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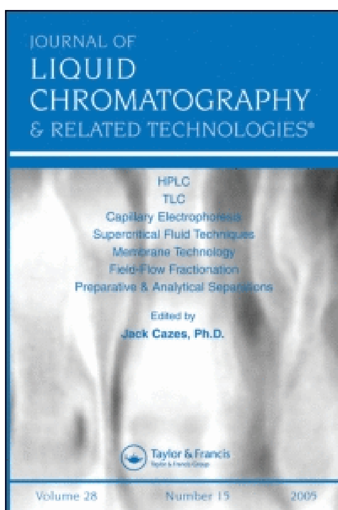

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Studies of Molecular Association of Glutenins and Maltodextrin in Solution by Capillary Electrophoresis

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ABSTRACT

Molecular associations of wheat glutenins and carbohydrates are important in bread systems and believed to contribute to differences of a number of dough and baking quality characteristics. In this investigation, the study of molecular association of glutenins with carbohydrates in solution was performed by capillary zone electrophoresis (CZE). The carbohydrate used was a maltodextrin of average molecular weight 2000. To follow the molecular association of major glutenin subunits with the maltodextrin of interest, the extracted proteins from hard red winter wheat (cv. Scout 66) were fractionated first by reversed-phase chromatography (RPC). Four

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major RPC fractions were collected, each of which still exhibited more than one glutenin component when reanalyzed by CZE. These RPC fractions were then incubated with the maltodextrin of interest, and the pre-equilibrated glutenin-maltodextrin complexes thus obtained were analyzed by CZE. Since maltodextrin is a neutral polymer, its binding to the glutenins results in decreasing the charge-to-mass ratio of the glutenins, and consequently, the effective electrophoretic mobility of protein–maltodextrin complex decreases with increasing maltodextrin concentration in the incubation solution. The evaluation of glutenin–maltodextrin interactions was only qualitative and the calculation of binding constants was hampered by various factors including the heterogeneity of the glutenin RPC fractions. The components of each RPC fraction yielded mobility lines (i.e., plots of electrophoretic mobility vs. percentage of maltodextrin in the glutenin sample) that paralleled each other indicating that these components have the same binding energetics magnitude with maltodextrin. This observation was substantiated by the sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) analysis, which showed that the various components of each RPC fraction are probably formed from one kind or one group of glutenin subunit with very similar properties. In addition, the mobility curves did not exhibit the usual rectangular hyperbolic forms of 1 : 1 binding isotherms.

Key Words: Wheat glutenin; Capillary electrophoresis; High performance liquid chromatography; Glutenin–maltodextrin associations.

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INTRODUCTION

Cereal grains are important sources of energy and a primary provider of proteins. Cereal proteins have been typically separated into classes based on their solubility in hydro-organic solvents (e.g., alcohol–water mixtures) in the presence/absence of reducing agents (e.g., dithiothreitol), as described by the early work of Osborne.[1] Among these important proteins are the high molecular weight glutenin subunits (HMW-GS), which are the subject of this investigation. Higher molecular weight glutenin subunits are polymeric proteins soluble in alcohol and reducing agents, and they can be subdivided into subclasses based on differences in structure, size, solubility, or reactivity.[2] The association/aggregation of these polymers form complexes of masses up to 10^6 Da, forming one of the most complex proteins.[3]

Because of the nutritional importance and functional role of cereal proteins including HMW-GS, the relations of the amount, composition, and structure of these proteins to baked product properties have been examined.[4–8] Total proteins content can vary from ~7 to ~15 wt% in wheat and wheat flour,[9]

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whereby gliadin and glutenin polypeptides represent the major protein components (~80% of the total protein in the grain).[10] Also, starch is another important and major component of cereal grains. For instance, in wheat flour, carbohydrates can account for ~68–76% of total flour weight, whereby starch constitutes the major component (~63–72%) and pentosan (~2–3%) and cellulose (~0.6%) represent minor components.[9] Proteins and carbohydrates, being the major components of wheat and wheat flour, have made the topic of protein–carbohydrate interactions an important research undertaking for many years. The understanding of the interactions of proteins and starch in wheat flour is a major goal in wheat biology, physiology, and processing. These interactions could explain key properties such as the endosperm texture in wheat of different classes, formation of transient structures in dough, and performance during baking.[9] Evidences of protein–carbohydrate interactions have been shown in several studies, including the elucidation by microscopy and rheological measurements of dough systems of the interactions of the extensible protein films inter-dispersed by starch granules.[11] Microscopic observations of regions of protein films associated with starch granules during mixing, fermentation, and baking, have revealed that the structure formed in these systems have important contributions from protein–starch interactions.[12,13] Protein–starch (non-covalent hydrogen bonds) and protein–protein interactions (non-covalent plus covalent disulfide bonds) are formed in the dough during mixing, fermentation and oven rise.[14] Protein–starch interactions are also relevant in the bread staling phenomenon. A chelation-type bonding was proposed for the interaction of glutenin and gliadin with starch chains. The amide of glutamine could interact with C-2 and C-3 hydroxyl groups of a glucose unit in amylopectin or amylose chains.[15] Martin and Hoseney[16] proposed a theory that involves structure stabilization by hydrogen bonds between protein and remnants of starch granules.

The occurrence of wheat protein–carbohydrate interactions has been revealed in studies involving wheat gluten (i.e., gliadin and glutenin) stabilization in the presence of carbohydrate by differential scanning calorimetry, turbidity, viscosity, and titration methods.[17–19] Sugars and polyols stabilized the proteins against heat denaturation.[19] Stabilization of the films formed by gluten proteins in the presence of polysaccharides may play a significant role in functional properties of the gluten in dough such as extensibility, gas retention, and bread volume.[20]

Other studies evaluated the dextrinization of gelatinized starch that occurs during the baking step due to the action of α- and β-amylases.[21] Dextrins in the breadcrumb contribute to gentle mouth feel and silky texture of the crumb and they also retard the staling phenomenon. When excess of dextrins are formed from starch hydrolysis, it results in sticky crumb, unpleasant to chew, and bread difficult to slice.[21] Jones and Erlander[22] studied interactions via

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turbidity measurements of glutenin and gliadin with dextrans having molecular weights ranging from 10 to 150×10^6, and found that the interaction curves of glutenin showed an optimum concentration for maximum interaction at higher concentration with dextrans compared to the gliadins.

As emphasized above, the association of wheat proteins including that of HMW-GS with starch is an important phenomenon and would require advanced methodology for its elucidation. Thus far, the techniques that have been used in such an investigation (e.g., turbidity, rheology, microscopy, viscosity), which are summarized above, lack selectivity, sensitivity, and reproducibility. Capillary electrophoresis (CE) is an advanced instrumental technique for studying the association of HMW-GS with starch. In fact, CE has been the method of choice in the fields of binding constant determination for molecular associations in solution (e.g., to micelles, cyclodextrins, antibiotics, proteins, DNA, RNA, etc.), for recent review articles see Refs.[23–25] The aim of this study is to evaluate the association of HMW-GS with maltodextrin by CE. Maltodextrin of molecular weight 2000 was selected as a model polysaccharide that mimics the behavior of starch. Indeed, maltodextrins are the hydrolysis products of starch, thus making them suitable for studies that require soluble components.

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EXPERIMENTAL

Chemicals

Phosphate buffer, glycine, and HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA). Hydroxymethylpropylcellulose (HMPC) and dithiothreitol (DTT) were from Sigma (St. Louis, MO). Ultra pure water for the preparation of solutions was obtained from a milliQ water purification system (Millipore, Milford, MA). Foundation seed of hard red winter wheat, cv. Scout 66 grown in Nebraska in 1998, was a gift from Dr. R. Graybosch (USDA-ARS). The wheat was milled on a Brabender Quadrumat Sr. mill (C.W. Brabender, Hackensack, NJ). The flour was sieved through a mesh with nominal opening of 0.149 mm. The content of proteins was 12.69% and 0.38% ash (14% moisture basis). Maltodextrins were a gift from Grain Processing Corporation (Muscatine, IA).

Glutenin Extraction

The HMW-GS from *Triticum aestivum* L. Scout 66 wheat flour were extracted using a modification of previously described procedures.[1,5,26]

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Albumins and globulins were extracted from the flour by three consecutive treatments with deionized water (1 g flour/4 mL solvent), 5 min vortex Genie 2, speed #4. After centrifugation (385 × *g*, 5 min, 4°C), the pellet was treated two times with 50% *n*-propanol to recover the gliadins. Glutenins were extracted by continuous stirring of the gliadin free pellet with 50% *n*-propanol containing 1% (w/v) DTT (for 45 min, vortex Genie 2, speed #4) to reduce the disulfide bonds and solubilize the proteins, and then by centrifugation (385 × *g*, 5 min, 4°C). High molecular weight glutenin subunits were then precipitated by the addition of *n*-propanol containing 1% DTT to bring the final propanol concentration to 60% v/v. The mixture was vortexed for 60 min, and then kept at 4°C for 72 hrs. The proteins were then collected by centrifugation (1951 × *g*, 5 min, 4°C). After drying and pulverizing, the HMW-GS were re-suspended in 50% *n*-propanol with 1% DTT and sonicated for 45 min before being analyzed by CE or HPLC. For the analysis with soluble maltodextrin (M.W. 2000), the sample, prepared as described above, was mixed with maltodextrin solutions, sonicated for 5 min, and incubated at room temperature for 4 hours before injection.

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HPLC Instrument

The HPLC analysis was carried out on a Waters Alliance instrument (Milford, MA) equipped with a photodiode array detector and a fraction collector from Gilson (Middleton, WI). The separation was performed with a Bio Rad Hi-Pore RP 304 column 250 mm × 4.6 mm I.D. (Hercules, CA). The mobile phases A and B were water and acetonitrile at 0.06% (v/v) TFA, respectively. The linear gradient was run from 25% B to 50% B in 30 min. Four major fractions were collected, speed vacuum dried, and then reconstituted in 50% *n*-propanol and 1% DTT for CE analysis.

Capillary Electrophoresis Instrument

The CE analyses were performed on a Beckman P/ACE 2000 and Beckman MDQ instruments (San Ramon, CA). In capillary zone electrophoresis (CZE), the separation was carried out at an operating voltage of 15 kV on a 27 cm (20 cm to the detector × 50 μm I.D.), using fused-silica capillary from Polymicro Technologies Inc. (Phoenix, AZ) thermostated at 45°C. The running buffer was freshly prepared before use and consisted of 100 mM phosphate (pH 2.4) at 20% (v/v) acetonitrile, 0.4% (w/v) glycine, and 0.05% (w/v) hydroxypropylmethylcellulose (HPMC of 3500–5600 cps), and the detection was performed in the UV at 200 nm. In SDS-CE, a polyacrylamide-coated capillary was used, which was filled with polyethylene oxides and gel

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buffer from Beckman (San Ramon, CA), and the separation was carried out at an operating voltage of 9 kV on a 27 cm (20 cm to the detector × 100 μm I.D.). The sample buffer was a solution of 0.12 M Tris/HCl containing 1% SDS (w/v), pH 6.6 (Beckman) and the detection was performed in the UV at 214 nm.

RESULTS AND DISCUSSION

Figure 1 illustrates the CZE map of the HMW-GS extract using a running electrolyte previously described by Lookhart and Bean.[6,27] Figure 1 shows the multicomponent nature of this essential wheat protein group. This is not surprising since glutenin molecules are highly heterogeneous comprising different polypeptide subunits. These genetically controlled subunits are linked

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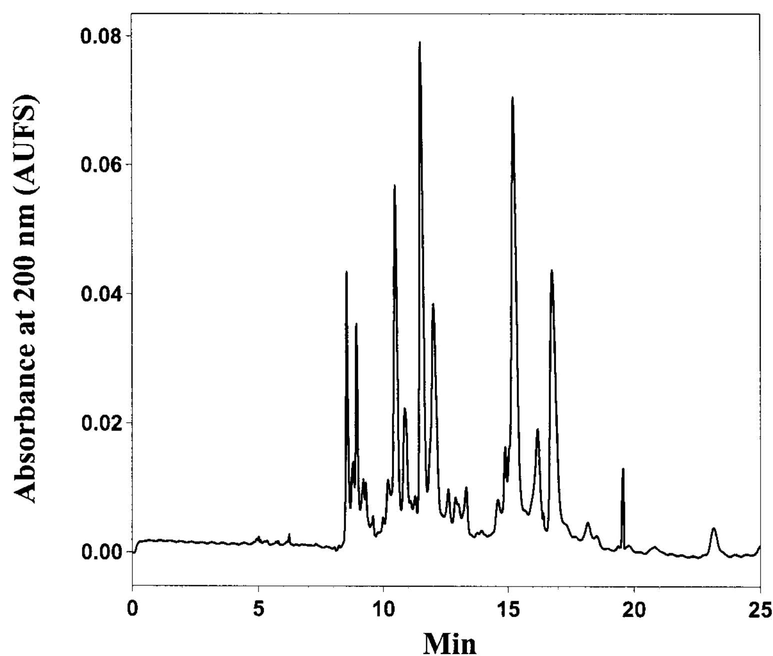

Figure 1. Electropherogram of soluble Scout 66 glutenins extracted with 50% (v/v) *n*-propanol containing 1% (w/v) DTT. Capillary, uncoated fused-silica, 27 cm × 50 μm i.d.; voltage, 15 kV; temperature, 30°C; running buffer, 100 mM phosphate, pH 2.5, containing 20% acetonitrile (v/v), 0.05% (w/v) HPMC, and 0.4% (w/v) glycine; injection, pressure (0.5 psi) for 5 s; detection, 200 nm.

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together by disulphide bonds.[7] Therefore, it was necessary to fractionate the extract in order to investigate the interaction of the individual components, or group of components, of glutenins with a standard maltodextrin of M.W. 2000 in solution. Reversed-phase chromatography (RPC), with its preparative capability was the chromatographic technique of choice to achieve this goal. Figure 2 is a typical chromatogram obtained by RPC of the same glutenin extract as that in Fig. 1. This chromatogram shows few separated peaks, most of which are of low intensities. Usually, HMW-GS are better separated in RPC as reduced and alkylated proteins, than in their native state.[28,29] In the present work, the aim was to isolate fractions of glutenins in the native forms for subsequent molecular association studies of the isolated glutenin fractions with maltodextrin by CZE. In this regard, four major peaks in the RPC map (see Fig. 2) were selected and collected to perform the CZE studies. While fractions #1 and #2 are single peaks each, the peaks of fractions #3 and #4 show shouldering (see inset of Fig. 2). To ensure sufficient amount of protein in the collected RPC fractions, each collected RPC fraction was pooled from at least five analytical injections of the HMW-GS extract.

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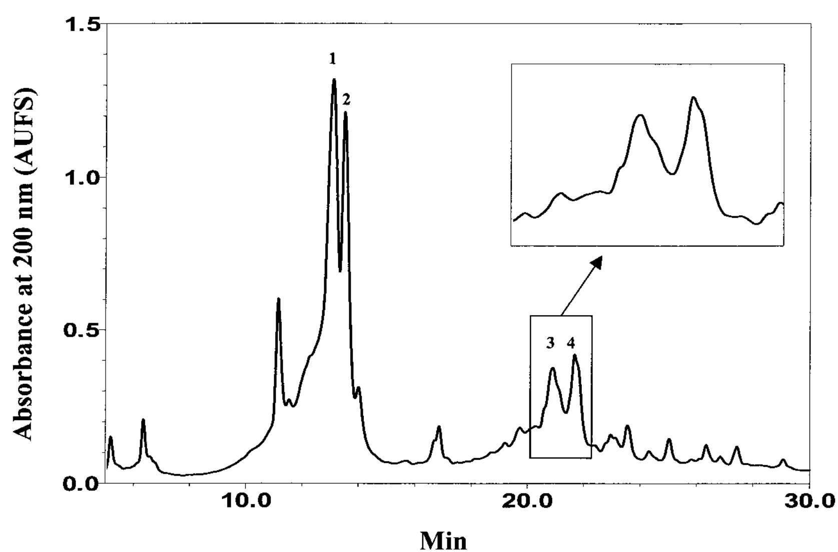

Figure 2. RP-HPLC map of Scout 66 glutenins extracted with 50% (v/v) *n*-propanol containing 1% (w/v) DTT. Column, Bio Rad C4, 250 × 4.6 mm i.d.; flow rate, 1.5 mL min^{-1}; 30 min linear gradient from 25 to 50% (v/v) acetonitrile in water containing 0.05% (w/v) trifluoroacetic acid; temperature 60°C; detection, 200 nm. Four major fractions were collected for CE analysis.

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The four RPC fractions were first analyzed by CZE in the absence of maltodextrin in order to assess the homogeneity of each fraction. The results are shown in Fig. 3 as four sub-maps, each showing the various components in the collected RPC fractions. While the RPC fractions #2 (Fig. 3b) and #3 (Fig. 3c) show four major peaks each, fractions #1 and #4 are more complex, containing six to seven major peaks each, as shown in Fig. 3a, d. Some of the peaks in RPC fraction #1 (indicated by asterisks) exhibited the same migration times as some peaks found in RPC fractions #2 and #4. Also, one major peak of RPC fraction #3 (indicated by an arrow) showed the same migration time as one peak found in RPC fractions #1 and #4. A second major peak in RPC fraction #3 (indicated by a check mark) is also found in RPC fraction #4. This may indicate that some of the peaks in the CZE map (see Fig. 1) are not a single component, and they are rather more than one component that co-migrated. These findings also indicate that some glutenin components that differ in their hydrophobicity (different retention time in RPC) have the same charge-to-mass ratio (i.e., same migration time in CZE). Inversely, components of different charge-to-mass ratios exhibited the same hydrophobicity and yielded a single peak in RPC fractionation.

Based on the above results, it was necessary to assess the molecular weight range of the various RPC fractions. To achieve this, sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) employing polyacrylamide coated capillary and in the presence of polyethylene oxide as the sieving media,[30,31] was performed on each fraction. Under these conditions, the apparent molecular weight range for each RPC fraction was calculated from the calibration curve obtained by plotting log M.W. of standard molecular weight protein markers vs. SDS–protein mobility. Reversed phase chromatography fraction #1, which showed at least six peaks when re-analyzed by CZE, yielded three peaks in SDS-CGE, with M.W. of 247,000, 318,000, and 393,000. Reversed phase chromatography fraction #2, which gave a CZE sub-map with five peaks yielded one major peak by SDS-CGE of a M.W. of 313,000. Also, RPC fractions #3 and #4 yielded one peak each with M.W. of 80,000 and 83,000, respectively. This may indicate that the various components of each RPC fraction have very close molecular weights and/or the SDS-CGE conditions dissociate the disulfide bonding between the subunits,[32–34] as well as des-aggregate the components into their actual monomeric forms. The net result of the dissociating and des-aggregating effect of the SDS-CGE conditions is to yield a single component as in the case of RPC fractions #2, #3, and #4, or a fewer components as in the case of RPC fraction #1. Since the HMW-GS subunits were reported to have a molecular weight in the 60,000 to 90,000,[12] one can assume that RPC fractions #3 and #4 have yielded their corresponding subunits upon exposure to SDS-CGE conditions, while RPC fractions #1 (M.W. 247,000, 318,000, and 393,000) and #2 (M.W. 313,000) have yielded glutenin macropolymers with disulfide bonds that are highly resistant to cleavage.[34]

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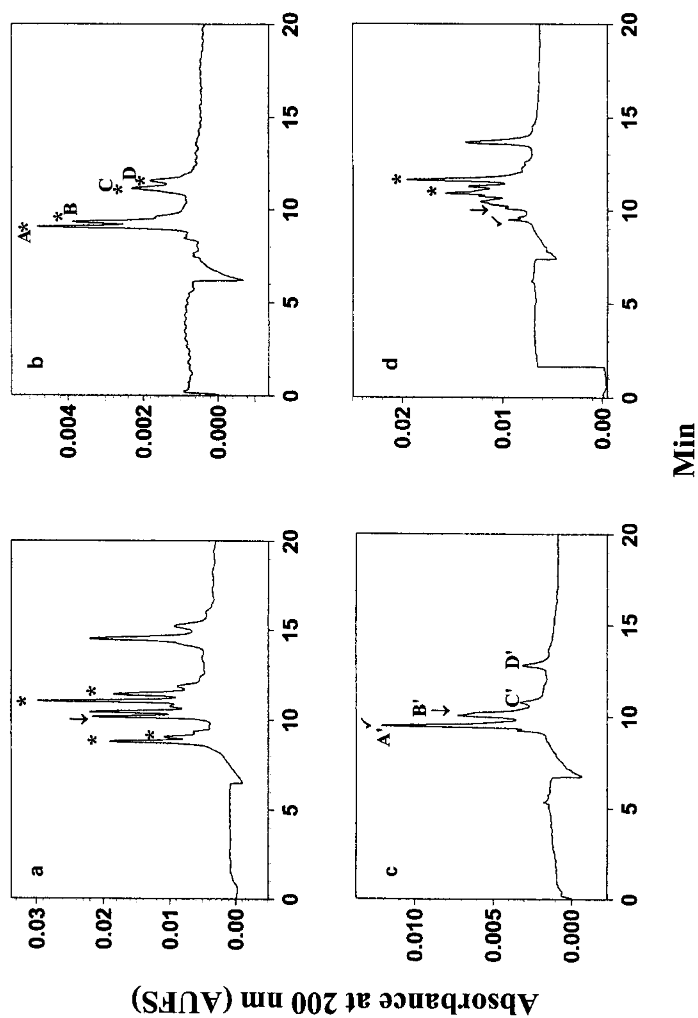

Figure 3. Capillary zone electrophoresis sub-maps of RPC fractions: RPC-1, RPC-2, RPC-3, and RPC-4 represented as a, b, c, and d, respectively. Conditions, as in Fig. 1.

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In our initial studies on the interactions of the various RPC fractions with maltodextrin, we have found that including the maltodextrin in the running electrolyte (at the concentration range 0.01–8% w/v) resulted in either an excessively viscous medium when concentration of maltodextrin was relatively high (1–8% w/v) or in a medium that did not exhibit significant interactions with glutenins when concentration of maltodextrin was relatively low (<0.15% w/v). These facts precluded the use of the most common approach for studying interactions by CE, which relies on measuring the change in electrophoretic mobility of a solute through electrolyte solutions containing dissolved complexation ligands.[23,24] Also, to use CE in this manner, the equilibrium time scale must be much faster than the CE separation time scale. This requirement could not be met in the case of glutenin–maltodextrin association, which turns out to be a slow process. In fact, when using the method based on solute and ligand pre-equilibration before injecting onto the CE capillary,[35,36] we have found that an incubation time higher than 2 hours was necessary to observe a detectable interaction between the glutenins and maltodextrins. No significant change in the effective electrophoretic mobility of glutenins was observed for incubation time of less than 2 hours. At 4 hours incubation time, the glutenin fractions underwent interaction with the maltodextrins, as was manifested by a significant decrease in the effective electrophoretic mobility of the individual proteins with increasing the percentage of maltodextrin in the incubated sample, see Fig. 4. In all cases, the amount of free proteins (i.e., unbound proteins) was negligible, such that no peaks for free proteins were detected. This may indicate that glutenins exhibit high affinity interactions with maltodextrin, which lead to stable complexes where the dissociation kinetics are slow compared to the separation time scale. These facts favors the use of the pre-equilibration method as also outlined in a recent review article on the use of CE in studying molecular association.[25]

At the pH of the CE measurement (pH 2.5) and the ionic strength used (100 mM sodium phosphate), the electroosmotic mobility is negligible, and as a result the apparent mobility is an accurate estimate of the effective electrophoretic mobility. Since maltodextrin is a neutral polymer, its binding to the glutenins results in decreasing the charge-to-mass ratio of the glutenins, and consequently, the effective electrophoretic mobility of protein–maltodextrin complex decreases with increasing maltodextrin concentration in the incubation solution (see Fig. 4), thus leading to longer migration time. This is clearly seen by inspecting Fig. 5, which shows electropherograms of the RPC fractions #3 and #4 in the absence and presence (1, 2, 4, and 8%) of maltodextrins in the maltodextrin incubated RPC fractions.

The evaluation of glutenin–maltodextrin interactions was only qualitative and the calculation of binding constants was hampered by various factors including the heterogeneity of the glutenins RPC fractions. As shown in Fig. 3, each RPC fraction yielded multiple peaks by CE, and only total protein

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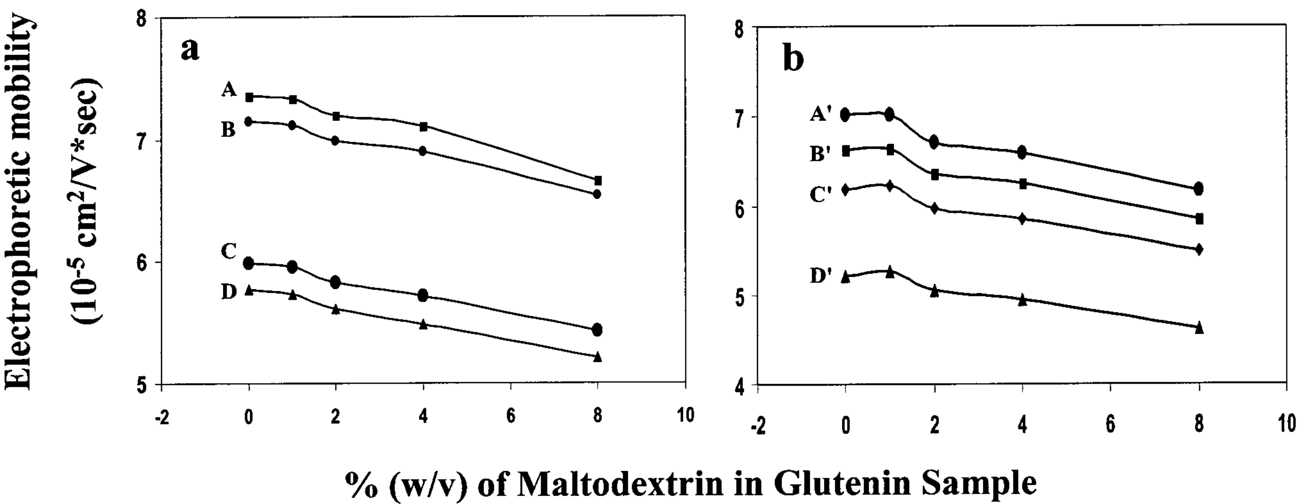

Figure 4. Electrophoretic mobility vs. wt% maltodextrin in solution for RPC fractions #2 and #3 represented as a and b, respectively. Conditions, as in Fig. 1.

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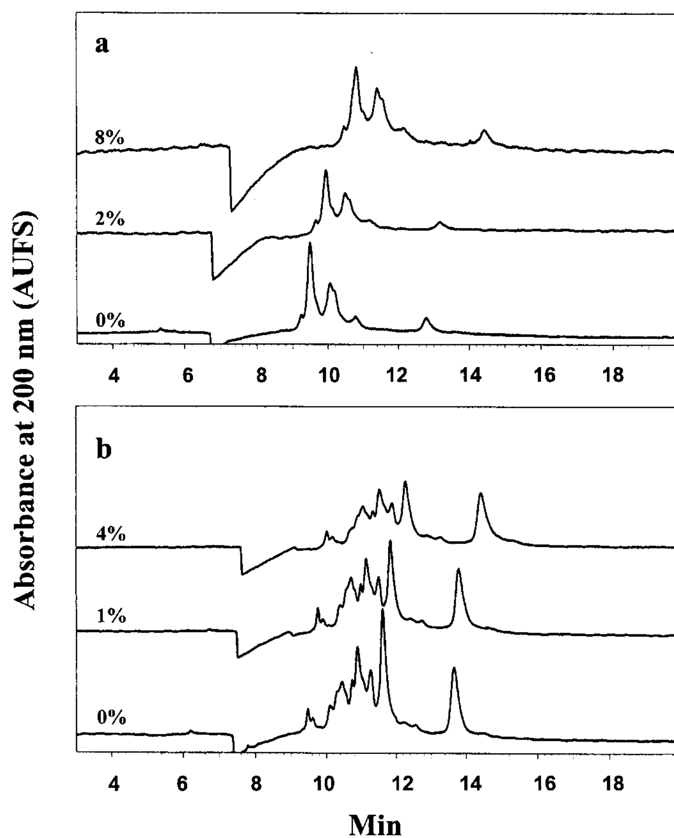

Figure 5. Electropherograms pertaining to the study of glutenin–maltodextrin association of RPC fractions #3 and #4 represented as a and b, respectively. The percentage on each electropherogram corresponds to the percentage of maltodextrin in the incubated sample. Conditions, as in Fig. 1.

concentration in each fraction can be determined. This condition allows only a qualitative assessment, at best, of the glutenin–maltodextrin interactions.

Returning to Fig. 4, one can see that the components of each fraction yielded mobility lines that parallel each other, indicating that these components have the same binding energetics magnitude. This observation is substantiated by the SDS-CGE results in that the various components of RPC fractions #2 and #3 are probably formed from one kind of subunit of M.W. 313,000 and 80,000, respectively. In addition, the mobility curves do not exhibit the usual rectangular hyperbolic forms of 1 : 1 binding isotherms.

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In conclusion, using RPC and CE in concert for the fractionation and analysis of glutenins, respectively, facilitate the investigation of molecular interactions of glutenin fractions and maltodextrin. The pre-equilibrium method for molecular interactions proved promising for assessing the presence of glutenin–maltodextrin associations. However, and due to the heterogeneity of RPC fractions of glutenin, the binding of maltodextrin to glutenin could only be studied qualitatively. Nevertheless, RPC-CE methodology advanced here may be regarded as a useful approach for the rapid monitoring and assessing of glutenins association with starch, an area of importance in the wheat and food technology fields.

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