACTIONS I AND II

Amino Acid	Protein Fraction I g.	Protein Fraction II g.	Amino Acid	Protein Fraction I g.	Protein Fraction II g.
Lysine	8.13	10.11	Glycine	5.77	5.06
Histidine	1.47	0.71	Alanine	4.00	3.65
Ammonia	1.18	1.99	Cysteine	5.37	8.31
Arginine	8.60	10.86	Valine	5.37	3.14
Aspartic acid	8.11	6.27	Methionine	2.51	1.11
Threonine	3.60	3.62	Isoleucine	4.49	2.53
Serine	5.35	5.43	Leucine	6.64	8.12
Glutamic acid	15.12	6.31	Tyrosine	2.93	3.04
Proline	5.48	4.61	Phenylalanine	5.30	3.96
			Total	99.32	88.86

relatively high values for lysine, arginine, and cysteine. In general, the amino acid values are similar to those reported by Fisher et al. (3).

Protein fraction II (22% of the total free lipids) contained about 30% protein (N × 5.7) and about 7.5% carbohydrate. Fraction II still contained a large quantity of lipid, 62.5% by difference, and was soluble in lipid solvents such as water-saturated butanol and chloroform-methanol (2:1), but insoluble in petroleum ether. Lipids of protein fraction II apparently were not covalently bound to protein, because they were separated by TLC (patterns 4 and 7, Fig. 2). Lipids bound to the protein II were practically all polar, and were tentatively identified as mono- and digalactosyl diglycerides, phosphatidyl ethanolamine, phosphatidyl choline, and several low-concentration and unidentified components. The four major components are identical with those recently reported by Redman and Fisher (5) in a lipopurothionin preparation. Starch-gel electrophoresis (Fig. 3) indicated that the protein in fraction II apparently was identical with the "fast-moving doublet" reported by Fisher et al. (3). Paper chromatography (Fig. 4) identified the carbohydrate as mainly arabinose along with a small quantity of galactose.

Although both protein fractions contained the same sugar residues, protein fraction I contained predominantly galactose and fraction II predominantly arabinose. Amino acid composition of fraction II (Table I) was similar to that of fraction I in that concentrations of lysine and arginine were high, and that of cysteine was extremely high. Fraction II had much lower concentrations of histidine, glutamic acid, and methionine than fraction I. The significantly lower recovery of amino acids from fraction II probably can be attributed to the high lipid content of the fraction.

Role of Lipoprotein in Baking

Recent studies (11,12,13) have demonstrated the importance of lipids in baking. The oil extracted from flour with petroleum ether (free lipids) is essential for normal baking characteristics. Fractionation of free lipids into nonpolar and polar lipids by silicic acid column chromatography, followed by reconstitution studies, has shown that free polar lipids were the effective agent.

Spot tests for protein have shown that free polar lipids contain significant amounts of protein. The presence of lipoproteins in the polar lipid fraction raised the question as to which was responsible for the beneficial effect in bread-baking. Therefore, the total free oil was fractionated as shown in Fig. 1. The oil soluble in acetone did not contain any protein. However, this fraction was shown (patterns 3 and 6, Fig. 2) to contain polar lipids, particularly the galactosyl diglycerides. The acetone-soluble oil was reconstituted with petroleum ether-defatted flour and baked into bread (Table II). Loaf volume, crumb grains, and mixing requirements were fully comparable to those of loaves baked from the original unfractionated flour. Therefore, the petroleum ether-soluble lipoproteins apparently had nothing to do with the beneficial effect of free polar lipids in breadmaking.

Distribution of Lipids and Lipoprotein in Flour Fractions

When flour is wetted and mixed into a dough, all polar lipids and lipoprotein become bound (no longer extractable with petroleum ether). Previously reported results (14) have shown that the bulk of the lipids became bound to the gluten when flour was fractionated into its components of gluten, starch, and

TABLE II. BAKING DATA FOR ACETONE-SOLUBLE AND TOTAL-FREE LIPIDS THAT WERE RECONSTITUTED WITH PETROLEUM ETHER-DEFATTED "RBS" FLOUR[a]

Flour	Lipid	Mixing Time min.	Loaf Volume[b] cc.
RBS[c]	None	3 7/8	82
RBS (defatted)	None	3 7/8	70
RBS (defatted)	0.8% Total-free	4	82
RBS (defatted)	0.8% Acetone-soluble	4	81

[a]All samples had absorption of 65% and potassium bromate requirement of 30 p.p.m.

[b]Crumb grains were satisfactory for all loaves.

[c]RBS, regional baking standard (1967).

water-solubles. Fractionation of the gluten by dissolution in dilute (0.005N) lactic acid followed by ultracentrifugation at 100,000 X g yields a sedimented fraction containing about 21% lipid. About 70% of the lipid in the sedimented fraction was extractable with petroleum ether and gave a positive spot test for protein, indicating the presence of lipoprotein.

Several similarities have been noted between proteins isolated from free flour oil and certain wheat flour globulin proteins (3,4,5). Although the sedimented fraction described above (14) is predominantly glutenin protein, it contains a significant quantity of globulin protein. Therefore, flour was fractionated into gluten, starch, and water-solubles. A 2% salt solution, instead of distilled water, was employed to increase the solubility of the globulin protein. The use of 2% salt in the wash water (Table III) decreased the amount of lipid in the gluten from 3.67 to 3.00%. The total amount of water-soluble components increased from 4.2 to 7.0 g., and the percent lipids in the water-soluble fraction increased from 0.19 to 0.80%. Most lipids in the water-soluble fraction were polar (Fig. 5). The water-solubles extracted

TABLE III. FRACTIONATION OF A COMPOSITE HRW WHEAT FLOUR INTO GLUTEN, STARCH, AND WATER-SOLUBLES WITH WATER AND A 2% NaCl SOLUTION[a]

Description of Flour Fraction	Weight g.	Protein %	Lipids %
Flour	100	12.9	1.34
Water fractionation			
Gluten	20.7	57.9	3.67
Starch	67.3	0.9	0.13
Water-solubles	4.2	23.0	0.19
2% NaCl fractionation			
Gluten	19.5	57.3	3.00
Starch	70.7	0.8	0.19
Solubles	7.0	12.8	0.80
Globulin	0.43	...	13.80
Dialyzed solubles	2.67	...	0.47

[a]All data on 14% moisture basis.

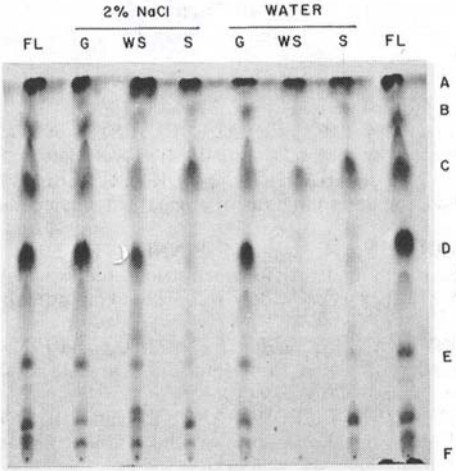


Fig. 5. Thin-layer chromatogram of lipids extracted from flour and certain of its fractions that were separated in 2% salt solution and distilled water. Fl, G, WS, and S are abbreviations for flour, gluten, water-solubles, and starch, respectively. Spots are tentatively identified as: A, unresolved nonpolar lipids; B, monogalactosyl diglycerides; C, phosphatidyl ethanolamine; D, digalactosyl diglycerides; E, phosphatidyl choline; and F, lipoprotein.

with 2% salt were dialyzed to precipitate the globulin proteins. The globulin fraction contained 13.8% lipid. Extracting the lipid with petroleum ether and spot-testing for protein indicated the presence of lipoprotein. Increasing the salt concentration in the wash water to 6% increased the lipid in the globulin fraction to 32.5%.

Thus, the two lipoproteins described above apparently are soluble in petroleum ether and salt solutions. Their apparent unimportance in breadmaking corroborates previous work (6), whereby the globulin proteins were found to have no apparent effect on baking characteristics.

Acknowledgment

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