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Fractionation of wheat proteins by counter-current distribution using aqueous two-phase systems containing propionic acid

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Abstract

Wheat proteins, soluble in diluted acid (glutenins), have been fractionated by counter-current distribution (CCD) using an aqueous two-phase system. The phase system is based on poly(ethylene glycol) and dextran but contains also 1% propionic acid and 6 m*M* magnesium sulfate. Approximately half of the bulk proteins partitioned to the upper phase while starch and other particles were recovered only into the lower phase. Whole wheat flour could be applied as sample for the CCD and 57 transfers were carried out. Starch and insoluble proteins remained stationary, while proteins followed the mobile phase to various degrees giving rise to a distribution pattern. The CCD pattern of the proteins showed distinct differences when various kinds of wheat flour were analysed. The patterns indicate that at least six subpopulations of proteins can be obtained by using two-phase extraction.

Keywords: Aqueous two-phase systems; Proteins; Propionic acid

1. Introduction

Baking studies employing flour constituents, which have been separated and reconstituted into doughs, have established that flour proteins are prime factors governing wheat baking qualities [1]. The wheat proteins are classified into five groups according to their solubilities: Albumins, soluble in water; globulins, soluble in salt solutions; gliadins, soluble in water-containing organic solvents; glutenins, soluble in diluted acids; and insoluble rest proteins. The glutenins and gliadins are described as the gluten proteins or wheat storage proteins, while the two first groups are known as (water) soluble proteins.

The wheat grain is composed of three parts: the embryo (germ), the endosperm and the bran. The albumins and globulins are present in both the embryo and the endosperm, while the gluten proteins only are found in the endosperm, where they are the main proteins [1]. The gluten proteins, gliadins and glutenins, confer extensibility and elasticity to the dough, respectively [2]. Gluten is a protein mixture with a molecular mass distribution from about 30 to about 20 000 kDa. These proteins are rich in glutamine and proline, but poor in lysine and arginine, which are present to a great degree in the water-soluble proteins. The high content of hydrophobic amino acids also explains the insolubility of gluten protein in water.

It is generally assumed that mainly the gluten proteins are responsible for variations in baking performance of different flours. Several explanations have been put forward: (1) The gliadin/glutenin ratio [3,4]; (2) the mean molecular mass distribution of glutenins [3]; (3) the presence of certain high-

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molecular-mass (HMW) glutenin subunits [2,5,6]; or (4) the presence of certain gliadin subunits [7].

The gliadin group is heterogeneous and the mobility in native aluminium lactate starch gel electrophoresis has been used to divide the gliadins in four groups: α-, β-, γ- and ω-gliadins, arranged in order of decreasing electrophoretic mobility. The molecular mass of most gliadins is in the range 30–40 kDa, but the ω-gliadins have a molecular mass between 60 and 80 kDa. The ω-gliadins differ from the other gliadins in that they do not contain α-helix or β-sheet structures. Instead, the ω-gliadins contains β-turns, which are stabilized by hydrophobic interactions between aromatic amino acid residues, and by hydrogen bonding involving glutamine residues [8].

The structure of the glutenin proteins is quite similar to that of the ω-gliadins. The conformation is characterized by a large portion of β-turns, but also some α-helix is seen in the terminal domains. The existence of β-turns can be the reason for the rather high elasticity of glutenins [9]. The glutenins are big molecules; molecular masses up to 20 000 kDa have been reported for native, non-reduced glutenins [3,10]. This high molecular mass can also explain the low solubility of the proteins of this group. Because of the big size of the native, non-reduced glutenin proteins, these are normally analyzed by studying the subunit pattern with electrophoresis. The subunits can be divided into three groups: low-molecular-mass (LMW) subunits, 30–51 kDa; medium-molecular-mass (MMW) subunits, 51–90 kDa; and high-molecular-mass (HMW) subunits, 90–150 kDa. These three groups of glutenin subunits differ in hydrophobicity, with the LMW subunits being the most hydrophobic and the MMW glutenins being the least hydrophobic [11].

In the present work an aqueous two-phase technique in combination with counter-current distribution was applied for fractionation of wheat proteins. One of the advantages of using aqueous two-phase systems for fractionation of storage proteins should be that the proteins can be separated in their native form instead of reducing them to subunits. This would also offer the possibility to study the influence of intact proteins on the baking performance by adding them to a standard dough.

Aqueous two-phase systems composed of two immiscible water-rich liquids can be used to separate biological material, e.g., proteins, by making use of a selective distribution of the components between the two phases [12–15]. The unequal partition between top- and bottom phase depends on the molecular structure and surface characteristics of the partitioned protein molecules. The two-phase systems are prepared by dissolving two polymers in water, i.e., poly(ethylene glycol) (PEG) and dextran. In these two-phase systems dextran is concentrated in the lower phase and PEG in the upper phase. The partitioning of a substance is usually expressed by a partition coefficient, K, defined as the ratio of the concentrations, C, of partitioned material in the two phases, i.e., $K = C_{top}/C_{bottom}$.

2. Experimental

2.1. Chemicals

Dextran T500 (500 kDa) was supplied by Pharmacia (Uppsala, Sweden) PEG 8000 was obtained from Union Carbide (New York, NY, USA). All other chemicals were of analytical grade.

2.2. Flour

Commercial wheat flour (Kungsörnen, Järna, Sweden) was used for solubility studies. Flour from six varieties of wheat were obtained from Svalöf Weibull (Svalöf, Sweden).

2.3. Gluten preparation

Gluten was prepared by treating 100 g of wheat flour with distilled water until no more starch could be washed away followed by washing with 0.5 M NaCl. Remaining proteins are gliadins and glutenins.

2.4. Solubility of gluten

Diluted acids were tested for their efficiency in dissolving gluten protein. Gluten (0.75 g) was treated with the diluted acid (99.25 g) by mechanical stirring at room temperature for 2 h. The mixture

was centrifuged for 10 min at 2700 *g*. The protein concentration in the supernatant was determined.

2.5. Assays

Protein was analysed according to Bradford [16] by absorbance measurement with an ELISA photometer at 595 nm using bovine serum albumin as standard. The true protein content in the samples deviates from the apparent protein concentration by a factor in the order of 10, due to less effective binding compared to globular proteins.

Particulate materials were measured via the light scattering as the apparent absorbance at 400 nm. Starch was measured as the absorbance at 600 nm after treating 25 μl of sample with 1.00 ml diluted aqueous iodine solution at 95°C for 5 min.

2.6. Two-phase systems and partitioning of proteins

Aqueous two-phase systems containing 5% (w/w) PEG 8000, 7% (w/w) dextran T-500, 1% (w/w) propionic acid and 6 m*M* salt, with or without sample (gluten or flour), were prepared from concentrated aqueous solutions of the polymers [40% (w/w) PEG, 20% (w/w) dextran] and solid salt. Propionic acid was added as 2% (w/w) solution with or without suspended sample. The partition of protein was determined by equilibrating the systems (10 g) at room temperature (23°C) by careful mixing for 1 min followed by centrifugation at 4500 *g* for 5 min. Samples (100 μl) were taken from each phase and the protein concentration was determined. The percentual amount of protein in the upper phase was calculated via the observed volume ratio of the phases.

2.7. Counter-current distribution

A thin-layer counter-current distribution (CCD) apparatus according to Albertsson [17] containing 60 chambers and with bottom phase cavities with a volume of 1.05 ml was used. Mixed systems containing 0.95 ml bottom phase and 1.20 ml top phase were added to each chamber. Systems with sample were applied in chamber 0-1. Sample was prepared by suspending the flour in 2% propionic acid (2.5 g flour per 10 ml acid), and then adding the concentrated polymers and $MgSO_4$ to get the systems, giving a protein concentration in the system of about 10 mg/ml. 57 distribution steps were carried out at 23°C including 50 s mixing and 10 min settling in each step. Finally the systems were broken by addition of 1.5 ml 2% (w/w) propionic acid to each chamber, and the obtained fractions were collected and analyzed for protein, light scattering and, in some cases, for starch. The obtained absorbance values are plotted as a function of tube numbers resulting in a CCD diagram.

3. Results

3.1. Diluted acids as solvent for protein

For almost all the diluted acids used to dissolve protein an increase in the acid concentration, from 1%, caused a decrease in the dissolving efficiency (Table 1). If the proteins are dissolved in fatty acids of varying carbon-chain length there is a maximum efficiency at 3 carbon atoms (propionic acid). Longer carbon chains show low dissolving efficiency. The introduction of an hydroxyl group (lactic acid) also reduces the solubility but the effect is less than what is achieved by adding an CH_2 group to the acid molecule. Based on these results 2% propionic acid was chosen for dissolving the proteins, and 1%

Table 1
Solubility of gluten proteins at 23°C in various diluted acids

Acid	Apparent concentration of dissolved proteins (mg/l)		
	1% Acid	5% Acid	25% Acid
Hydrochloric	16	10	27
Phosphoric	54	13	20
Acetic	58	46	16
Propionic	190	80	72
Butyric	12	16	14
Valeric	21	14	12
Lactic	76	60	40

Protein concentration was measured according to Bradford [16]. Percent values indicate volume concentrated acid per final volume when diluted with water.

propionic acid was included in the two-phase systems.

3.2. Selection of suitable two-phase system

Addition of salts influenced the partition of proteins. The amounts of proteins found in the top phase for systems containing different salts are shown in Table 2. The salts have the opposite effect on the partition than what is normally observed at neutral pH values [18]. It is known that the steering effect of salts on the partition of proteins is related to their net charge. At pH 2.7, as used in these systems, most proteins are positively charged. Therefore, a salt that increases the partition of a protein towards the lower phase (at neutral pH) may move it towards the upper phase at low pH. The adjustment of the partition of proteins is of importance when the system is to be used for counter-current distribution. With this multi-step fractionation procedure the best separation occurs when the average distribution ratio, i.e., material in upper phase/material in lower phase+interface, is close to one. Both lithium propionate and $MgSO_4$ gave this distribution of protein. The latter salt was chosen because it is a neutral non-buffering salt which does not effect the pH value of the system. The propionate increases pH and this will eventually decrease the solubility of protein.

Therefore, a system with the following composition was chosen: 5% (w/w) PEG 8000, 7% (w/w) Dextran 500, 1% (v/v) propionic acid and 6 m*M* $MgSO_4$.

Table 2
Effect of salts (6 m*M*) on the partitioning of proteins in the two-phase system containing 1% propionic acid at 23°C

Salt	Content of protein in upper phase (%)
None	56
KSCN	78
$KClO_4$	70
KCl	68
LiCl	61
NH_4CH_3COO	57
Li propionate	50
$MgSO_4$	49
$(NH_4)_2SO_4$	37

3.3. Counter-current distribution

The liquid–liquid partitioning can be made more effective by the so-called counter-current distribution (CCD). The principle of CCD is that two sets of liquid phases (upper and lower phase, respectively) stepwise are coming into contact with each other in a chromatographic manner. The sample to be analyzed is initially contained in one or several of the first systems (numbered 0 and upwards). The partitioning of a homogeneous compound between mobile and stationary part of the two-phase system is expressed by a partition ratio, where G=mass in mobile phase/mass in stationary phase. The amount of the substance in each tube, numbered from 0 to n, where n is the number of transfers, is given by Eq. 1, for the case of a sample applied in tube number 0 [17]:

$$T = \frac{i!}{n!(n-i)!} \frac{G^i}{(1+G)^n} \quad (1)$$

To get an idea of the heterogeneity of the proteins the CCD patterns were analysed by determining the lowest number of components (with different G values) necessary to give a reasonable fit. This was done by using a simple computer program based on Microsoft Excel.

By treating the flour directly in the acid the time-consuming gluten-preparation step can be omitted. Considerable amounts of proteins may be lost during the gluten-preparation procedure. Suspending the flour directly in the propionic acid solution therefore has the advantage of eliminating loss of protein. Starch and other substances present as particles (insoluble in acids) partition to the bottom phase or to the interface. As can be seen from the CCD diagram (Fig. 1A) particles (measured as light scattering) remain to the left close to the site of sample application. Practically all particles are found in tubes number 0–10, and most of it is present in the position of sample application (tube 0 and 1) and the following four tubes (2–5). Especially the starch, the blue iodine colour at 600 nm, was found in tube number 0–8.

A CCD of flour is shown in Fig. 1B. As predicted from the batch experiments the proteins distribute equally around the middle of the CCD diagram because of the presence of $MgSO_4$ (6 m*M*). Two-

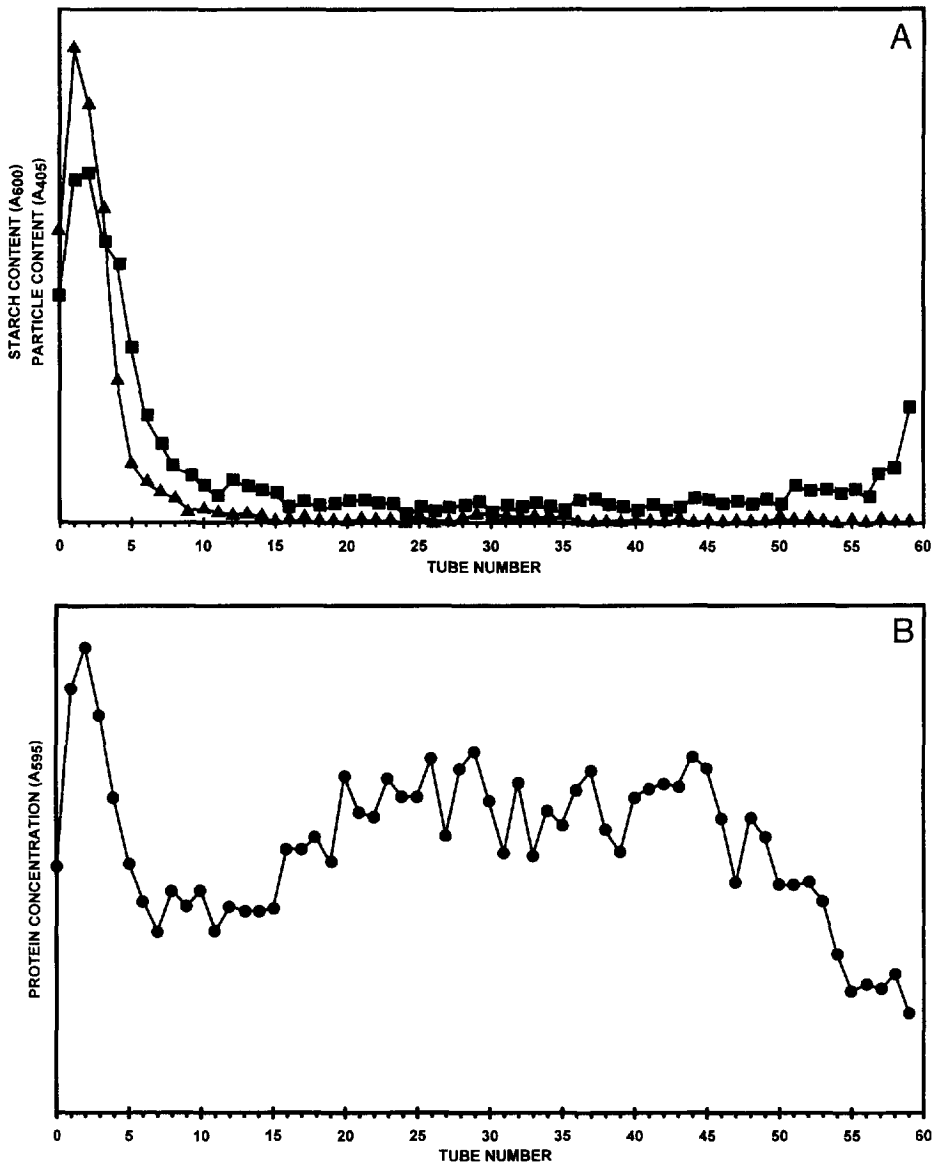

Fig. 1. CCD of commercial flour using 57 transfers. (A) Distribution of particles (■) and of starch (▲). (B) Distribution of proteins measured according to Bradford [16] at 595 nm (●).

phase partitioning of proteins have so far mainly been applied at physiological pH values (6.5–8) using potassium, sodium or lithium salt for steering of the partition. Because of the high acidity in the system, divalent cations, such as Mg^{2+} can be used, which at higher pH values would give rise to insoluble hydroxides or basic salts.

Fig. 2A–F shows the protein distribution after CCD analyses of six different kinds of flour: Turbo A6, Contra B4, Haven C2, Hussar C2, Cadenza B5 and Urban A9, varying in their baking characteristics.

Since the acid-soluble proteins are a mixture of many components the experimental CCD curves

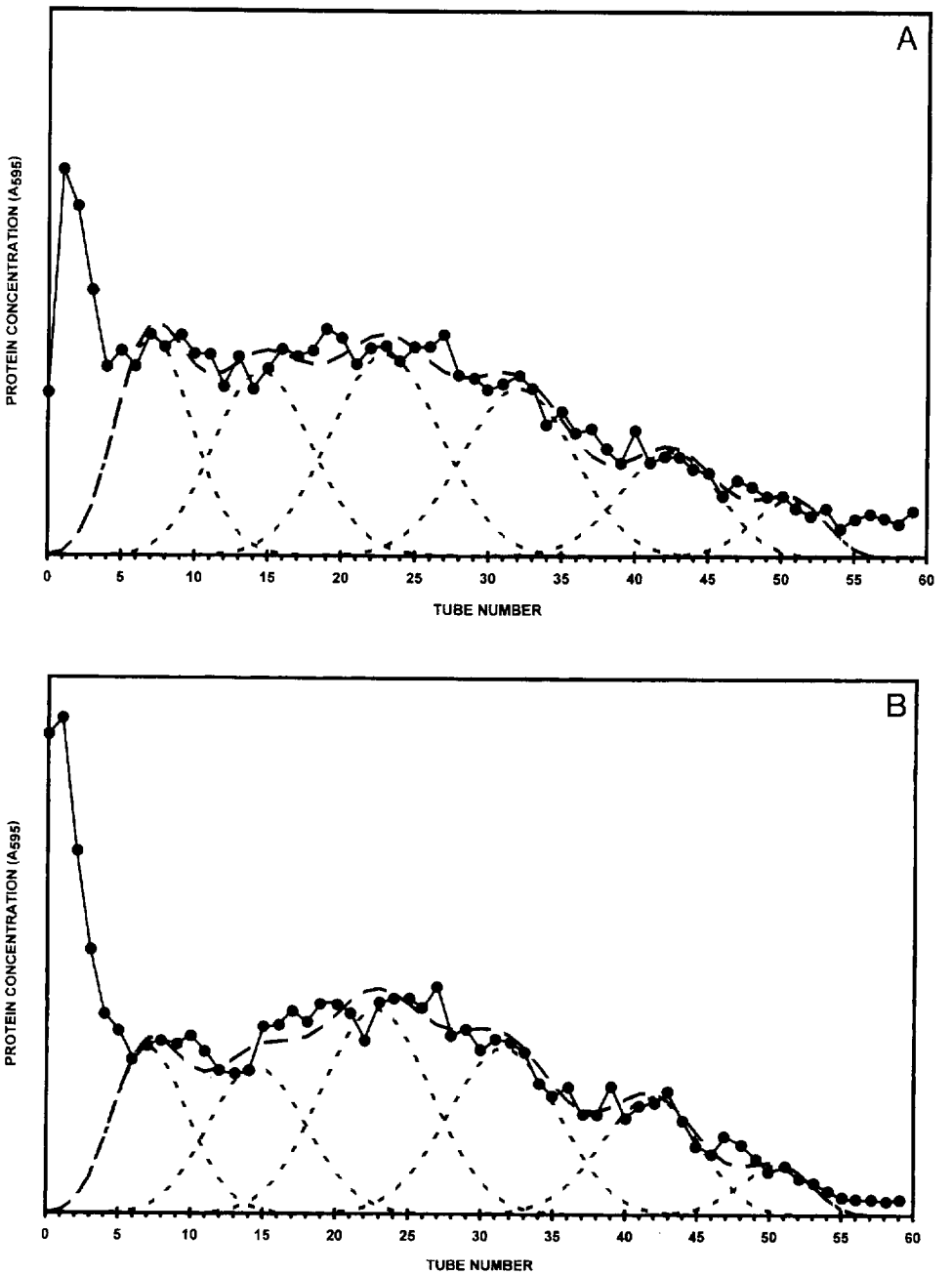

Fig. 2. CCD diagrams of six varieties of wheat. Dots (●) represent the experimental curve, – – – represents the theoretical curves and – · – represents the sum of the theoretical curves. The varieties are: (A) Turbo A6, (B) Contra B4, (C) Haven C2, (D) Hussar C2, (E) Cadenza B5 and (F) Urban A9.

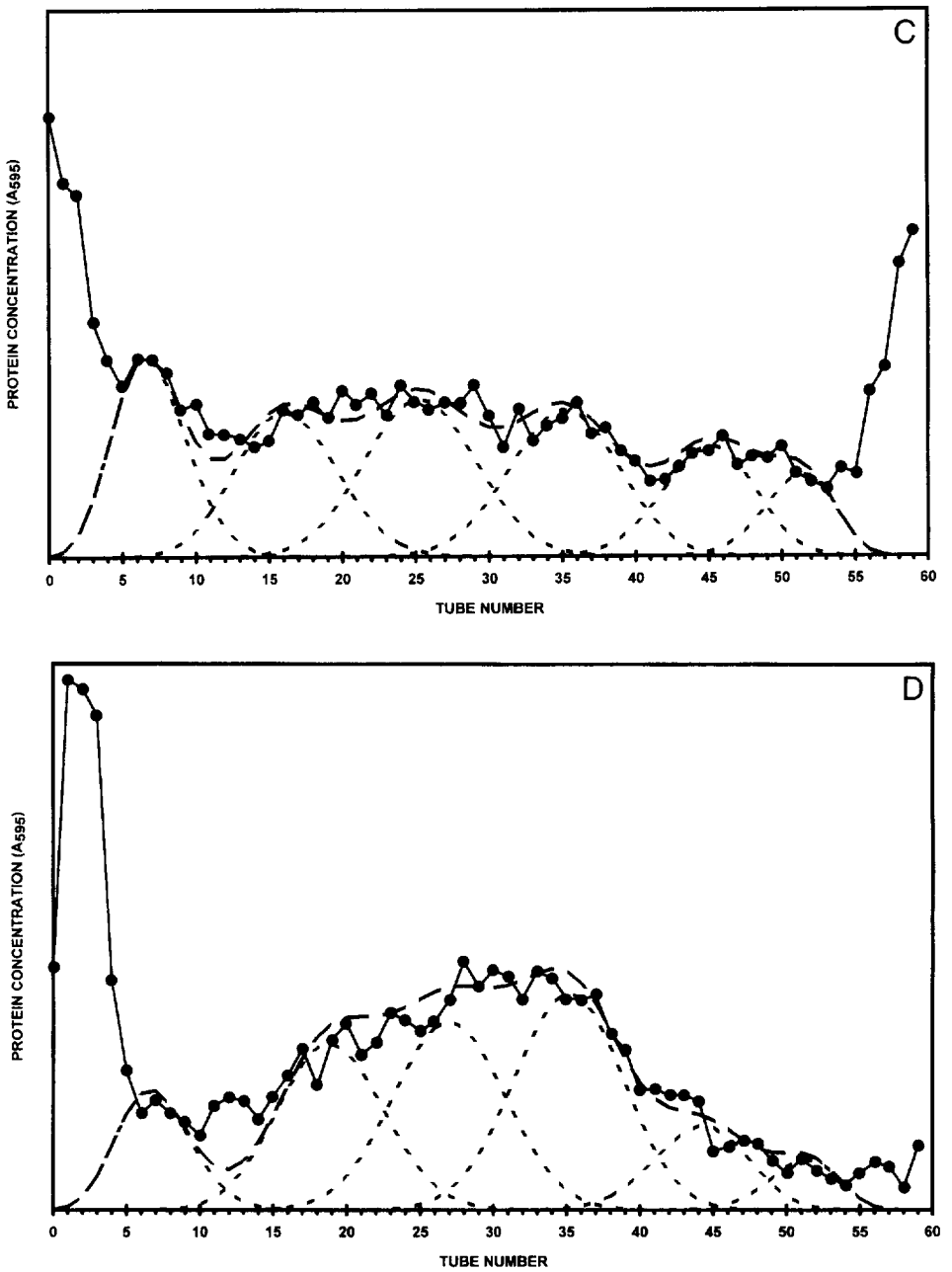

Fig. 2. (*Continued*).

have a complex appearance. A complete resolution could not be obtained with the number of steps used (57 transfers or "plates") and the proteins overlap. The distribution curves can, however, be analysed by calculation of theoretical curves for the various components and subsequently fitting a weight sum of these theoretical curves to the observed data. By fitting the sum of the theoretical curves to the experimental curve one can calculate the minimum number of components, and their relative apparent

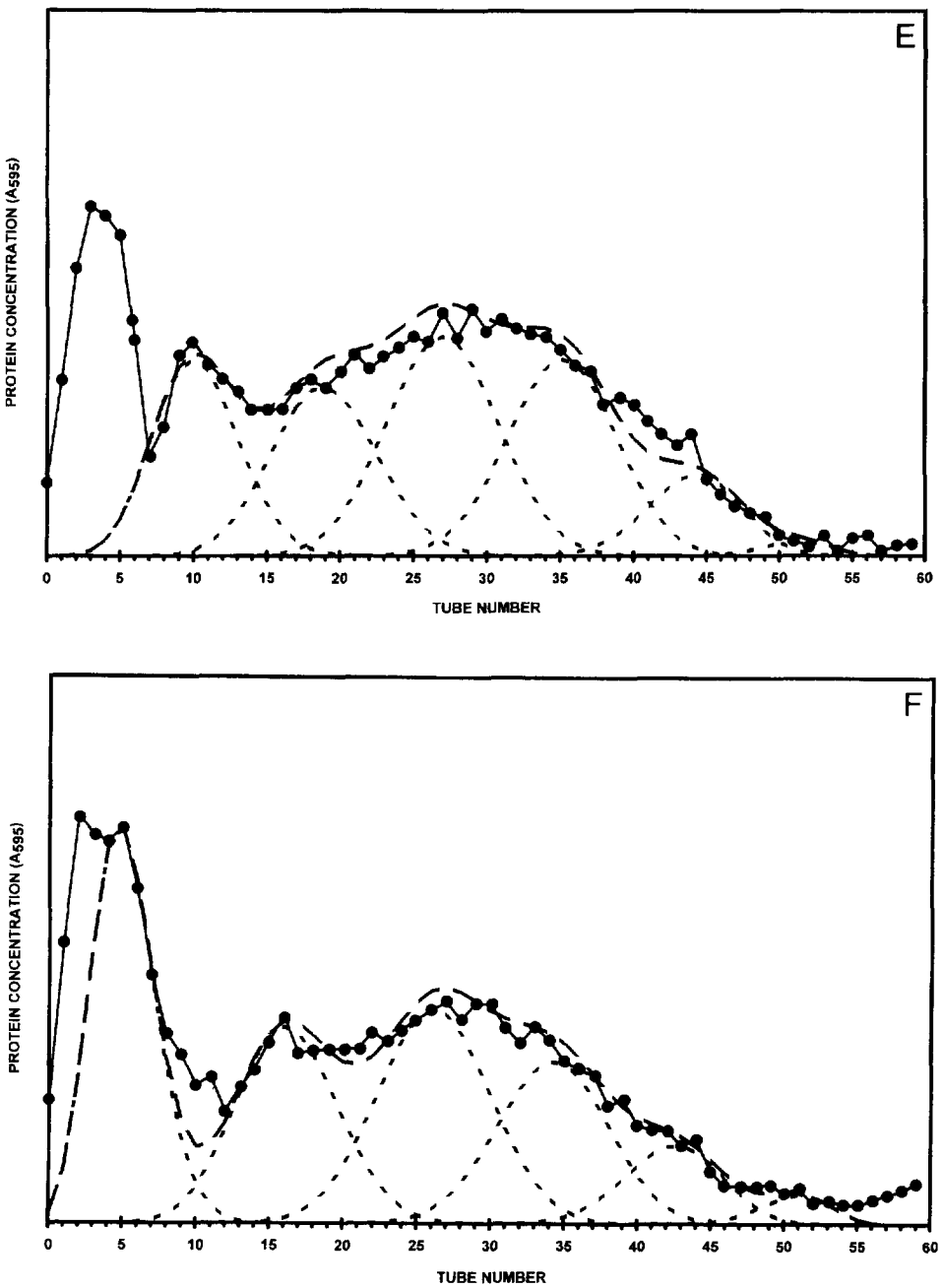

Fig. 2. (*Continued*)

amounts can be estimated. By assuming 6 protein components a reasonable fit with the experimental curve is obtained in all the six cases, indicating that the flour samples contain at least six, but probably many more, acid-soluble proteins.

Table 3 shows the percentage of each theoretical

Table 3
Percentual distribution of the six hypothetical proteins, represented by the theoretical curves for the various types of wheat in Fig. 2A–F

Wheat type	Hypothetical protein component					
	1 (G=0.1–0.2)	2 (G=0.4–0.5)	3 (G=0.7–0.9)	4 (G=1.2–1.6)	5 (G=2.7–3.7)	6 (G=7.4–8.5)
Turbo A6	18.5	20.0	24.6	20.8	11.5	4.6
Contra B4	15.1	17.3	27.0	22.2	14.0	4.3
Haven C2	18.6	18.6	22.3	20.3	12.9	7.2
Hussar C2	10.6	21.2	25.5	28.7	9.6	4.3
Cadenza B5	18.9	20.0	27.4	24.0	8.6	1.1
Urban A9	26.3	20.5	24.7	18.4	7.9	2.1

Protein components are numbered from left to right.

component in the sum of all the assumed protein components. The large peak to the left in the experimental curve has not been included in the analysis, since, to a great deal, it is a result of light scattering from particles influencing the absorbance measurements. The six (theoretical) components differ in their relative amounts, e.g., subfraction 1 for Hussar C2 forms a smaller part of the total amount of protein than is the case for the other five flours, while the same fraction forms a very big part for Urban A9. Subfraction 6 for Cadenza B5 is only about one-fourth as big, compared to the same fraction for the other wheat flours.

4. Discussion

The experiments show that acid-soluble proteins can be fractionated by partitioning in two-phase systems containing propionic acid, especially by using counter-current distribution. Since the proteins are charged (mainly having a positive net charge due to the low pH value in the systems) their partitioning can be adjusted by addition of various salts. The low pH also allows use of many more kinds of salt than the traditionally used ones [18] without risk of precipitation.

Counter-current distribution shows marked differences in the distribution pattern for flour of various wheat cultivars. This offers a possibility to relate the pattern to other properties of the wheat flour, e.g., it could be compared with SDS-gel electrophoresis, which is often used as a standard method for analysis of pattern recognition for wheat proteins, while the CCD technique gives rise to other patterns which are based on the surface properties of the intact proteins. This may be related to the baking properties. The wheats studied were classified with regards to their gluten strength which increases in the order C2-B4-B5-A6-A9, but no clear correlation between this property and the CCD pattern can be seen among this limited numbers of flours (Table 3).

One of the advances of using aqueous two-phase systems for fractionation of gluten proteins is that the proteins can be separated in their native forms instead of reducing them to subunits. This makes it possible to add purified intact proteins to flours and by that study their influence on the baking performance as is the case in SDS-gel electrophoresis.

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