Characterization of Protein Extracted from Flaked, Defatted, Whole Corn by the Sequential Extraction Process[1]

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An investigation was conducted to identify and characterize protein extracted by 45% ethanol:55% 0.1 M NaOH from flaked, defatted, undegermed corn (*Zea mays* L.) during Sequential Extraction Processing (SEP). This new approach to corn milling, SEP, recycles the ethanol produced from the fermentation of cornstarch to upstream steps of protein extraction and the simultaneous extraction of corn oil and dehydration of the ethanol. About 10% of the protein was extracted by ethanol during countercurrent-percolation oil extraction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid analysis identified this protein as zein. Nearly 65% of the total protein was recovered by the process in the protein extraction step from soft dent corn (Pioneer 3377), medium-hard dent corn (Pioneer 3732) and high-lysine corn. The freeze-dried solids of the ethanol/alkali extracts from these corn hybrids contained about 80% crude protein (db). The amino acids were present in quantities similar to those in whole corn and markedly higher than those in corn gluten meal. These results indicate that SEP produces high-quality protein suitable for food and industrial uses.

KEY WORDS: Corn, corn oil, corn protein, ethanol, extraction, maize, protein concentrate.

Wet grain milling has been the preferred method for recovering starch from corn (*Zea mays* L.) for more than a century. The process also produces oil and feed coproducts, such as protein (corn gluten meal) and fiber (corn gluten feed). The wet-milling industry experienced enormous growth in the 1970s because of the increased demand for starch to be converted into high-fructose corn syrup and into ethanol as an alternative fuel source. Consequently, traditional livestock feed markets are rapidly becoming saturated with the coproducts from wet corn mills, resulting in lower prices and reduced milling returns. New markets for corn coproducts are critical for the expansion of the wet-milling-related industries. The continued instability of world petroleum prices has also encouraged the exploration of new low-cost approaches for fractionating corn to produce novel coproducts with higher values than are obtained by either current wet and dry corn milling processes.

The Sequential Extraction Process (SEP) (1) is a radical new approach to fractionating dried, flaked, undegermed corn by using ethanol (Fig. 1). It involves recycling the ethanol produced by cornstarch fermentation to upstream steps of protein extraction and the simultaneous extraction of corn oil and drying of the alcohol. This process has several advantages over the current wet-milling methods: (i) It eliminates the expensive time-consuming step of steeping

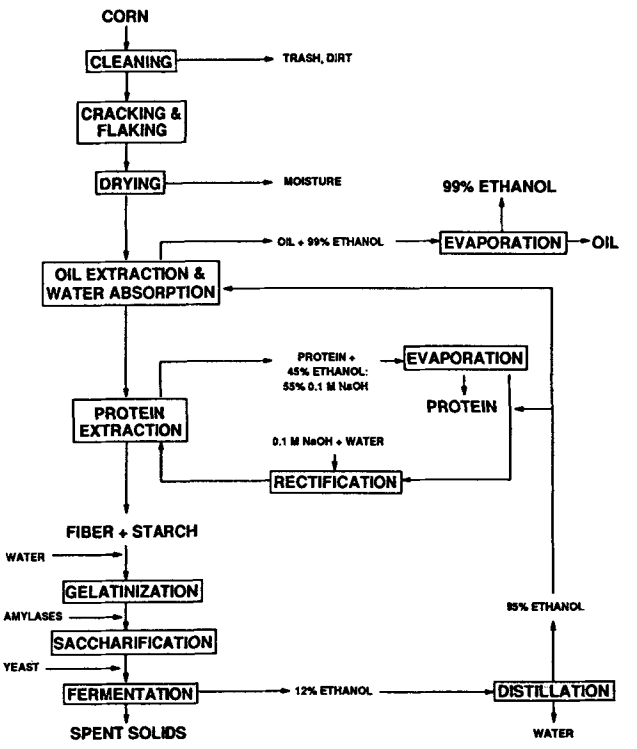

FIG. 1. Sequential Extraction Processing of corn.

corn in the presence of sulfur dioxide, which adversely affects the functional and edible properties of the protein; (ii) oil of higher yield is recovered during the process, eliminating the need to ship the germ to a separate crushing facility and minimizing deterioration in oil quality; (iii) expensive ternary distillation for recovering anhydrous ethanol is replaced with a simple water-extraction step with dried corn as the adsorbent; and (iv) a food-grade protein concentrate is produced, compared with the relatively low-value gluten meal or feed from traditional wet milling. The protein is expected to have a higher nutritional value than gluten meal because the germ proteins are also extracted.

This study is concerned with the corn protein fractions produced by SEP and their potential uses. Zein is currently the only corn protein that has industrial applications. It has properties that make it useful for the manufacture of industrial products such as linoleum tiles, textile fibers and packaging films (2), but its utilization for these purposes has not been exploited for the past 40 years because of cheaper petroleum-based substitutes (3). Finding new uses for zein and other corn proteins will benefit both farmers and processors. This paper presents the results of our efforts to identify the proteins produced by SEP and to characterize them with respect to their molecular weights and amino acid compositions.

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EXPERIMENTAL PROCEDURES

Preparation of corn. Soft dent corn (Pioneer 3377) was provided by Pioneer Hi-Bred International, Inc. (Johnston, IA), and medium-hard dent corn (Pioneer 3732) was provided by the Agricultural Engineering Grain Quality Laboratory, Iowa State University (Ames, IA). High-lysine corn was supplied by Crow's Hybrid Seed Co. (Milford, IL).

Twenty-five batches of undegermed corn, each weighing 300 g, were individually cracked, then flaked to 0.5 mm (0.02″) thickness by using the Roskamp rollermill (Model K, Roskamp Mfg., Inc., Waterloo, IA). The flaked corn samples were placed in aluminum pans and dried at 50°C in a forced-air convection oven to a moisture content of <2%. Each dried sample was stored in a resealable polyethylene bag and kept in a desiccator until used.

Proximate analyses. All batches were analyzed for crude free fat and crude protein by using AACC standard methods 30-20 and 46-08 (4), respectively, and for initial moisture content by Karl Fischer titration by using ASTM standard method E 203-75 (5). These methods were also used to analyze the fractions produced by SEP of corn.

Extraction system. The countercurrent percolation-type oil/moisture extractor (Fig. 2) consisted of multiple jacketed glass vessels covered with rubber stoppers for holding the solvents and the dried, flaked corn. The system was maintained at 75°C by circulating hot water through the jacketed glass vessels. Temperatures were monitored by thermometers inserted through the stoppers. Solvent evaporation was minimized by cold-water condensers. Desiccants attached to the condenser vents and dried nitrogen gas flushed through the system prevented entry of atmospheric moisture. A diaphragm pump circulated the solvent through a heat exchanger and then into the bed of flaked corn. The rotary evaporator separated the dry ethanol from the oil without exposure to air. A peristaltic pump brought the recovered dry ethanol from the evaporator into the graduated separatory funnel. The extraction system was designed to simulate the use of percolation-type extractors used by the soybean extraction industry with little retrofitting.

Sequential Extraction Process(ing). The seven ethanol concentrations at start-up of the countercurrent extraction were 97.2% (newest solvent, vessel 7), 98.4%, 99.0%, 99.2%, 99.5%, 99.5% and 99.5% (oldest solvent, vessel 1). These values were calculated based on (i) the exponential relationship between oil extractability and alcohol concentration; (ii) the solvent hold-up, which was experimentally determined to be 65% of the weight of the corn; and (iii) the amount of ethanol produced from the fermentation of one bushel of corn (15% moisture content), which is 2.5 gal or 35 g ethanol/100 g corn at 2% moisture content.

Six hundred milliliters of each solvent was placed in the appropriate jacketed, glass solvent-holding vessel. This amount was sufficient for a 2:1 solvent:corn (w:w) ratio. Dried, flaked corn was placed in the extraction vessel and was subjected to seven extraction stages. One extraction stage was completed when the solvent had been pumped through the heat exchanger, allowed to percolate through the flakes for 10 min, and then drained by gravity for 5 min into the previously emptied vessel: *i.e.*, solvent 2 is drained into vessel 1, solvent 3 into vessel 2, and so on until solvent 7. This series of steps advanced the miscella and simulated countercurrent solvent flow. In the case of solvent 1, the miscella was drained into the recovery vessel and drawn by vacuum into the preweighed sample flask of the rotary evaporator. The alcohol was evaporated, condensed, and then pumped into the graduated funnel where its volume was measured. Volume equivalent to 35 g ethanol/100 g corn extracted was taken out to represent the amount of ethanol produced from the fermentation of 300 g corn. The equivalent of 100 g of dry ethanol/100 g flaked corn was then drained into solvent vessel 7. The rest of the dry ethanol was emptied into a preweighed screw-capped vial and analyzed for moisture content. Volume equivalent to 100 g of 95% ethanol/100 g flaked corn was mixed with the dry ethanol in vessel 7. This amount of 95% ethanol represented the solvent hold-up (65%) and the 35 g of dry ethanol/100 g corn taken out earlier.

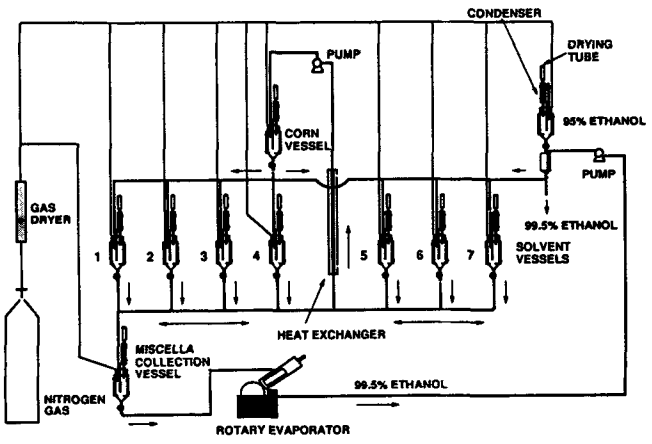

FIG. 2. Countercurrent oil/moisture extraction system.

The corn extraction vessel was disconnected from the system. Small portions of the defatted flakes were set aside for moisture, volatiles, residual oil and crude protein analyses. The remaining flakes were weighed into six blender cups in amounts equivalent to 25 g of dry corn. The mixture of 45% ethanol: 55% 0.1 M NaOH was added at a ratio of 1.5 mL/g dry flaked corn. The corn/solvent mixture was ground at full speed in a Waring Blendor for 1.5 min and then allowed to stand for 2 hr. After soaking, the rest of the ethanol/alkali was added for a total ratio of 15 mL/g dry flaked corn. The mixture was blended for another 30 sec; then the contents of each blender cup were transferred to a centrifuge bottle. The bottles were capped tightly, placed in racks, and then immersed in a water bath maintained at 55°C. The bottles were shaken for 2 hr at 130 rpm. After protein extraction, the bottles were centrifuged at 1050 × *g* for 5 min. The supernatant was analyzed for crude protein content, and the extraction efficiency was calculated. The residue was analyzed for moisture content and then dried overnight in an oven at 105°C before analysis of residual oil and crude protein contents.

The sample flask from the rotary evaporator was also removed and set aside for measuring oil recovery. The oil

and solids were separated by washing with petroleum ether, filtering the washings into a preweighed flask and evaporating the ether by using a hot-water bath. Of the 20 extraction trials done, the first 14 were used to establish system equilibrium. Protein extraction began on the 13th run. Data from the last six runs were used to evaluate the process.

Polyacrylamide gel electrophoresis. Commercial zein (Sigma Chemical Co., St. Louis, MO) and protein extracted by 99.5% ethanol with oil were solubilized in 70% ethanol/30% sample buffer (50 mM Tris-HCl, 0.2% SDS, 5 M urea, 10 mM 2-mercaptoethanol) by stirring overnight at room temperature. The solution was freeze-dried and then redissolved in the sample buffer. Tracking dye (1 M MES, 10% SDS, 0.4% bromophenol blue, sucrose) was added to the sample mixture, which was then placed in a boiling-water bath for 10 min. Samples were loaded onto vertical slab gels containing an 8–15% gradient of polyacrylamide overlaid with 4% stacking gel and electrophoresed according to the method of Laemmli (6) for 16 hr at constant current (8 mA). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol: 10% acetic acid, then destained with the same solution without the dye.

Protein extracted by ethanol:alkali was dialyzed against distilled water, ultrafiltered through a 10-kDa membrane, and then freeze-dried. Ten milligrams of the freeze-dried protein was solubilized in 1 mL of extracting solution (3% SDS, 1% 2-mercaptoethanol, 0.01% bromophenol blue) and incubated overnight in a water bath maintained at 30°C. After addition of 20% (w/v) sucrose, the samples were placed in a boiling-water bath for 3 min and then loaded onto 6%–15% gradient SDS-polyacrylamide vertical slab gels overlaid with 4% stacking gel. Electrophoresis was performed as already described.

Amino acid analysis. About 2 mg of the freeze-dried ethanol:NaOH extracts and 5 mg of the residues coextracted with oil were transferred to individual hydrolysis vessels. Two hundred microliters of 6 N HCl was added to each vial; then, the dead air space was blown with Ar to displace the oxygen. The reaction vials were sealed and placed in an oven at 110°C for 24 hr. After hydrolysis, the vials were dried in the Speed-Vac to remove all residual acid. The entire sample hydrolysate was redissolved in 1.0 mL of lithium citrate buffer. From each sample, 20 μL was further diluted to 100 μL with the same buffer. The hydrolysate dilutions were placed on a Beckman 6300 high-performance ion-exchange analyzer (Beckman Instruments, Inc., Fullerton, CA). The injection loop size was 50 μL/sample. The separation of amino acids was achieved by using a 25-cm physiological column. Analysis was postcolumn by ninhydrin detection.

Samples were prepared for cystine and methionine determinations by transferring 1.5 mg to individual hydrolysis vials already containing 10 nmol of the alpha-amino butyric acid (AABA) internal standard. A 50-μL aliquot of performic acid solution was added to each reaction vial, and the reaction was allowed to proceed at room temperature for 30 min. Excess acid was removed from the vials by vacuum-induced evaporation in the Speed-Vac. For tryptophan determination, about 2 mg of each sample was transferred into the hydrolysis vessels, followed by addition of 100 μL of 4 N methane sulfonic acid. Hydrolysis conditions were identical to those that used HCl. Analyses and detection of these amino acids were performed as described for all other amino acids.

RESULTS AND DISCUSSION

Oil extraction and ethanol dehydration. Little residual oil remained in the defatted, flaked corn, demonstrating the excellent oil extraction efficiency by the countercurrent method (Table 1). The oil recovery of 90% was far superior to the 72% estimated for conventional prepress hexane extraction (80% of the oil in corn is in the germ × 90% extraction efficiency). The moisture content of the flaked corn substantially increased after oil extraction (Table 1). This increase indicated the absorption of water from the miscella by the flaked corn bed. The marked reduction in the moisture content of the ethanol recovered from the miscella verified the dehydration of the alcohol during oil extraction. The ethanol initially had 5% water (volume basis), which was reduced to nearly 1% after oil removal, representing 74% absorption efficiency.

Protein extracted with oil. Some nonoil solids, particularly protein, were coextracted with the crude corn oil, with more being taken out of soft dent corn than either high-lysine or medium-hard dent corn (Table 2). The solids appeared as yellow, flaky residues after rotary evaporation of the ethanol from the miscella and were separated from the oil by washing with a small volume of petroleum ether and filtration. The recovered solids contained 25–30% protein.

TABLE 1

Oil Extraction and Ethanol Dehydration Data[a]

A. Oil extraction	
Residual oil in corn (% db)	0.30 ± 0.09
Oil recovery (%)	91.6 ± 2.7
B. Ethanol drying	
Initial corn MC[b] (%)	1.16 ± 0.13
Corn MC after oil extraction (%)	3.14 ± 0.12
Ethanol MC after oil extraction (% vb[c])	1.31 ± 0.05

[a]Grand mean of fifteen extraction trials with three different types of corn.

[b]MC denotes moisture content.

[c]Vb denotes volume basis.

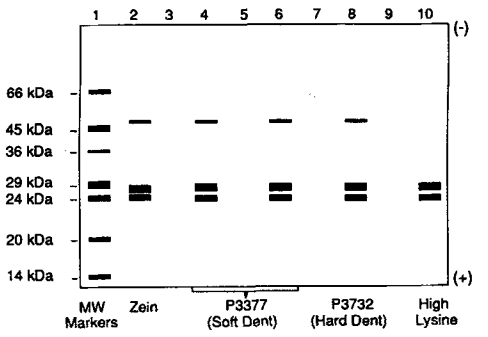

FIG. 3. Graphical representation of band patterns obtained by SDS-PAGE on protein residues coextracted with oil.

TABLE 2

Crude Protein (CP) Yields with Oil[a]

Corn type	Initial CP in flaked corn (% db)	Residue with oil (g/100 g dry flaked corn)	Crude protein with residue (% db)	% of total CP with oil (db)
Soft dent	8.3 ± 0.2[a]	3.2 ± 0.1[a]	30.5 ± 2.5[a]	11.6 ± 1.2[a]
Medium-hard dent	8.6 ± 0.8[a]	2.8 ± 0.4[b]	26.5 ± 3.6[b]	8.7 ± 2.1[b]
High-lysine	8.7 ± 0.4[a]	3.1 ± 0.1[a,b]	25.4 ± 2.0[b]	9.0 ± 1.1[b]

[a]Grand mean of five extraction trials. Means within a column with the same superscript are not significantly different at $p<0.05$.

TABLE 3

Amino Acid Composition of Protein Extracted by Ethanol with Oil

	Amount present (g amino acid/100 g protein)			
		SEP solids with oil		
Amino acid	Zein[a]	Soft dent	Hard dent	High-lysine
A. Essential				
Histidine	1.3	1.0	1.0	1.0
Isoleucine	3.7	5.6	5.5	5.2
Leucine	19.8	25.8	26.4	23.1
Lysine	0.1	0.1	0.1	0.2
Methionine	1.5	1.6	1.3	1.2
Phenylalanine	6.6	6.5	6.5	6.1
Threonine	2.4	3.1	3.2	2.6
Tyrosine	5.0	4.8	4.9	4.8
Valine	4.0	3.1	3.1	2.9
B. Nonessential				
Alanine	9.6	7.7	7.6	7.6
Arginine	1.6	1.2	1.4	1.4
Asparagine + glutamine	23.2	23.8	24.6	24.3
Cysteine	1.0	0.7	0.7	0.8
Glycine	1.4	1.0	0.9	0.9
Proline	9.4	9.4	9.2	14.0
Serine	4.5	4.4	4.5	4.2

[a]Zein data from Wall and Paulis (11).

TABLE 4

Crude Protein (CP) Extracted by Ethanol:NaOH and in Freeze-dried Extracts[a]

Corn type	Initial CP (% db)	CP in ethanol: NaOH (% db)	% of total CP extracted (db)	CP in freeze-dried extract (% db)
Soft dent	8.30 ± 0.25[a]	5.14 ± 0.16[a]	66.1 ± 1.1[a]	79.6 ± 3.1[a]
Medium-hard dent	8.55 ± 0.80[a]	5.57 ± 0.79[a]	60.1 ± 5.9[b]	75.3 ± 2.7[b]
High-lysine	8.74 ± 0.44[a]	5.60 ± 0.39[a]	57.6 ± 2.5[b]	78.5 ± 3.9[a]

[a]Grand mean of five extraction trials. Means within a column with the same superscript are not significantly different at $p<0.05$.

This protein was initially assumed to be zein because of its solubility in ethanol. The results of electrophoresis supported this assumption. The band patterns of the coextracted solids from the three types of corn were identical with that of commercial zein (Fig. 3). The approximate molecular weights of 22, 24 and 45 kDa are within reported literature values for zein (7–10). The amino acid profiles of the coextracted protein from the three corn types showed that the amounts of amino acids in the protein were similar to the literature values for the amino acids in zein (Table 3). This is further evidence that the coextracted protein was predominantly zein.

Protein extracted by ethanol/alkali. The solvent mixture of ethanol and 0.1 M NaOH extracted approximately two-thirds of the total protein in the original corn. Slightly more protein was recovered from soft dent corn than from medium-hard dent and high-lysine corns. In addition, the freeze-dried protein concentrates of the ethanol/alkali extracts from both dent corn types and high-lysine corn contained almost 80% crude protein (db, Table 4). This amount of protein is significantly greater than the typical 60–62% protein content of corn gluten meal. SEP concentrate could be made in a food-grade manner and has a very mild corn flavor. It is white (compared with corn gluten

TABLE 5

Amino Acid Compositions of Freeze-dried Ethanol:NaOH Corn Protein Extracts, Whole Dent Corn, Whole Opaque-2 Corn and Corn Gluten Meal

Amino acid	Amount present (g amino acid/100 g protein)					
	Whole dent corn[a]	Whole opaque-2 corn[b]	Corn gluten meal[c]	SEP protein concentrates		
				Soft dent corn	Hard dent corn	High-lysine corn
A. Essential						
Histidine	2.8	3.4	1.3	3.3	2.8	2.4
Isoleucine	3.7	3.9	2.6	5.0	5.3	5.0
Leucine	13.6	11.6	11.1	16.8	16.0	14.0
Lysine	2.6	3.4	1.1	2.4	2.8	2.8
Methionine	1.8	2.0	2.1	3.8	4.2	3.4
Phenylalanine	5.1	4.7	4.2	4.7	4.6	4.6
Threonine	3.6	3.9	2.2	3.2	3.6	3.6
Tyrosine	4.4	4.7	3.2	4.5	3.6	3.9
Valine	5.3	5.0	3.0	4.8	5.0	5.8
B. Nonessential						
Alanine	7.9	7.0	5.8	6.5	6.4	6.0
Arginine	3.8	5.1	2.1	4.3	3.8	5.6
Asparagine + glutamine	21.7	27.6	19.3	24.7	25.8	25.6
Cysteine	1.1	2.4	1.2	0.7	0.7	0.8
Glycine	3.4	4.0	1.8	3.6	4.0	4.4
Proline	8.3	9.4	6.1	7.6	7.1	8.0
Serine	4.8	5.0	3.4	4.1	4.4	4.2

[a]Data from Wall and Paulis (11).
[b]Data from Mertz *et al.* (13).
[c]Data from Wright (12).

TABLE 6

Residual Crude Protein (CP) Contents in Fiber/Starch[a]

Corn type	Initial CP (% db)	Residual CP (% db)	Unrecovered CP (%)
Soft dent corn	8.30 ± 0.25^{a}	1.69 ± 0.08^{a}	21.8 ± 0.9^{a}
Medium-hard dent corn	8.55 ± 0.80^{a}	2.86 ± 0.61^{b}	31.2 ± 7.6^{b}
High-lysine corn	8.74 ± 0.44^{a}	3.24 ± 0.05^{b}	33.4 ± 1.5^{b}

[a]Grand mean of five extraction trials. Means within a column with the same superscript are not significantly different at $p<0.05$.

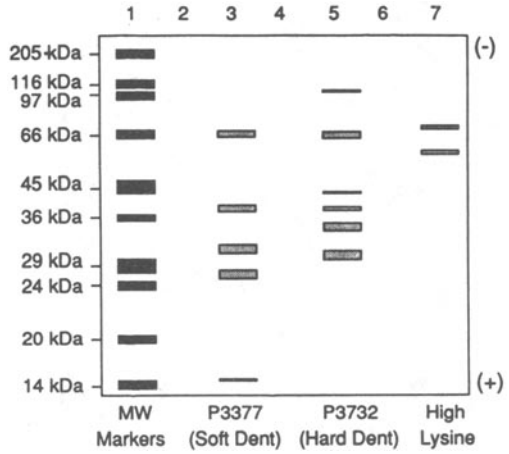

FIG. 4. Graphical representation of band patterns obtained by SDS-PAGE on freeze-dried ethanol:NaOH protein extracts from three types of corn.

meal's yellow), a potential advantage in food applications because no added color will be imparted to the product.

The amino acid profiles of the SEP protein concentrates from the dent and high-lysine corns are shown in Table 5 and are compared with those in whole dent corn, whole opaque-2 corn and typical corn gluten meal. The amounts of essential amino acids in the concentrates did not differ markedly from those found in their respective whole-corn values, but were much improved over those of corn gluten meal. A similar trend was observed for the nonessential amino acids. There was, however, less cysteine in the SEP protein concentrate from high-lysine corn compared with the literature value for whole opaque-2 corn. There was also no detectable amount of tryptophan in the protein concentrates. Nevertheless, these results suggest that the SEP was not detrimental to the corn amino acids, especially to lysine.

There were four major fractions in the SEP protein concentrates of the two types of dent corn and only two fractions in the SEP protein concentrate of the high-lysine corn (Fig. 4). Zein, glutelins and albumins were the frac-

tions expected to be recovered by the ethanol:NaOH solvent mixture. Although the amino acid compositions and identities of these fractions are still being established, their approximate molecular weights seem to indicate the presence of these three protein classes in the freeze-dried concentrates. The differences in the band patterns also suggest that the protein fractions from each corn type were not identical. It is not yet known if these differences were caused by corn type or processing factors.

Unrecovered protein. There was still a small amount of protein remaining in the fiber and starch residue after extraction with ethanol/alkali (Table 6). Although 20–30% of the protein was not recovered and remains with the fiber-rich, starch-rich fraction, 70–80% of the total protein was recovered by the two protein-extraction steps. The starch from this new process is expected to be of poorer quality than the starch from conventional wet milling because of its high residual protein content (about 2% compared with <0.3% in wet-milled starch), but it can be ideally used as substrate for ethanol fermentation.

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