CURTAIN ELECTROPHORETIC SEPARATION OF THE WATER-SOLUBLE CONSTITUENTS OF BLEACHED CAKE FLOUR[1]

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ABSTRACT

The constituents of bleached cake flour which are extractable by water (2 parts water:1 part flour) have been fractionated by means of curtain electrophoresis in acetic acid-formic acid, pH 2.27, into carbohydrate, protein, and mineral fractions.

The single carbohydrate fraction and two of the four protein fractions have been examined by moving-boundary electrophoresis and ultracentrifugation. Comparison of these materials with the original aqueous extracts and fractions derived by salt-precipitation techniques has been carried out. Migration characteristics in aluminum lactate buffers as well as solubility properties suggest that the major protein fraction, P_3, consists of a mixture of gliadin and albumins.

The carbohydrate fraction, as shown by various staining techniques as well as electrophoretic behavior on the curtain and in the moving boundary, has been obtained essentially free of protein.

The curtain electrophoretic technique has proved of value in yielding fractions suitable for further chemical characterization.

The application of salt-fractionation techniques to separation of the proteins and polysaccharides extracted from flour by aqueous solvents has been described by several workers (7,9). It was reported in earlier work (9), confirming reports for other flours (7), that ammonium sulfate fractionation did not produce a pentosan fraction free of protein. This pentosan fraction was therefore considered to be unsuitable for further characterization.

The use of new fractionation techniques for determining the composition of various of the protein fractions of flour is increasing rapidly (4,17,18). Coates and Simmonds (4) realized partial separation of protein from pentosan by DEAE-cellulose column chromatography of the flour constituents soluble in 0.01*M* sodium pyrophosphate, pH 7. The paper curtain electrophoresis apparatus has been used to separate the acetic acid-soluble proteins of flour (18). A study of the factors

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affecting separation of proteins on the curtain electrophoresis apparatus has been given recently by Peterson and Nauman (10). The instrument used in the present work has been described by Hannig (6), and the technical aspects of separation on this curtain have been discussed by Grassman and Hannig (5) and Weber (15).

The present work has involved fractionation by preparative curtain electrophoresis of the constituents of bleached cake flour which are soluble in water. The results of moving-boundary electrophoresis studies in previous work (7) have shown that the proteinaceous constituents of aqueous extracts of flour separate readily from the immobile pentosan fraction by migration at pH 3.8. This result prompted the use of acidic solvents for the curtain electrophoretic fractionation.

This work can be clearly distinguished from that of others by three features. Firstly, we have worked with the total soluble material which includes proteins, carbohydrates, salts, sugars, etc. of a wide spectrum of molecular weight. Secondly, no attempt has been made to inactivate enzyme systems by such means as heat-treatment, since other work in this laboratory suggests such procedures lead to the formation of anomalous components. Thirdly, chemical characterization has been delayed until the fractions were obtained in a relatively uncomplicated form. This is particularly true regarding the carbohydrate fraction.

Materials and Methods

A commercial bleached cake flour was used throughout this study. Analytically, the flour contained 1.3% nitrogen (14% moisture basis) and 0.7% lipid (ethyl ether-extractable). Reagent-grade chemicals were used throughout.

Isolation of the Total Water-Soluble Constituents. One part flour was mixed with two volumes of water in a Sunbeam Mixmaster at medium speed for 5 min. The slurry was centrifuged in an International centrifuge at 1,800 r.p.m. for 30 min. and the supernatant was freeze-dried to yield 3.6 g. of total water-solubles (TWS) per 100 g. flour. The protein content of a typical preparation of TWS was 49% (micro-Kjeldahl nitrogen $\times$ 5.7), assuming all nitrogen to be protein nitrogen.

Preparation of the Gliadin-Free Water-Soluble Fraction. A gliadin-free sample of the water-solubles was prepared by adding crystalline sodium chloride to the water extract to a concentration of 0.3*M*, allowing the precipitate to form on standing 16 to 24 hr. at 4°C., and removing the precipitated gliadin by centrifugation at 1,800 r.p.m. for 15

min. The supernatant was dialyzed against frequent changes of distilled water for 72 hr. at 4°C. and dried by lyophilization. An average yield of 1.1 g. per 100 g. flour was obtained. The protein content of a typical preparation was 42%.

Curtain Electrophoresis. The instrument used in this study was the Elphor VaP produced by the Bender and Hobein Co., Munich, Germany. The paper curtains employed were Binzer No. 230 and Schleicher and Schüll Nos. 2043b and 2030-I. Experimental details are given in the appropriate sections.

Qualitative fractionation of the components was carried out first on curtains of S&S No. 2043b. The curtain from each run was dried and cut into horizontal strips. Staining procedures, listed below, applied to these strips revealed the location of the proteinaceous, carbohydrate, and lipid fractions and hence the success of electrophoretic separation. Preparative curtain electrophoresis of the solutions under similar experimental conditions was then carried out. The curtain was rinsed with buffer for 16 hr. prior to application of the solution and for 10 hr. at the end of fractionation, for purposes of quantitative recovery. The mouths of the tared collection tubes were covered with small pieces of nylon hosiery prior to freezing of the contents in an acetone-dry ice bath. Lyophilization was carried out in a Virtis mechanically refrigerated freeze-drying apparatus. The tubes were weighed and yields in each tube and in groups of tubes corresponding to chosen fractions were calculated.

Staining Procedures. Specific staining methods for proteins, carbohydrates, and lipids were used. The procedures employed included the following: 1) direct staining of 3-cm. horizontal strips cut from the thick preparative curtain, 2) direct staining of strips cut from a "replica" of the thick curtain (which was prepared by pressing a thin sheet of chromatographic paper, by means of a roller, against the wet curtain), and 3) spot tests of five lambda quantities of the contents of the collection tubes.

a. Proteins, Peptides, and Amino Acids. The chlorination method of Reindel and Hoppe (11) was the more sensitive precedure for proteinaceous materials. Excess chlorine was removed by the method of Wieland and Dose (15). The materials develop a blue color varying from light to dark, depending on concentration.

Ninhydrin staining procedures (1) were also used to detect the proteinaceous constituents.

b. Carbohydrates. Carbohydrate components were detected by alpha-naphthol (2) and aniline-diphenylamine (3) procedures.

c. Lipids. Lipids were detected with 0.1% Sodium Black B in 60% ethanol (13,14).

Moving-Boundary Electrophoresis. Solutions of components to be examined were subjected to electrophoresis in a Model 38A Perkin-Elmer instrument at 2°C.

Ultracentrifugation. The Spinco Model E instrument with the RITC constant temperature control unit was used for ultracentrifugal examinations.

Results and Discussion

Electrophoretic Fractionation of the Gliadin-Free Water-Solubles. A 2% solution of the gliadin-free water-solubles in pyridine/acetic acid/water (10:3:750), pH 4.1, was applied to a Binzer No. 230 paper curtain at a rate of 7.5 mg. per hr. under conditions of 14.3 volts per cm. field strength. The separation achieved is shown in Fig. 1. Two

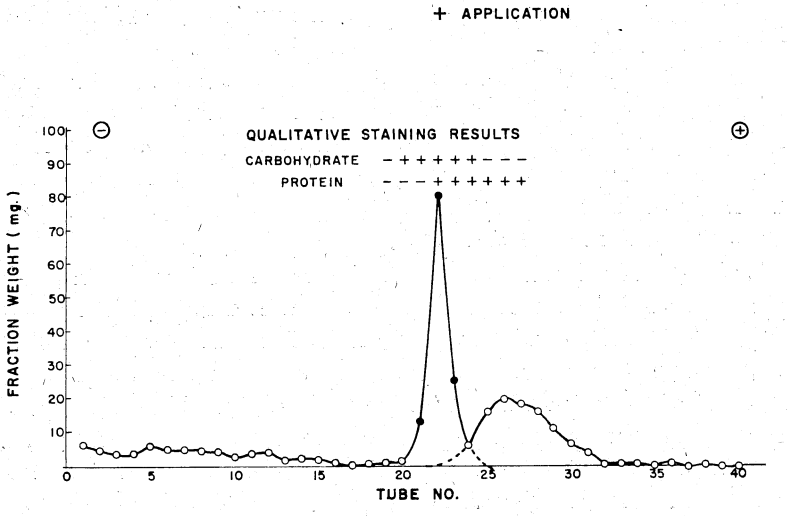

Fig. 1. Curtain electrophoretic separation pattern of the gliadin-free water-solubles (experimental conditions as given in text).

fractions were collected, a mixture of protein and carbohydrate from tubes 21–27 and a protein fraction from tubes 28–30. The protein-carbohydrate mixture was subjected to further electrophoretic fractionation in formic acid, pH 1.9, on a Binzer No. 230 curtain at a field strength of 37.0 volts per cm. Excellent resolution of the protein from the carbohydrate was achieved under these conditions. Nitrogen analysis revealed less than 1% protein in the carbohydrate fraction.

Figure 2 shows the moving-boundary electrophoretic patterns of the gliadin-free water-solubles used as the starting material and of the fractions obtained from the curtain electrophoretic separation.

The results show that separation of protein from carbohydrate was successful and indicate that this procedure should be of value for fractionation of the total water-solubles.

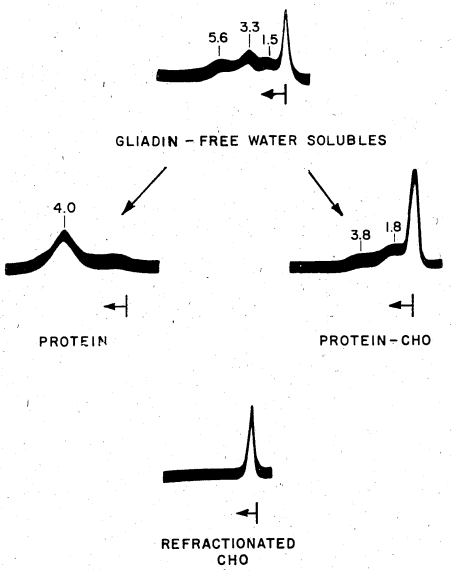

Fig. 2. Descending moving-boundary electrophoretic patterns of the gliadin-free water-solubles (5,400 sec., 6.50 volts/cm.); protein fraction from curtain (7,200 sec., 6.77 volts/cm.); protein carbohydrate mixture from curtain (7,200 sec., 6.83 volts/cm.); and refractionated carbohydrate fraction (7,200 sec., 6.54 volts/cm.). All runs in acetate buffer, pH 3.8, 0.1 ionic strength. Mobilities ($\times 10^5$) are shown above peaks.

Electrophoretic Fractionation of the Total Water-Solubles. Qualitative electrophoretic separations of 1% solutions of the total water-solubles (TWS) in a number of acidic solutions were carried out to determine the optimum solvent for preparative runs. These conditions included solutions of formic acid, acetic acid, mixtures of acetic acid and pyridine, and mixtures of formic acid and acetic acid. Optimum separation of protein from carbohydrate was achieved in a solvent of 0.15*N* formic acid:0.15*N* acetic acid, pH 2.27. Figure 3 shows the separation pattern obtained for 100 mg. TWS applied at a rate of 7.5 mg. per hr. at a field strength of 19.8 volts per cm. Staining procedures and chemical analyses revealed a carbohydrate fraction (CHO), essentially free of protein (~1%), in tubes 27–31, and proteinaceous

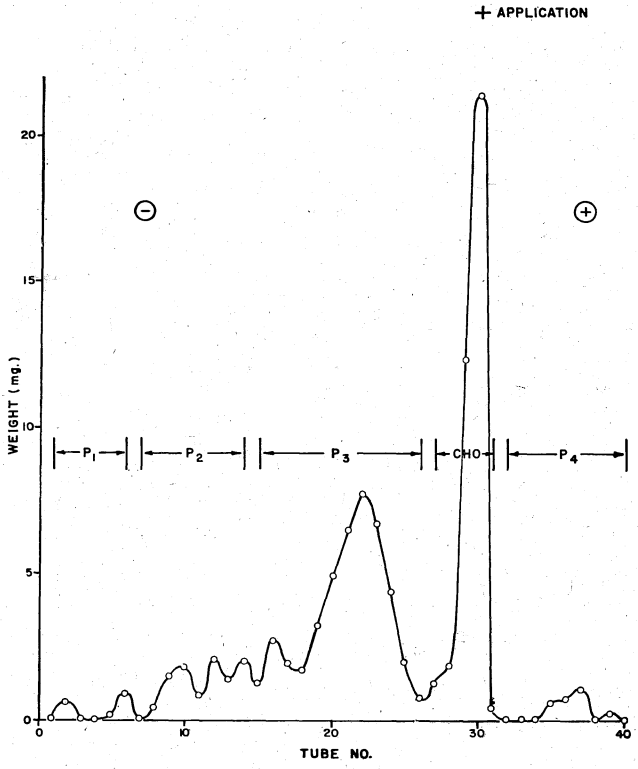

Fig. 3. Curtain electrophoretic separation pattern of total water-solubles (TWS).

components in all other tubes. A small amount of proteinaceous material, P_4, was anionic at this pH. Four protein fractions, P_1 to P_4, were arbitrarily collected from the tubes as shown in Fig. 3. The yields of each of these fractions are given in the table below. These results are averages of five separate runs. These protein fractions should not be considered as pure proteins. The accompanying paper (12) will reveal the chemical compositions.

	%
P_1	4 ± 2
P_2	9 ± 2
P_3	46 ± 3
CHO	37 ± 2
P_4	4 ± 2

Moving-Boundary Electrophoretic Analyses of the Fractions. The electrophoretic patterns of the carbohydrate fraction and two protein fractions, P_2 and P_3, were determined in two buffer systems; acetate, pH 3.8, 0.1 ionic strength, and aluminum lactate, pH 3.0, 0.6 ionic

strength. Previous work (7) has indicated that about one-half of the protein extracted by two volumes of water from this bleached cake flour is gliadin. Since gliadin has a low solubility in acetate buffers of 0.1 ionic strength, it will not be revealed by electrophoresis in this buffer system. However, the soluble polysaccharides and the albumin components can be examined in this buffer and therefore recognized in the various fractions collected from the curtain. Gliadin is soluble to a much greater extent in the aluminum lactate buffer system (8).

Figure 4 shows the electrophoretic patterns of the nondialyzable components of the total water-solubles, TWS, and of three fractions

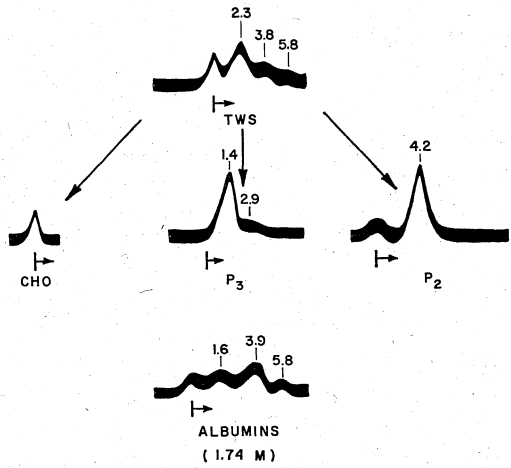

Fig. 4. Ascending moving-boundary electrophoretic patterns of the total water-solubles (TWS) (7,200 sec., 5.95 volts/cm.); carbohydrate fraction (CHO) (5,400 sec., 6.19 volts/cm.); P_3 (7,200 sec., 6.36 volts/cm.); P_2 (5,400 sec., 6.67 volts/cm.) and albumins (1.74*M* precipitate) (7,200 sec., 6.80 volts/cm.). All runs in acetate buffer, pH 3.8, 0.1 ionic strength. Mobilities ($\times 10^5$) shown above peaks.

from the curtain, carbohydrate, P_2, and P_3, in acetate buffer. Mobilities (ascending) are indicated by the numbers above the peaks. The electrophoretic pattern of an albumin preparation from the water extract of this same flour, which was obtained by precipitation at 1.74*M* ammonium sulfate, is also shown in Fig. 4. The carbohydrate fraction revealed no peaks corresponding to mobile protein constituents. Comparison of the patterns in Fig. 4 reveals the peaks in the starting material from which the fractions P_2 and P_3 (or that portion of P_3 soluble in this buffer) arise, and shows also that peaks of similar mobility are present in the total albumin fraction prepared by an entirely different procedure.

Figure 5 shows the moving-boundary electrophoretic patterns in aluminum lactate, pH 3.0, $\mu = 0.6$ of TWS, P_2, P_3, and a preparation of gliadin obtained from the water-extract of this same flour by precipitation at 0.3*M* sodium chloride. These patterns reveal that P_3 resembles gliadin as obtained from this flour.

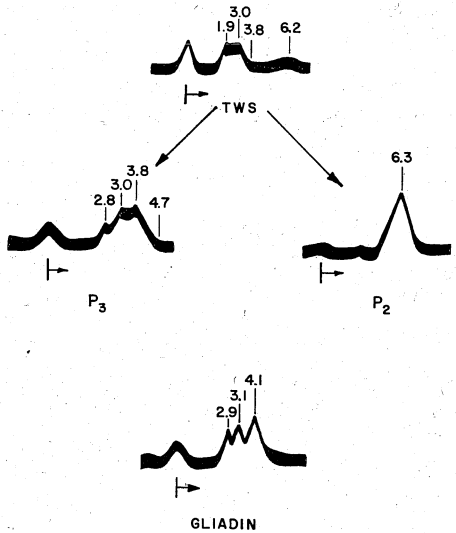

Fig. 5. Ascending moving-boundary electrophoretic patterns of the total water-solubles (TWS) (5,400 sec., 9.01 volts/cm.); P_3 (7,200 sec., 11.1 volts/cm.); P_2 (3,600 sec., 8.67 volts/cm.) and gliadin (0.3*M* sodium chloride precipitate from TWS) (5,400 sec., 11.65 volts/cm.). All runs in aluminum lactate buffer, pH 3.0, 0.6 ionic strength. Mobilities ($\times 10^5$) shown above peaks.

The results of the electrophoretic experiments demonstrate that the fractions obtained by curtain electrophoresis correspond to those we might expect on the basis of the moving-boundary behavior of the starting material. The results also strongly suggest that P_3 is made up of a mixture of some of the albumin components (which are soluble in both the acetate and aluminum lactate buffers) and gliadin (which is much more soluble in lactate buffer). This conclusion is also supported by the fact that P_3 has a higher solubility in aluminum lactate than in acetate and by the fact that the addition of sodium chloride to a water solution of P_3 results in precipitation of a majority of the protein. Additional evidence for this being the composition of P_3 is given in the accompanying paper (12).

Ultracentrifugal Examination of the Fractions. Table I gives uncorrected sedimentation coefficients determined for a number of flour

TABLE I

SEDIMENTATION COEFFICIENTS OF FLOUR FRACTIONS

FRACTION	SEDIMENTATION SOLVENT	S (UNCORRECTED)
Water solubles	Acetate, pH 3.8	1.9[a]
Acetate, pH 3.8, extract	Acetate, pH 3.8	1.6[a]
0.1*M* sodium chloride extract	Acetate, pH 3.8	1.8[a]
P_2	Acetate, pH 3.8	1.9
	Aluminum lactate, pH 3.0	1.2
P_3	Acetate, pH 3.8	1.9
	Aluminum lactate, pH 3.0	1.8
CHO	Acetate, pH 3.8	1.6

[a] Heavier components also present.

fractions obtained by simple extraction of the flour or from the curtain electrophoretic fractionation. In most cases the sedimentation patterns of such fractions as listed in Table I show heterogeneous dispersion (as skewness, rather than separate peaks) and reveal only small differences in sedimentation rates between the various materials (7). We therefore conclude that at our present state of knowledge ultracentrifugation does not reveal any really significant characterization results. As the fractions become more purified, this technique will likely be of more value.

In summary, then, the curtain electrophoretic technique has allowed fractionation of the water-soluble constituents of cake flour. The fractions have been analyzed more completely and these results are in the accompanying paper (12). It is certainly possible that other arbitrary choices of fractions could be made such as to fractionate P_3 further into the gliadin and albumins of which it is composed. Preparative fractionation of the total albumins is also possible, as shown in the work here with the gliadin-free water-solubles. The electrophoretic mobilities of the protein fractions from this material agree well with P_2 and the albumin portion of P_3 shown in Fig. 4. The further characterization of subfractions of albumins obtained from the curtain and from salt-precipitation techniques can be carried out by these methods.

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