# NATURE OF THE RESIDUAL PROTEIN ASSOCIATED WITH STARCH FRACTIONS FROM AIR-CLASSIFIED FIELD PEAS[1,2]

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**ABSTRACT**

Smooth-seeded green field peas (*Pisum sativum*) were pin milled and air-classified to yield a green starch fraction and an off-white protein concentrate. Scanning electron microscopy of the starch fraction revealed protein bodies attached to starch granules, agglomerates, and an uneven granular surface. Repeated pin milling and air classification of this starch fraction removed the protein bodies and reduced the number of agglomerates. Partition of the chlorophyll, which is associated with chloroplast membrane structures, and a calculation based on the chlorophyll/starch ratio of the flour and air-classified starch fraction indicated that 84% of chloroplast membrane residues remained with the starch fraction after initial air classification. Water washing the air-classified starch suspended these membraneous residues and solubilized most of the remainder of the nitrogen. Amino acid analysis of adherent protein in air-classified starch indicated proportionately more acidic and less basic amino acids than in the air-classified protein concentrate. Polyacrylamide gel electrophoretic patterns and nitrogen solubility of the two protein types were markedly different.

Pin milling and air classification of field peas does not completely separate the protein from the starch fraction (1). Similar results have been obtained for wheat; the protein bound to the air-classified starch granules has been referred to as adherent protein, while the protein between starch granules has been referred to as wedge or interstitial protein (2). These adherent and wedge proteins in wheat have been found to be different in physiologic source (3–5) and in physical and chemical properties (6).

This study was initiated to elucidate the sources and chemical characteristics of the residual protein associated with air-classified pea starch. Peas in which the cotyledon remains green at maturity were used as well as a variety that is yellow at maturity. The partitioning of the chlorophyll in the former assisted in determining the physiologic components that adhere to air-classified pea starch.

## MATERIALS AND METHODS

### Processing

Figure 1 illustrates the process used to prepare air-classified and water-washed pea starch. Air-dried, smooth-seeded green peas (*Pisum sativum* L. cv. Triumph) were obtained from McCallister Pea and Seed Cleaners Ltd., Winnipeg, and dehulled in a Currier-type plate mill. The splits (7.7% water) were pin milled in an Alpine Pin Mill Model 250 CW. The counter-rotating pins were operating at approximately 5,000 and 12,000 rpm, and the feed rate was about 450 lb/hr. The flour was air-classified in an Alpine Air Classifier Type 132 MP (cut point of about 15 $\mu$m and throughput of about 100 lb/hr) to obtain crude protein (PI) and starch (SI) fractions. The SI fraction was purified by repeated pin milling and air

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classification as illustrated in Fig. 1.

The SIV fraction was water washed by mixing 100 g with 800 ml of glass-distilled water for 10 min in a VirTis mixer at its lowest setting. The pH of the mixture was 7.1. The mixture was allowed to settle for 50 min to sediment most of the starch. The supernatant was separated by aspiration and then left to settle for 2 hr. The supernatant was again separated by aspiration from a small quantity of starch and centrifuged at 16,000 × *g* for 15 min to yield a green sediment, which was termed the suspended fraction, and the clear supernatant, which was termed the water-soluble fraction. The washed starch was resuspended in 500 ml of glass-distilled water, and the entire procedure was repeated. The 500-ml washings were repeated until the last water wash contained less than 1% of the total nitrogen removed by water washing.

Suspended and water-soluble fractions were freeze-dried. Water-washed starch was dried at 60° C *in vacuo*.

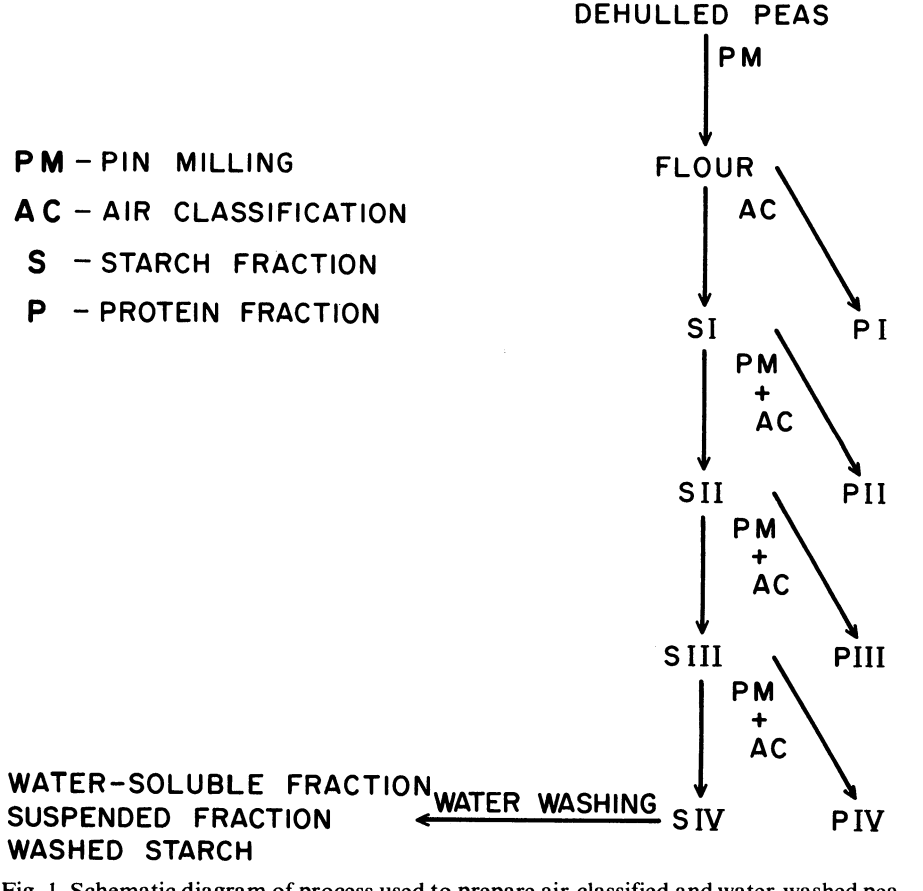


Fig. 1. Schematic diagram of process used to prepare air-classified and water-washed pea starch.

Yellow peas (*Pisum sativum* L. cv. Trapper) obtained from ProStar Mills Ltd, Saskatoon, were processed in the same manner. Initial moisture content of the splits was 6.9%.

**Isolation of Agglomerates**

Agglomerates (particles greater than 37 μm) were quantitatively isolated from the flour and starch fractions by sifting 2-g quantities on a 37-μm screen in an Allen-Bradley Sonic Sifter.

**Microscopy**

Flour fractions were sprinkled onto two-sided tape attached to circular (1.3 cm diameter) aluminum studs. Samples were coated with approximately 100 Å of gold and examined in a Cambridge Stereoscan Mark II scanning electron microscope (SEM).

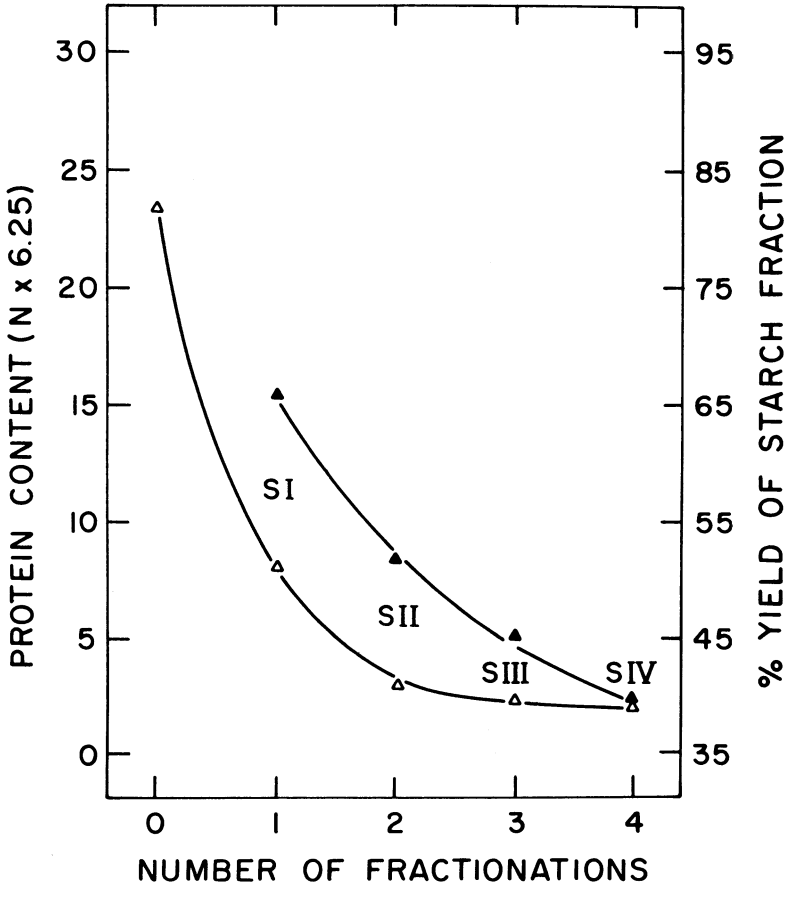


Fig. 2. Purification of starch fraction by repeated pin milling and air classification. △, protein content; ▲, yield of starch fraction.

**Analytic Methods**

*Protein.* All nitrogen determinations were done using a Hewlett-Packard 185B CHN Analyzer and the factor 6.25 was used to calculate protein content.

Amino acids were determined in duplicate by hydrolyzing vacuum-oven–dried samples in a sealed tube under nitrogen atmosphere with 6*N* HCl for 20 hr at 99° C. Hydrolysates were evaporated to dryness *in vacuo* and dissolved in the appropriate volume in 0.01*N* HCl. Aliquots of these solutions were analyzed on a Technicon amino acid analyzer model TSM-IR using norleucine as the internal standard.

Nitrogen solubility profiles were determined by the official AOCS method Ba 11-65 (7).

Polyacrylamide gel electrophoresis was performed using the "standard" analytic disk system that Brewer (8) described. An upper gel was not used. The system employs a 7.5% acrylamide gel, pH 8.9 (running pH 9.5), and tris-glycine electrode buffer at pH 8.3. Each sample (approximately 2 g of SIV and 0.09 g of PI) was mixed with 10 ml of the electrode buffer for 2 hr. The solutions were left to stand for 1 hr for starch to sediment and then were centrifuged at 12,000 × *g* for 15 min. Between 20 and 50 μl of these solutions in 10% sucrose were applied to the gels.

*Chlorophyll.* The chlorophyll content was determined with the official AOAC method 3.107 (9).

*Reflectance Spectroscopy.* Measurements of flour reflectance were determined on a Hitachi Perkin-Elmer Spectrophotometer with a diffuse reflectance attachment. Samples were packed into glass cells as firmly as possible with a spatula and compared with a reference standard of MgO (10).

*Lipids.* Neutral lipids were extracted from samples with hexane for 8 hr in a Soxhlet apparatus. Polar lipids were similarly extracted from the dried hexane-extracted flours with chloroform/methanol (2:1, v/v).

*Starch.* The dual enzyme micromethod that Banks *et al.* (11) described was used to assay starch content.

Ash and moisture were determined with standard AOAC procedures (9).

All results are on a dry weight basis.

## RESULTS AND DISCUSSION

The air classification of green pea flour (23.50% protein) yielded an off-white protein concentrate (PI) and a green starch fraction (SI) at protein contents of 54.67 and 8.11%, respectively. Purification of the starch fraction by repeated pin milling and air classification reduced its protein content and yield (Fig. 2). The protein content of SIV was 2.16%; this was the purest starch fraction that could be prepared by pin milling and air classification. The protein contents of PII, PIII, and PIV, which were the light fractions obtained after regrinding and reclassifying the starch fraction, were 27.56, 8.14, and 4.64%, respectively.

**Sources of Residual Protein in Air-Classified Pea Starch**

The SEM photographs illustrate the pin-milled pea flour and its component starch grains and protein bodies (Fig. 3A-D). Some protein bodies (light-colored particles) adhered to the surface of starch granules in the SI fraction (Fig. 3C). These were removed by repeatedly regrinding and reclassifying this fraction; SIV

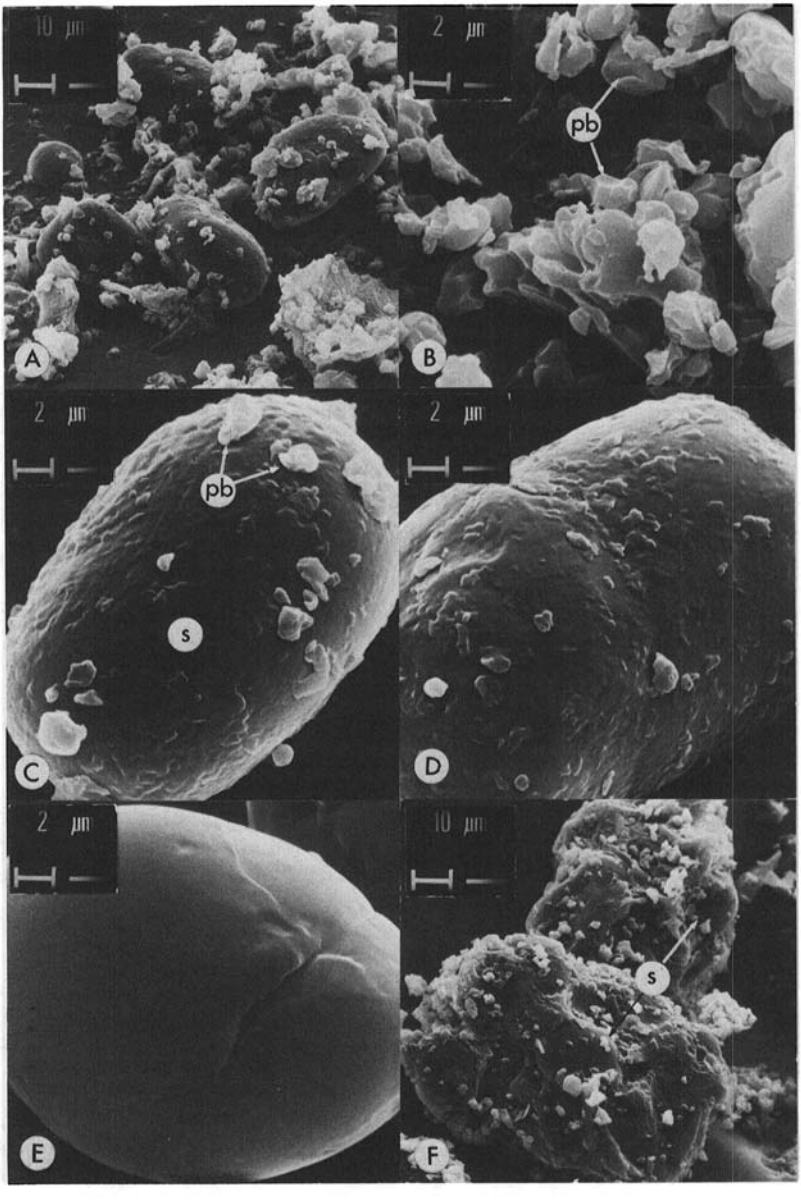


Fig. 3. Scanning electron micrographs of air-classified flour fractions. (A) Flour, (B) PI, (C) SI, (D) SIV, (E) water-washed pea starch, (F) agglomerates, (pb) protein bodies, (s) starch grains.

(Fig. 3D) contained only few protein bodies. The surface of air-classified starch granules appeared uneven in comparison with water-washed pea starch (Fig. 3E). Agglomerates (Fig. 3F) consisting of starch granules still embedded in a protein matrix were observed in the flour and initial starch fractions.

The flour and SI fractions from green and yellow peas contained 4–8% of agglomerates greater than 37 μm by weight (Table I); these contributed primarily to the residual protein of SI. The SII–SIV fractions contained less than 2% of these agglomerates. The protein content of agglomerates was higher than that of the flours. Whether these agglomerates originate from a particular physiologic area of the cotyledon or from discrete cells containing more protein matrix and less starch is not known.

The chlorophyll was partitioned in the air classification of green pea flour into the more dense fraction. The concentration of chlorophyll in SI is nearly double that in PI (Fig. 4A). Reflectance measurements, which quantitate apparent or surface chlorophyll content, were much higher in all starch fractions than in protein fractions (Fig. 4B). Regrinding and reclassifying the starch fractions resulted in a decreased chlorophyll content in the starch fractions and increased concentrations in the light fractions. Purification of SII and SIII resulted in light fractions containing more chlorophyll than the resulting starch fraction. After each fractionation, an average of 4.7% of the chlorophyll could not be accounted for, presumably due to its destruction during the pin-milling step. The ratio of the percentage of chlorophyll to the percentage of protein in SI and SIV were 11.6 and 37.0 times, respectively, the ratio in PI.

The partition of chlorophyll can be explained in reference to the physiology of the developing and mature pea cotyledon. Bain and Mercer (12) have shown that from 10 to 19 days after fertilization, a single starch granule is initiated in most pea chloroplasts in the embryo. Starch grains grow rapidly from the 20th to the 45th day after fertilization, disrupting the structure of the plastid and compressing the lamellae and grana, which are embedded in the stroma, against the limiting membranes of the chloroplast. The grana contain essentially all the chlorophyll of the chloroplast. Membrane remnants have been shown to persist around starch granules in the mature air-dried pea (13).

An estimate of the proportion of membrane remnants that remained with the starch granules on pin milling the cotyledon is calculated in Table II. If the ratio of the percentage of chlorophyll to the percentage of starch in the flour is taken as

**TABLE I**
**Yield and Protein Content of Agglomerates Greater Than 37 μm**

| Agglomerates Isolated From | Green Peas | | Yellow Peas | |
|---|---|---|---|---|
| | Weight (%) | Protein in Agglomerates (%) | Weight (%) | Protein in Agglomerates (%) |
| Flour[a] | 4.5 | 30.41 | 6.1 | 32.62 |
| SI | 6.2 | 25.68 | 8.2 | 32.72 |

[a]Protein content of green and yellow pea flour was 23.50 and 28.00%, respectively.

1.00, the ratio in SI is 0.84. That is, if one group of membranous residues is associated with each starch granule in the cotyledon, there is a fraction (0.84) of these remaining on starch granules of SI, air classification having shifted 77.7% of the protein into PI. Thus, 84% of the membrane remnants remained with the starch granules on pin milling the cotyledon. Regrinding and reclassifying removed some of the surface membranous residues and shifted them into the light fraction, thus accounting for the higher chlorophyll contents of PIII and PIV (Fig. 4A).

**TABLE II**
**Ratio of Percentage of Chlorophyll to Percentage of Starch in Green Pea Flour and SI Fraction**

| | Chlorophyll (%) | Starch (%) | % of Chlorophyll/% of Starch Ratio | |
|---|---|---|---|---|
| | | | $\times 10^4$ | $\div 2.01 \times 10^{-4}$ |
| Flour | 0.00960 | 47.7 | 2.01 | 1.00 |
| SI | 0.01110 | 65.6 | 1.69 | 0.84 |

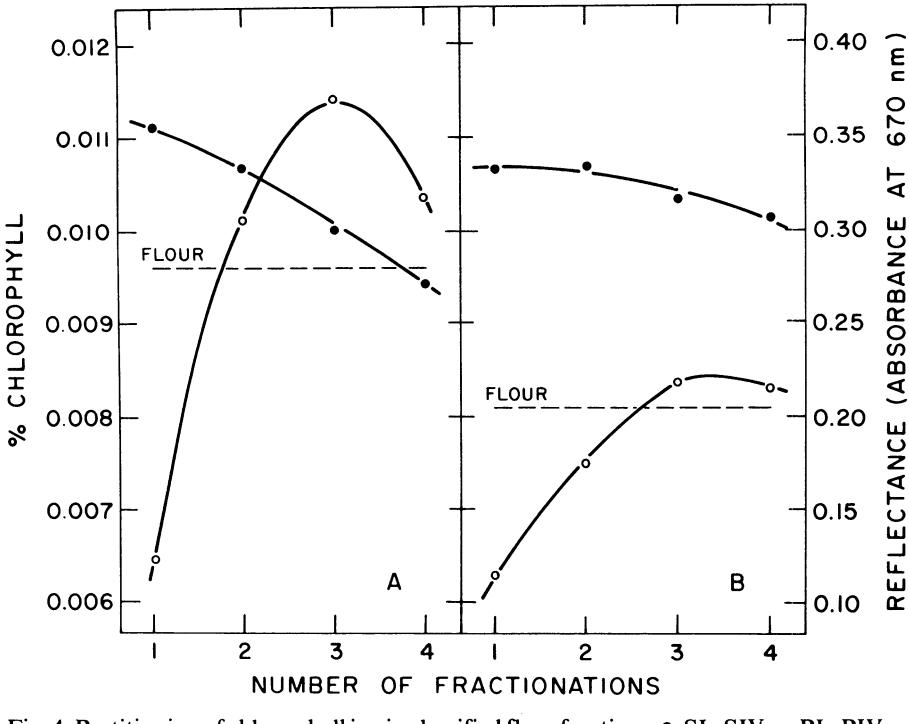


Fig. 4. Partitioning of chlorophyll in air-classified flour fractions. •, SI–SIV; o, PI–PIV.

**Chemical Nature of Adherent Protein in Comparison With Air-Classified Protein Concentrate**

The nitrogen solubility characteristics of the PI fraction, which contains mainly the globulin storage proteins legumin and vicilin (14), and the SIV fraction, containing adherent protein, are compared in Fig. 5. At pH 4.4, the

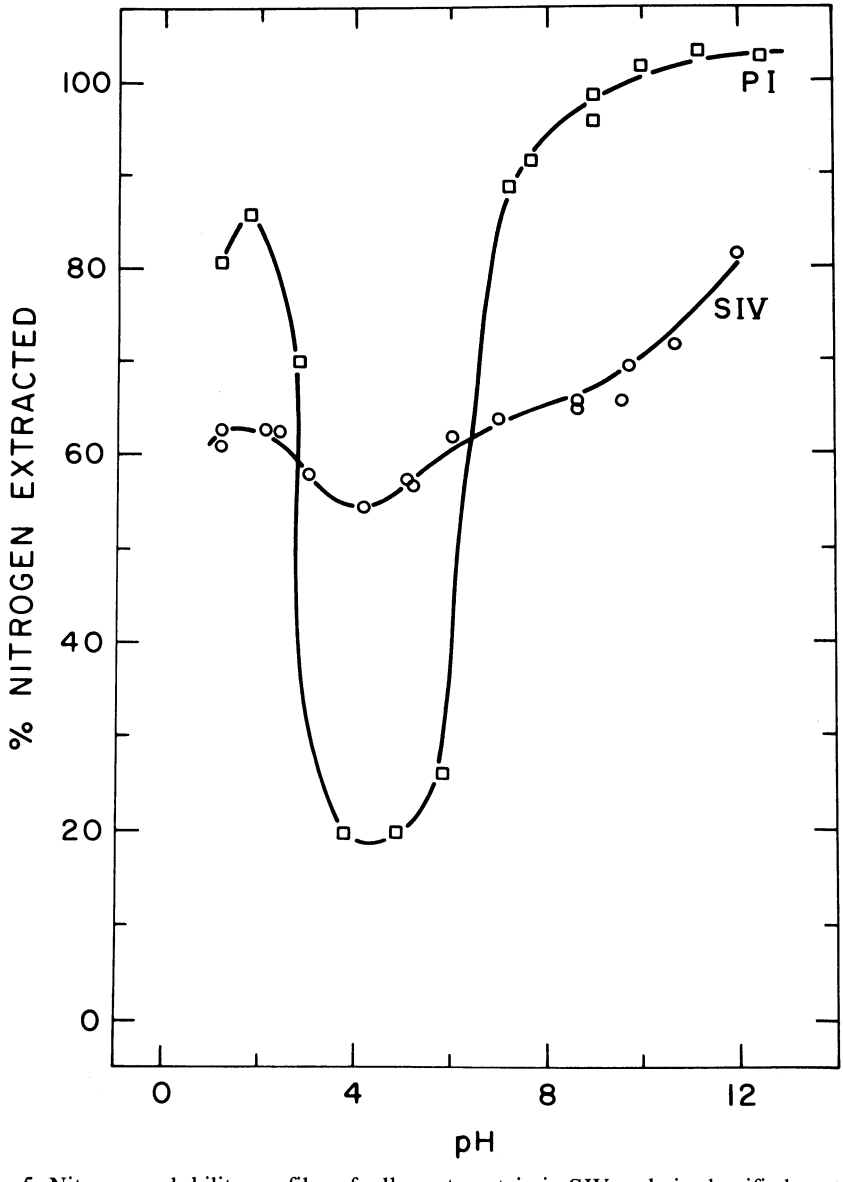


Fig. 5. Nitrogen solubility profiles of adherent protein in SIV and air-classified protein concentrate (PI).

nitrogen in SIV is more soluble than is nitrogen in PI, whereas the reverse is true at more acidic pH and alkaline pH.

The adhering material in SIV was fractionated into water-soluble and suspended fractions after water washing and the white starch was removed by sedimentation. Yields of the freeze-dried fractions and their composition in comparison with PI are given in Table III. From 100 g of SIV, 10.96 g and 0.90 g

**TABLE III**
**Composition of SIV and its Water-Washed Fractions Compared With PI**

| | PI | SIV⟶ (100 g) | Water-Soluble Fraction (10.96 g) | + Suspended Fraction (0.90 g) | + Washed Starch (88.14 g) |
|---|---|---|---|---|---|
| % Chlorophyll | 0.00645 | 0.00943 | 0.0367[a] | 0.601 | Negligible |
| % Protein | 54.67 | 2.16 | 12.73 | 49.93 | 0.26 |
| % Neutral lipids | 2.23 | 0.14 | 0.20 | 9.96 | 0.13 |
| % Polar lipids | 3.40 | 0.42 | 4.75 | 14.90 | 0.18 |
| % Ash | 5.26 | 0.73 | 5.58 | 2.15 | 0.02 |

[a]Calculated by difference assuming washed starch has no chlorophyll content.

**TABLE IV**
**Amino Acid[a] Composition of Green Pea Flour Fractions, PI, and Adhering Protein of SIV**

| Amino Acid | PI | SIV[b] | Suspended Fraction | Water-Soluble Fraction |
|---|---|---|---|---|
| Aspartic acid | 9.83 | 17.97 | 10.99 | 20.22 |
| Threonine | 3.43 | 3.30 | 4.92 | 2.78 |
| Serine | 4.58 | 3.09 | 5.13 | 2.43 |
| Glutamic acid | 12.81 | 22.26 | 9.23 | 26.46 |
| Proline | 4.08 | 2.50 | 5.40 | 1.56 |
| Glycine | 3.67 | 6.17 | 4.86 | 6.59 |
| Alanine | 3.89 | 3.98 | 5.58 | 3.47 |
| Cystine | 1.15 | 2.89 | 0.65 | 3.61 |
| Valine | 3.79 | 2.56 | 4.36 | 1.98 |
| Methionine | 0.96 | 1.16 | 1.42 | 1.07 |
| Isoleucine | 3.40 | 1.96 | 4.27 | 1.21 |
| Leucine | 6.53 | 3.36 | 7.70 | 1.96 |
| Tyrosine | 3.49 | 2.23 | 4.57 | 1.47 |
| Phenylalanine | 4.86 | 2.23 | 5.60 | 1.14 |
| Lysine | 6.43 | 3.95 | 6.10 | 3.26 |
| Histidine | 2.36 | 1.29 | 1.90 | 1.10 |
| Arginine | 8.45 | 2.70 | 4.67 | 2.07 |
| $NH_3$ | 1.55 | 2.39 | 1.28 | 2.75 |
| Total | 85.26 | 85.99 | 88.63 | 85.13 |

[a]G/16 g of nitrogen.
[b]Calculated as sum of suspended fraction and water-soluble fraction in ratio of their nitrogen recovery from SIV, 24.36:75.64.

of water solubles and suspended material, respectively, were obtained; these fractions contributed 67.28 and 21.67%, respectively, to the residual nitrogen in SIV. The suspended fraction was bright green in color and contained the majority of the chlorophyll, 49.93% protein, and a total of 24.86% lipids; by virtue of its composition, this fraction is composed of chloroplast membrane remnants. Protein, lipids, and ash accounted for only 23.26% of the composition of the soluble fraction, the remainder by difference being carbohydrate. The origin of this fraction is likely the dehydrated stroma of the pea chloroplast. Barlow *et al.* (4) stated that the dehydrated stroma surrounding starch grains in mature wheat grains would be expected to contain mainly some proteins, enzymes, small sugars, incompletely synthesized amylose and amylopectin chains and amino acids.

Water washing the SIV fraction from yellow pea flour yielded comparable suspended and water-soluble fractions. The suspended fraction lacked chlorophyll and was yellow-brown in color.

The amino acid compositions of PI and of the adherent protein in SIV were markedly different (Table IV). The total of acidic amino acids (glutamic acid plus aspartic acid) was 40.23 in SIV and 22.64 in PI. The total of basic amino acids (lysine plus histidine plus arginine) was 7.94 in SIV and 17.24 in PI. The content

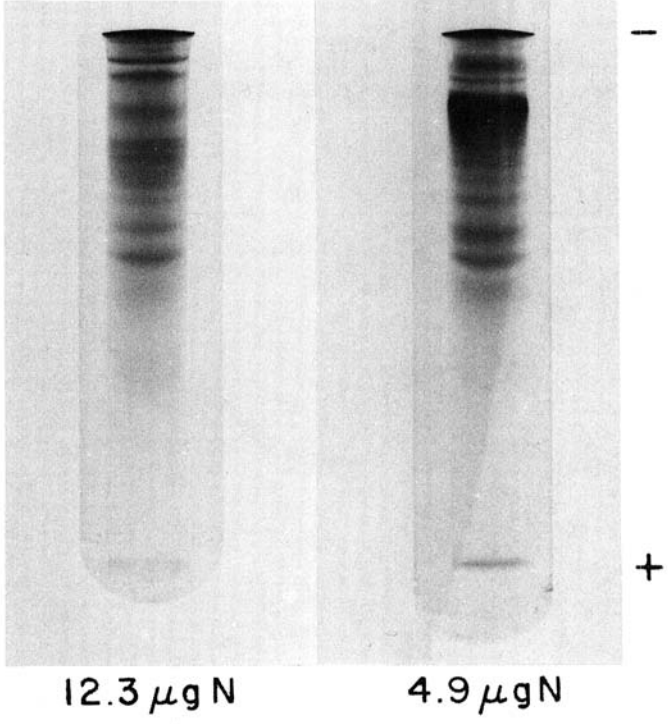


Fig. 6. Polyacrylamide gel electrophoresis of adhering protein in SIV (left) and air-classified protein concentrate (PI) (right).

of aromatic amino acids (tyrosine plus phenylalanine) was higher in PI than in SIV, as was leucine, isoleucine, serine, valine, and proline. The amino acid composition of the suspended fraction was markedly different from the water-soluble fraction, but in some respects similar to PI. The amino acid composition of pea albumins that Grant *et al.* (15) prepared and these water-solubles were markedly different. The amino acid compositions and observed trends for yellow pea flour fractions were similar to those for green pea flour fractions.

The polyacrylamide gel electrophoretic patterns (Fig. 6) are of adherent protein (SIV) and the PI protein. Nitrogen solubilities in the extracting buffer were 99.7 and 75.5% for PI and SIV, respectively; membrane proteins in SIV were not likely solubilized at pH 8.3. Applying about three times the nitrogen for the adhering protein was necessary to obtain distinctly staining bands comparable to protein bands from PI. This may be due to the much higher basic amino acid content of PI. Proteins with similar mobilities are located in SIV and PI electrophoresis patterns. Bands also occur, however, in the electrophoretic patterns of each preparation that are absent or faint in the pattern of the other, most notably the predominant protein in PI.

## CONCLUSIONS

The results of this study demonstrate that protein bodies, agglomerates, chloroplast membrane remnants, and a water-soluble fraction, which is presumably derived from the dehydrated stroma, contribute to the residual protein in air-classified pea starch. Protein bodies and agglomerates are removed by remilling and reclassifying the starch fraction. Water washing the air-classified starch suspends the membraneous material and solubilizes most of the remainder of the residual protein. The chemical properties of adherent protein and protein in the air-classified protein concentrate are markedly different.

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