

# Effect of Morphology of Mechanically Developed Wheat Flour and Water on Starch from Gluten Separation Using Cold Ethanol Displacement

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## ABSTRACT

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The mechanical development of wheat flour and water creates micro and macro structures in dough or batter that critically influence the ability to separate starch from protein by fluid displacement. This study sought to identify specific structural and rheological features and to relate these to separation as indexed by the separation factor. Structural features, especially protein and starch distributions, were examined using visible light microscopy applied to dough samples that had been exposed to a protein dye. Flour and water samples were developed in a Brabender microfarinograph at conditions (water content and time of development) generally suitable for use of the USDA Western Regional Research Center, cold-ethanol fluid-displacement method. No truly homogenous structures were observed. However, distinct segregation of protein and starch were apparent at all conditions. Structural features correlated qualitatively with the success of separation indexed by the overall separation factor ( $\alpha_{p/s}$ ) for the separation process. Highly segregated states characterized by large protein

bands, clustered starch, and large open spaces were obtained with intermediate development ( $25 \pm 5$  min) and were most readily separated ( $\alpha_{p/s} = 118 \pm 7$ ). Segregated states with relatively thin protein bands ( $\leq 10 \mu\text{m}$  dia) in complex networks entrapping starch were obtained after additional development ( $\geq 45$  min) and were less completely separable ( $\alpha_{p/s} = 32 \pm 2$ ). Segregated states with irregularly organized protein in the form of clumps and bands were obtained with minimal development and were partially separable ( $\alpha_{p/s} = 65 \pm 4$ ). Consistency indicated on the microfarinograph increases monotonically throughout and beyond the period of maximum separability. However, elasticity changes and a high rate of increase in consistency evident in the microfarinogram may reflect changes in the structure that also reduce separability. The study demonstrated the use of the ethanol method to isolate development from displacement phenomena for independent study.

The cold ethanol method for separating wheat starch from protein displaces mobile starch from wheat dough provided the dough is properly hydrated and developed. Specifically, if the water-to-flour ratio is  $\approx 0.9$  and the dough is developed for 25 min in a microfarinograph, successful separation is achieved with fluid displacement of starch by 55–100% ethanol at  $-10^\circ\text{C}$  (Robertson and Cao 1998a,b). The method is operationally similar to the Martin method (Grace 1989) in which starch is displaced from the developed protein matrix, except that ethanol replaces water as the displacement fluid. In both aqueous and ethyl alcohol technologies, hydrated, developed, and conditioned dough is manipulated in the presence of excess washing or displacement fluid over a porous support or screen. Manipulation before displacement creates a protein structure from which the starch is displaced. However, because ethanol immediately begins to remove water from the structure, the development process that would ordinarily continue during water displacement is reduced and halted. Furthermore, a gluten that is open, sponge-like or curd-like results from ethanol displacement; a gluten that is condensed, cohesive, and gummy results from the water displacement.

The role of development on the microstructure of dough and the ultimate quality of baked foods such as bread has been studied in detail. However, there has been no detailed discussion of mechanical development and the subsequent capacity of the dough for starch displacement from the protein. AACC Approved Method 38-10, a hand-washing method for laboratory separation, describes manipulation of the dough under running water (AACC 2000). A review has stated that separation requires a dough that is “smooth, uniform, rather stiff” (Knight and Olson 1984). A laboratory inves-

tigation of the water displacement method employed 19 min of development followed by  $\leq 20$  min of resting. In general, the literature currently suggests that a separable mixture can be obtained with more development and more water than is usually used in developing dough for bread.

The microstructure of dough prepared for manufacture of bread has been reported (Amend and Belitz 1993), but very rarely correlated or even reported with either the conditions of development or a quantitative measure of resultant dough quality. In a study of high-speed development (unspecified developing equipment and speed), the steps of development included destruction of the native protein matrix enclosing starch; development of the gluten into compact masses surrounded by starch granules free of supporting protein; development of a continuous fibrillar network up to optimum development; and, finally, development of a veiling mantle of protein that surrounds all of the starch granules (Moss 1972). Reported microscopy methods generally introduce artifacts (principally removal of water) that significantly affect the organization of the resulting matrix and make interpretation difficult. No attempt has been made to correlate dough or batter structures with separation of the protein and starch.

This study is the first to correlate dough structure with quantitatively determined separability when the separation method is cold ethanol displacement. This separation method currently is practiced only at bench scale but may have commercial large-scale potential. The method can be used for basic studies on protein-starch interactions because development processes are isolated from displacement processes.

## MATERIALS AND METHODS

Unbleached flour with 13.5% (flour dry basis) protein ( $5.7 \times N$ ) from a commercial supplier (Giusto, San Francisco, CA) was employed in all experiments. This flour was stored at  $-30^\circ\text{C}$  and subsampled for the experiments. Reagent grade, anhydrous ethyl alcohol was diluted with distilled water to the desired volumetric concentration. Flour moisture content was  $11.4 \pm 0.4\%$  wb.

Dough was developed in a Brabender microfarinograph (model 8110, C.W. Brabender, Duisburg, Germany). A 10-g sample of flour at 11.4% moisture was placed in the farinograph chamber, the development initiated, and water added quickly at a rate of 20 mL/min from the farinograph burette. To record a characteristic devel-

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opment farinograph, the chamber was maintained at 30°C. In the region of 500 BU, the rate of change of consistency is  $\approx 20$  BU/0.05 mL of added water. The amount of water needed was determined by a standard titration of the flour using drop-wise addition of water to produce a response centered on  $500 \pm 10$  BU. For all other experiments, water was added in the amount to give the best separation as determined in earlier experiments (Robertson and Cao 1998a,b) and in the present study.

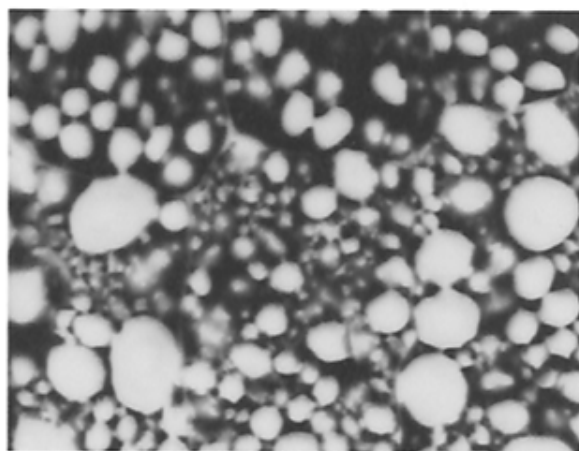
### Dough Microscopy

After development times of 1–75 min, a sample for microscopic evaluation was drawn from the farinograph and spread on a glass slide. Each examined sample represented  $\approx 0.5$ –1.0% of the total developed mass. On the glass slide, the sample covered  $\approx 0.5$ –1 cm<sup>2</sup> area to a depth of 0.5–1.0 mm. When protein dyeing was required, a solution of 0.05% bromophenol blue in water (Eastman Kodak Co., Rochester, NY) was added from a micropipette to the surface of the dough on the microscope slide. The dye stained the protein and displaced starch to reveal the protein structure at the dough surface. Samples were not cross-sectioned. Transfer, display, and dyeing normally were completed in  $\approx 1$ –2 min. Examination of the sample was completed within 5 min. Sample imaging employed a laboratory microscope (Meiji, ML2000) with a color video camera (Cole Parmer model 49901-20). Digital images were captured directly to the computer (Macintosh G3, Avid Cinema software) from five to ten regions on the surface of the displayed sample. Three to five samples were prepared from freshly developed mixtures for imaging at each development time. Reported images were chosen to best represent the set of sample images obtained for each development time. Each image represented  $\approx 0.2$ –0.4% of the total displayed surface.

Continuous video recordings of the separation were made through a laboratory microscope. For this, developed samples of dough drawn from the farinograph were displayed on a slide as above and gently manipulated with the addition of water drop-wise until the starch was displaced and the protein network was clearly visible. No dye was employed.

### Dry Wheat Endosperm Microscopy

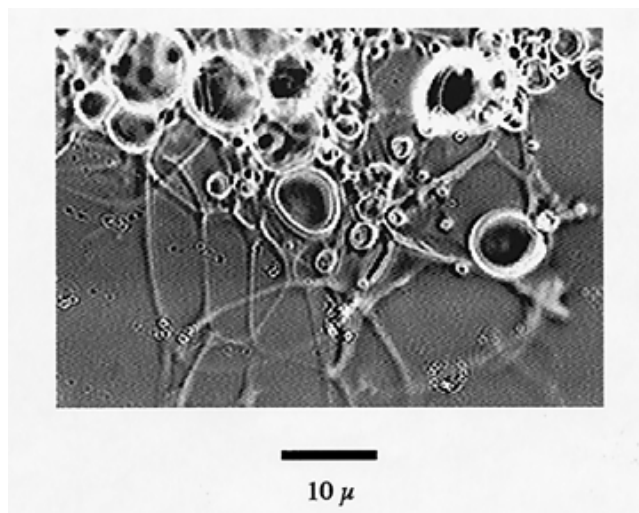
Wheat caryopses were cut transectionally into pieces  $\approx 1.5$  mm thick and fixed in a mixture of 3% glutaraldehyde and 2% formaldehyde (from paraformaldehyde) in 0.05M sodium phosphate buffer, pH 6.9, overnight at 0–4°C. Tissues were dehydrated in an



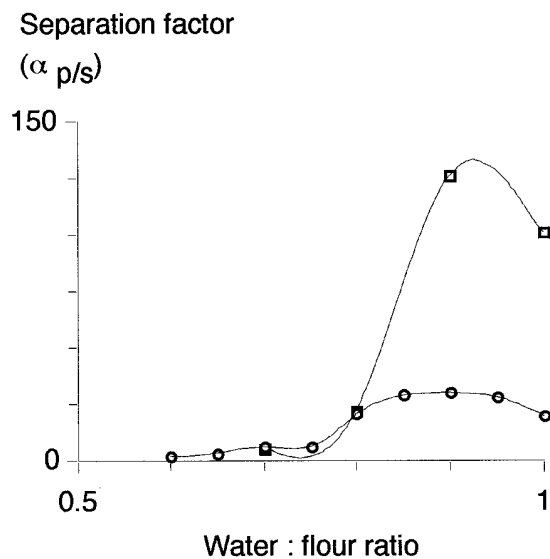
**Fig. 1.** Plastic section of wheat kernel starchy endosperm illustrating the distribution of wheat starch (white) and protein (dark) using a protein-sensitive dye (acid fuchsin) and a digitally enhanced negative image.

ethanol-butanol graded series (Jensen 1962) and infiltrated with glycol methacrylate (JB-4, Polysciences, Warrington, PA) over several days at 23°C. Tissues were embedded in glycol methacrylate and sectioned at 2  $\mu$ m using an MT-2 Porter-Blum ultramicrotome. Sections were collected on slides and stained in 0.01% acid fuchsin in 1.0% acetic acid for 1 min. After slides were rinsed in running tap water, they were air-dried and the sections were mounted in oil. The sections were viewed and photographed using a Zeiss universal research microscope equipped with a 100W mercury illuminator (Osram HBO 100W, Berlin) and an exciter-barrier filter combination which excited in the green region of the spectrum (exciter filter, BP 546; beam splitter, FT 580; barrier, LP 590) (Fulcher and Wong 1980, Fulcher et al 1989). Images were captured photographically on Ektachrome 400 transparency film developed by the E6 process. The resulting images were scanned (Polaroid Sprint Scan 35), the image was digitally inverted (negative), and the colors were enhanced to increase the contrast between the starch and protein matrix using Corel 9 photo paint.

Separation factors ( $\alpha_{p/s}$ ) were determined for the cold ethanol separation process (Robertson and Cao 1998a,b). This method may be summarized as flour hydration and development in a 10-g



**Fig. 2.** Wheat protein fibrils formed through contact of flour particle with water and seen in light microscopy (unstained).

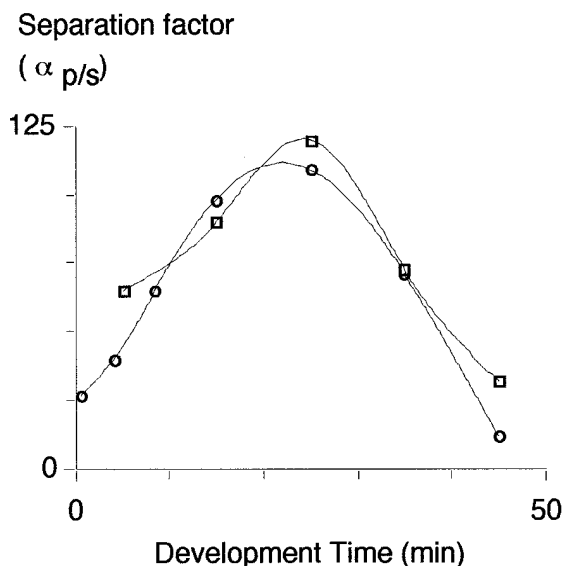


**Fig. 3.** Water-content control of separation factor for cold-ethanol, displacement separation of wheat flour into starch and protein. First-round optimization (○) at 5 min of development, 90% ethanol at  $-7^{\circ}\text{C}$  and second-round optimization (□) at 25 min of development, 90% ethanol at  $-12^{\circ}\text{C}$ .

microfarinograph, chilling, and finally displacement separation in a Glutomatic device (model 2200, Perten Instruments). The sigmoidal mixing elements in the microfarinograph rotate in opposite directions and at different speeds (65 and 96 rpm). The displacement occurs in the Glutomatic by a Martin-like process (Godon et al 1983, Grace 1989) in which the bulk mixture is manipulated while being bathed in excess displacing fluid. The fluid carries starch from the protein matrix through a supporting screen and is collected in a beaker. In the published experiments (Robertson and Cao 1998a), the separation efficiency was determined for the entire process as a function of individual variables in each process step. The separation efficiency was indexed to the separation factor: the ratio of protein to nonprotein in the protein-rich, starch-poor gluten fraction to the same ratio in the protein-poor, starch-rich, or nonprotein fraction. In this mathematical representation, a high value of the separation factor is associated with better separation. A value of 1 characterizes no separation. It should be noted that small changes in the minor component (starch in protein-rich gluten and protein in the starch-rich fraction) cause very large changes in the separation factor. The earlier report of this method used a sequential search method to identify operating conditions that yielded product similar in concentration to that of aqueous methods. The report described evaluation of level of flour hydration (using best estimate values of development time, temperature, and concentration), development time (using best hydration value, and best estimate values of temperature and concentration), temperature of the displacement fluid (using best hydration and development time, and best estimate values of concentration), and ethanol concentration (using best hydration, development time, and temperature). Best estimate values were assigned on the basis of preliminary experiment. This report includes the results of a second round of experiments in which hydration was reevaluated using the best first-round values (Robertson and Cao 1998a) and development time was reevaluated using the best first- and second-round values.

## RESULTS AND DISCUSSION

In its native form, wheat starch is distributed in protein-rich areas shown in thin section (Fig. 1). The separation objective is to convert this into enriched starch and enriched protein. Milling disassembles this structure and creates a distribution of particles some of which



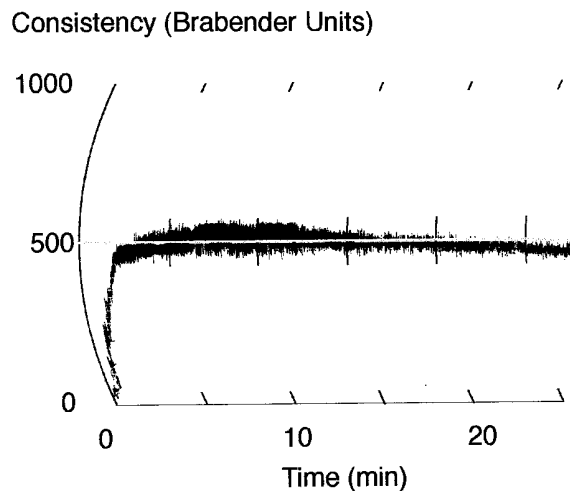
**Fig. 4.** Development time control of separation for cold-ethanol displacement separation of wheat flour into starch and protein fractions. First-round optimization (○) with 0.9 g of water/g of flour, 90% ethanol at  $-7^{\circ}\text{C}$ ; second-round optimization (□) with 0.9 g of water/g of flour, 90% ethanol at  $-12^{\circ}\text{C}$ .

are enriched protein and some enriched starch, but most containing both protein and starch (Jones et al 1959). Virtually all separation techniques employ water added to milled flour to activate the protein's ability to extend and form continuous matrices and, with mechanical development, to release previously entrapped starch. Development of wheat protein networks begins on first contact of wheat particles with water and generally at the conjunction of solid, air, and water interfaces. This development is the rapid formation and extension of fibrils of protein in excess of water. This is attributed to hydration of the protein and mechanical drag of surface tension at the surface (Bernardin and Kasarda 1973a,b; Amend and Belitz 1993). Its occurrence was confirmed here for the flour used in this experimental program (Fig. 2). Because air-water interfaces abound in flour as it is being hydrated, the phenomenon undoubtedly occurs at the beginning of development and should result in similar structures or related structures or complexes in the dough.

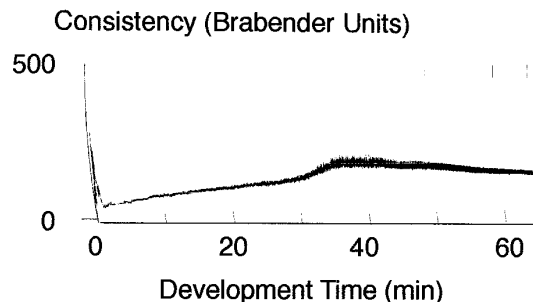
The creation of separable structures also involves the application of external forces to stretch and relax the dough and thereby bring the individual protein elements into contact, allow the formation of binding interactions, and reduce the entrapment of starch. This mechanical development occurs in mixers of many types and creates a dough with a spectrum of states: well-mixed, homogenous, uniform, demixed, unmixed, segregated, developed, and undeveloped. When the mixer is the microfarinograph, as in the present work, extensive and compressive actions are created by the differential-rotation and counter-rotation of the mixing blades.

### Separation Quality of Dough

In earlier research of the cold-ethanol separation method, we evaluated variables that we anticipated as important to the ultimate sep-

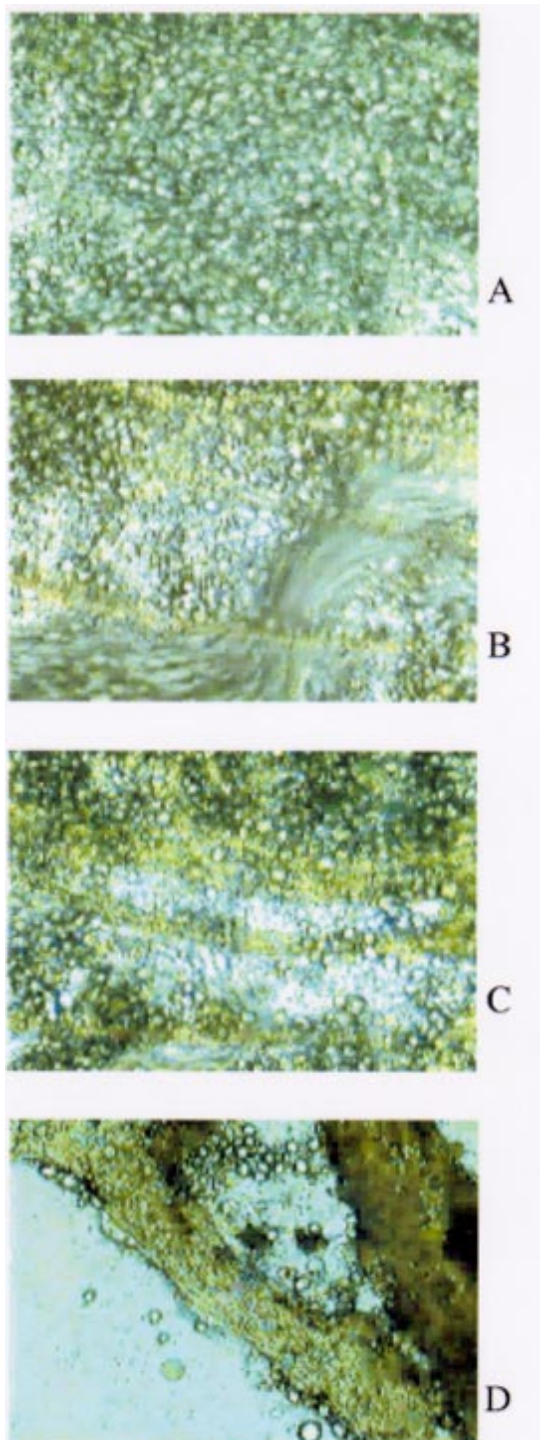


**Fig. 5.** Characteristic farinogram for wheat flour in this investigation. Water (0.675 g/g of flour) added to achieve a consistency of  $500 \pm 10$  BU at peak consistency.



**Fig. 6.** Farinogram for wheat flour and water matrix illustrating consistency changes during development (0.9 g of water/g of flour) at  $25^{\circ}\text{C}$ .

aration. The separation system involved separate development and displacement operations in separate apparatus. But the criterion of effective separation, the separation factor was computed for the entire separation system. Water content of the dough, development time, temperature of the ethanol, and water content of the ethanol were independently varied. To verify the appropriateness of the conditions determined in the first evaluation (Robertson and Cao 1998a,b), a second evaluation (using only 90% w/w ethanol) was performed employing conditions from the first evaluation. The present evaluation demonstrates a fourfold increase in the separation factor to  $\approx 120$  during



**Fig. 7.** Progressive fluid displacement of starch (B to C) from developed dough (0.9 g of water/g of flour and 25 min in the microfarinograph) (A) leading to protein concentrate (D) using visible light microscopy. Diameter for circular starch particles is  $\approx 20$ – $25 \mu\text{m}$  (image internal reference). Protein is yellow to tan color.

the hydration evaluation reflecting the use of a longer development time (25 min vs. 5 min) and lower temperature ( $-12^\circ\text{C}$  vs.  $-5^\circ\text{C}$ ) (Fig. 3). The experiments also demonstrated most of the observed effect could be attributed to the hydration of the dough and that little additional improvement was obtained when the temperature was reduced by  $7^\circ\text{C}$  ( $-12^\circ\text{C}$  vs.  $-7^\circ\text{C}$ ) (Fig. 4). However, the evaluation suggests that best separation is for a water-to-flour mass ratio in the range of 0.85–0.95 and a development time of  $25 \pm 5$  min. Furthermore, development beyond the period of best separation leads to a mixture with a separation factor of 32 at 45 min that is less separable than one mixed for 5 min (separation factor of 65). Note that the standard deviation for the separation factor in repeated experiments was 5.8% of the mean value.

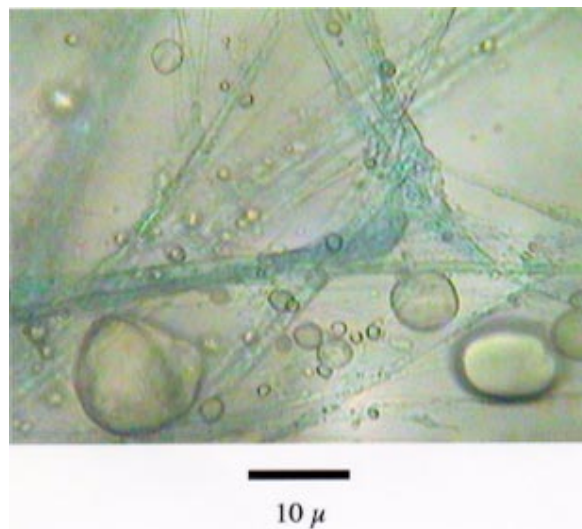
### Dough Consistency

Development of water and flour matrices for this study and all the previous separation studies was performed in a microfarinograph. A standard or characteristic farinogram for the flour used here is shown in Fig. 5. This sample was hydrated to 0.675 g of water/g of flour, an amount sufficient to give a consistency of 500 BU and tested at  $30^\circ\text{C}$  as recommended by the manufacturer. The data is presented as a functional characterization of the flour employed in this study. The farinogram is similar to that for a strong flour suitable for breadmaking and would be expected to give the best performance (highest loaf volume) at the time of peak consistency or  $\approx 7$  min. However, dough formed at this level of moisture would be poorly separable by the criterion of Fig. 3.

A development farinogram employing 0.9 g of water/g of flour is shown in Fig. 6. This flour and water matrix reached a maximum consistency of 200 at 35–40 min. The steady increase in consistency may be related to the increased separation shown in Fig. 4, but the consistency continues to increase until well beyond the time of best separation. The period of greatest rate of increase of consistency was observed at 30–35 min when the separation is degrading. The period of greatest elasticity, as evidenced by the high vertical spread of the consistency data, was 35–40 min and coincided with diminished separability.

### Structural Features of Dough

We initiated the microscopic investigation with video micro-recordings of the displacement phenomenon (using water as the displacing fluid). A sequence of images (separated in time by a few seconds) from one of these recordings is shown in Fig. 7. These depict the initial starch dominated state (Fig. 7A) and the protein dominated final state (Fig. 7D). Transition images with fluid-

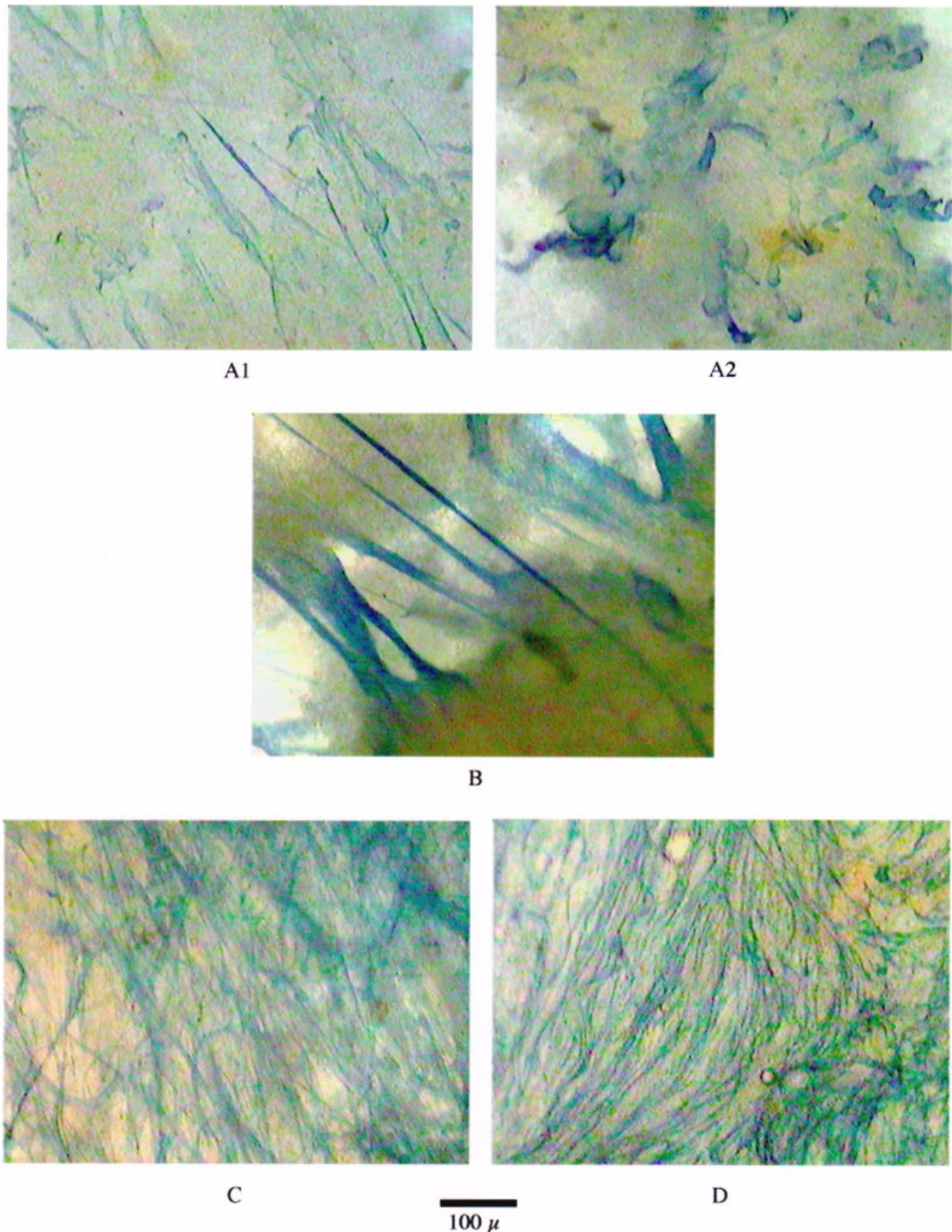


**Fig. 8.** Protein structures in dough developed in a farinograph from a water flour matrix (0.9 g of water/g of flour), developed for 45 min and stained with bromophenol blue (0.05%) immediately before image capture.

ized starch displaced by the water (Fig. 7B and C) reveal rapid movement of the starch (Fig. 7B), a high degree of protein and starch segregation, and a high degree of openness in the developed protein structure.

The protein structure in these images resembled an open-celled sponge. This sponge-like structure and segregation of the starch into a fluid phase can explain why the separation of wheat protein and

starch proceeds with relative facility whether the displacing fluid is water or refrigerated ethanol. In the separation phase of the experiments, a cohesive wetted flour mass is repeatedly forced through a constricting space during which the flour mass is compressed and the fluid starch-rich phase is expressed. After the constriction, the flour mass decompresses, relaxes, and imbibes fresh fluid that is supplied dropwise to surfaces by the device. Repeated expres-



**Fig. 9.** Protein structures in dough developed in a microfarinograph from water flour matrix (0.9 g of water/g of flour) and stained with bromophenol blue (0.05%) immediately before image capture. Dough developed for 5 min (A1 and A2), 25 min (B), 45 min (C), and 75 min (D).

sion of starch-rich fluid and uptake of new displacement fluid continues until no additional starch can be displaced.

Microscopic images of developed, wetted flour for different times of development reveal additional structural detail that may explain why separation is most successful at 25 min of development in the microfarinograph (Fig. 9). Images were taken for as little as 1 min and as late as 75 min of development. The 1-min examples were very irregularly developed. Extremes of structure at 5, 25, and 45 (or 75) min were noted and these correspond to separation factors for ethanol displacement of 65, 118, and 32. At 5 min (Fig. 9A and 9B), a variety of structures including partially segregated, globular, but unnetworked protein as well as banded and networked protein were observed. A more uniform, highly segregated and thick-banded network was seen at 25 min of development. A very uniform, interlaced, segregated and thin-banded network was observed after 45 and 75 min of development. The openness and unique character of the 25-min structure was rapidly revealed by displacement of starch during the staining. Less open structures in the 5- and 45-min samples led to less successful displacement of starch. Because the mechanism of starch removal is by fluid displacement, the structure with the most space for flow created by coalescence of protein into large structures was also that from which starch was most effectively displaced. Furthermore, the structure with the most dispersed and interlaced protein was the least successfully separated, suggesting entrapment of starch.

In addition to the formation of an interlaced network, high development times also were observed to produce disaggregation into fine threads of protein (Fig. 8). This detail was not observed in images at the shorter development times. The transition to this fine structure may contribute to the loss of separability not only by entrapment of starch particles but also by fragmentation of the protein. The latter effect is hypothesized to occur by progressive embrittlement and loss of elasticity of the protein threads as water is removed by the dehydrating alcohol. The proposed explanation is based on observation of bulk property changes to wet gluten that is washed in 100% ethanol.

## CONCLUSIONS

This manuscript is the first to relate structural features of wheat dough to the effectiveness of separation. The attainment of a highly coalesced protein network with large open spaces is a feature that correlates to the most effective separation. Development in a microfarinograph achieves a state like this after 25 min but continuation of development alters this structure and limits the dough separation. A farinogram indicates increased consistency that correlates with improved separability but a consistency rate change and elasticity increase that may correlate with loss of

separability. The development of complex networks of thin-stranded protein ultimately may be responsible for both loss of separation and high consistency and elasticity in the development of wetted wheat flour. Loss of separability may be due to starch entrapment and protein fragmentation.

Single word descriptions of wetted flour structures such as uniform, mixed, demixed, unmixed, developed, or even dough and batter are inadequate depictions of reality and may mislead. The use of mixing when applied to wheat flour and water clearly is especially misleading because states of highly aggregated protein and starch may result when wetted flour is "mixed" in a "mixer" as described here.

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