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Surface-Associated Proteins of Wheat Starch Granules: Suitability of Wheat Starch for Celiac Patients

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Wheat starch is used to make baked products for celiac patients in several European countries but is avoided in the United States because of uncertainty about the amounts of associated grain storage (gluten) proteins. People with celiac disease (CD) must avoid wheat, rye, and barley proteins and products that contain them. These proteins are capable of initiating damage to the absorptive lining of the small intestine in CD patients, apparently as a consequence of undesirable interactions with the innate and adaptive immune systems. In this study, starch surface-associated proteins were extracted from four commercial wheat starches, fractionated by high-performance liquid chromatography and gel electrophoresis, and identified by tandem mass spectrometry analysis. More than 150 proteins were identified, many of which (for example, histones, purothionins, and glutenins) had not been recognized previously as starch-associated. The commercial starches were analyzed by the R-5 enzyme-linked immunosorbent assay method to estimate the amount of harmful gluten protein present. One of these starches had a low gluten content of 7 ppm and actually fell within the range proposed as a new Codex Alimentarius Standard for naturally gluten-free foods (maximum 20 ppm). This low level of gluten indicates that the starch should be especially suitable for use by celiac patients, although wheat starches with levels up to 100 ppm are deemed safe in the proposed Codex standards.

KEYWORDS: Wheat starch; wheat proteins; celiac disease; ELISA test; starch synthase; proteomics; mass spectrometry

INTRODUCTION

People with celiac disease (gluten-sensitive enteropathy) must avoid eating wheat, rye, and barley storage proteins and foods containing them. Strictly speaking, gluten is derived only from wheat flour (grain endosperm). However, in relation to celiac disease, it is common to use the term gluten for the main storage proteins of all three harmful grains (*1*). The consumption of gluten-containing foods by celiac patients may result in damage to the absorptive lining of the small intestine, giving rise to a general malabsorption syndrome, which is often represented by wide-ranging symptoms. Although the basis for celiac disease is not completely understood, it appears likely that a combination of an abnormal innate immune response to gluten peptides coupled with a necessary adaptive immune response is responsible (*1*−*3*). In celiac disease, the adaptive immune response is known to involve presentation of gluten peptides by proteins of the major histocompatibility complex found on the surface of antigen-presenting cells (probably dendritic cells) to T-cell receptors of CD-4 subepithelial lymphocytes. When stimulated, these lymphocytes produce inflammatory cytokines.

Wheat starch is not inherently harmful to celiac patients but has been avoided as a food ingredient in the United States because it has long been recognized that small amounts of gluten proteins remain adsorbed on the surfaces of the washed starch granules. It might be argued that gluten protein is a particulate contaminant, rather than truly starch surface-associated, but we think this unlikely for very low protein starches because of the highly adsorptive nature of wheat starch granules. In this paper, we shall refer to the proteins as surface-associated. The amount of gluten associated with the starch varies considerably among starches depending on the method of preparation (*4, 5*). Wheat starch is, however, commonly used for gluten-free or low-gluten products in some European countries and is generally well-accepted by celiac patients in those countries (*6*). Although there is little supporting literature, the properties of wheat starch appear to be more favorable for good baked product quality than other starches (*7, 8*).

Starch is synthesized and deposited during grain development as granules within specialized organelles called amyloplasts.

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Starch granules contain intrinsic proteins that are embedded in the starch matrix and proteins associated with the granule surface. Evidence suggests that the intrinsic proteins are almost entirely enzymes involved in starch synthesis (*9*). The gluten proteins are synthesized and aggregate as protein bodies in another subcellular compartment and are spatially separated from amyloplasts and starch granules during grain development; yet, most starch preparations contain some gluten, in large part as a consequence of the breakdown of organelle structure that occurs during grain maturation. Although some starch surface proteins have been identified and characterized (for a review, see ref *4*), further characterization of the amounts and types of gluten and nongluten proteins associated with the surfaces of both commercial and laboratory-prepared starches would be helpful in assessing the validity of the avoidance of wheat starch in the United States. We emphasized the proteins associated with the granule surface, because our analyses (this paper) of intrinsic proteins indicated, in agreement with the work of Rahman et al. (*9*), that the intrinsic proteins are mainly enzymes involved in starch synthesis and hence not likely to contain the amino acid sequences that cause problems for celiac patients. These sequences are found in significant amounts only in wheat gluten or related storage proteins from rye, barley, and triticale (*1*).

In this study, we characterize the surface-associated proteins extracted from three commercial starches produced in the United States or Canada and a commercial starch produced in Germany that is used in gluten-free products. Proteins were fractionated by high-performance liquid chromatography (HPLC) and gel electrophoresis and identified by tandem mass spectrometry (MS/MS) analysis of tryptic peptides. The proteins identified were classed into 54 general types, many of them not previously recognized as being associated with wheat starch granules. The commercial starches were also analyzed by the R-5 enzyme-linked immunosorbent assay (ELISA) method for gluten content (*10*), a method that is currently being used to determine if food gluten levels are safe for celiac patients.

MATERIALS AND METHODS

Commercial Wheat Starches. Samples of three commercial starches from North American production facilities were donated for our studies by ADM (Archer-Daniels-Midland Co., Decatur, IL). Two of the starches, Whetstar 4 and Aytex P, were food grade starches. The other starch from ADM, Keestar 328, was an unmodified, prime wheat starch that had been refined to contain a higher percentage of A type starch granules in the 20−32 μm size range. Keestar 328 was intended for use in making carbonless paper. A fourth wheat starch, Sanostar, was donated by Kröner Stärke, (Hermann Kröner GMBH, Ibbenbüren, Germany). Sanostar was indicated in the product description as being suitable for baking mixtures and as having less than 10 mg/100 g of gliadin (<100 ppm). Sanostar has sometimes been used in making low-gluten baked products for celiac patients. The Codex Alimentarius standard for designating products as safe for celiac patients is currently 200 ppm, although this may be revised to 100 ppm in the near future. All of the commercial starches were used as received from the manufacturer.

Protein, Moisture, and Phosphorus Determination. Protein was determined by means of nitrogen content, which in turn was determined by an oxidative/combustion method with 5.7 used as the conversion factor. Nitrogen was determined with a Leco 2000 instrument (Leco, St. Joseph, MI.) by Eurofins Laboratories (Des Moines, IA), AOAC method 990.03. Nitrogen determinations by similar oxidative methods were also donated by the Leco Corp., Elementar Americas, Inc., (Mt. Laurel, NJ), and C.E. Elantech (Lakewood, NJ), all using different standards for their instrument calibrations. Moisture contents were also determined by Eurofins Laboratories using the standard AOAC method 930.15 (2 h at 130 °C). Phosphorus was determined by Eurofins Laboratories using an ashing procedure and emission spectroscopy (AOAC methods 965.17 and 985.01).

Extraction of Surface and Internal Proteins. Fifty milligrams of starch was incubated for 5 min with 0.4 mL of 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol (DTT), and 50 mM Tris-HCl, pH 6.8 (SDS/DTT buffer) and centrifuged in a microfuge (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY) for 15 min at 14000*g*, and the supernatant solutions were collected, divided into aliquots, and placed in microfuge tubes for protein determinations and gel analysis. Proteins were precipitated with 4 volumes of cold acetone at −20 °C overnight. The starch pellets were washed twice by mixing with 0.4 mL of H_2O for 5 min followed by centrifugation as above. The supernatant solutions were collected and divided, and the proteins in these water washes were precipitated as above. The starch pellet was then gelatinized by adding 150 μL of water, mixing well, heating at 100 °C for 5 min, and then cooling. The starch gel was frozen in liquid nitrogen and ground into powder with a mortar and pestle. Interior proteins were then extracted with 300 μL of SDS/DTT buffer for 30 min followed by centrifugation. The supernatant solution was divided, and proteins were precipitated as above. For protein identifications, a larger quantity of protein was extracted in the same manner from the starches, using 1 g of starch and 4 mL of SDS/DTT buffer to extract the surface proteins, 2 mL of H_2O for each water wash, 3 mL of H_2O to gelatinize the starch, and 6 mL of SDS/DTT to extract the interior proteins from the starch gel. Four volumes of acetone were added to the final SDS/DTT buffer extracts, and samples were held overnight at −20 °C to precipitate the proteins prior to preparation for analysis by one-dimensional (1D) SDS−polyacrylamide gel electrophoresis (PAGE) (*11*) (Novex NuPAGE 4−12%, Invitrogen, Carlsbad, CA) and HPLC.

Two-Dimensional Electrophoresis (2DE). Proteins were extracted from 6 g of starch with 6 mL of 2% SDS, 10% glycerol, and 50 mM DTT in 40 mM Tris (pH 8.6). Starch and the extracting solution were mixed on a platform rocker (Stovall Life Sciences, Greensboro, NC) for 30 min with intermittent vortexing. The mixture was centrifuged at 12000*g* for 15 min at room temperature (Sorvall RC5C, DuPont Co., Wilmington, DE) to pellet the starch. The supernatant solution was centrifuged again in a microfuge at 16000*g* for 10 min at room temperature to remove any residual starch. The proteins were precipitated from the supernatant solution by the addition of 4 volumes of cold (−20 °C) acetone with incubation overnight at −20 °C and recovered by centrifugation. The pellet was air-dried and solubilized in urea buffer (9 M urea, 4% Nonidet P-40, 1% DTT, and 2% Servalyte 3-10 Isodalt grade). Proteins were separated by 2DE as described by Hurkman and Tanaka (*12*).

HPLC and Electrophoretic Separation of Starch Proteins for MS/MS. Starch protein samples obtained after acetone precipitation of the proteins from SDS solutions were centrifuged and dried. Samples of the dried protein were dissolved in 6 M guanidinium chloride and 50 mM DTT, adjusted to pH 8.0 with TRIS buffer, by heating at 60 °C for 1 h. The solution was returned to room temperature, and 4-vinylpyridine was added to approximately four times the expected total amount of thiol. The samples were flushed with nitrogen gas, and the alkylation was allowed to proceed for 1 h in the dark. Cysteine alkylation was terminated by the addition of formic acid. Each sample was filtered through a Millipore, UFC40H00 Ultrafree-CL HVPP Low binding Durapore 0.45 μm filtration device (Millipore Corp., Burlington, MA) and loaded by manual injection onto a Jupiter C18 semi preparative HPLC column (Phenomenex, Torrance, CA). The elution pattern from the HPLC was divided into "peaks". The protein fractions corresponding to the peaks were dried in a SpeedVac (Savant, Thermo-Fisher Scientific, Waltham, MA), and each fraction was then separated by 1D SDS-PAGE (*11*).

MS-Based Identification of Starch Proteins. Bands from the 1D SDS-PAGE gels for MS/MS analysis were selected based on staining intensity and cut from the gel. Reduction, alkylation, reagent removal, and tryptic digestion were carried out automatically by a DigestPro xyz robot (INTAVIS Bioanalytical Instruments AG, Bergish Gladbach, Germany). The DigestPro collection tray containing the tryptic peptides was placed in an autosampler that was interfaced with a QSTAR pulsar

Table 1. Compositional Information for Commercial Starches

starch type	moisture (%)	nitrogen[a] (%)	phosphorus	protein[b] (%)	corrected protein[c] (%)
Keestar 328	12.1	0.034 (0.027−0.043)	0.032	0.194	0.112
Sanostar	12.7	0.039 (0.031−0.048)	0.035	0.222	0.133
Aytex P	11.5	0.042 (0.037−0.054)	0.039	0.239	0.139
Whetstar 4	8.4	0.051 (0.049−0.063)	0.036	0.291	0.198

[a] Average of 10 determinations from three different companies, all using different reference standards. The range is given in parentheses. [b] Nitrogen × 5.7. [c] Corrected for lipid nitrogen in starches by assuming one nitrogen atom per phosphorus atom, multiplying by the ratio of the nitrogen atomic weight:phosphorus atomic weight (0.45) × % phosphorus and subtracting the result from the total % nitrogen to give nitrogen in protein form, and then multiplying the result by 5.7 to give the corrected protein. Although some companies do not guarantee nitrogen determinations to more than two decimal places (% nitrogen), we report values to three places to provide an indication of reproducibility.

i hybrid quadrupole-TOF instrument (Applied Biosystems/MDX SciEx, Toronto, Canada) configured with an electrospray ionization (ESI) source. Data were acquired using the Analyst QS version 1.1 software (Applied Biosystems, Foster City, CA). From an initial survey scan of mass range *m*/*z* 400−2000, the most abundant doubly or triply charged ion above a threshold of 20 counts was selected for fragmentation. Collision-induced dissociation of the mass-selected ion in the collision cell was carried out using UHP nitrogen as the collision gas. Following the 3 s MS/MS fragmentation period, the MS survey scan was repeated until another MS/MS period was triggered. Wiff data files were created for each sample by the QSTAR Analyst QS software. The resulting AnalystQS wiff files were converted to MGF text files using Mascot Daemon (http://www/matrixscience.com/) and submitted in batch mode to a locally installed copy of X!Tandem (*13*) using a script provided by Jayson Faulkner (University of Michigan). The results were visualized using a locally installed copy of the Global Proteome Machine (http://thegpm.org/). The NCBI nonredundant green plant database (June, 2005) that contained 185000 protein sequence entries was created by parsing the NCBI nonredundant (nr) database (01/06/2005) into taxonomic groupings using the Sequence Database Management Wizard from Genomic Solutions. This local version of the green plant database contained nr-Arabidopsis-thaliana.fasta, nr-other-Viridiplantae.fasta, and nr-Oryza-sativa.fasta. Trypsin was selected as the cleavage enzyme. The results were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 0.40 Da. Oxidation of methionine was specified as a variable modification (see also refs *14* and *15*). Identifications accepted as valid had log *e* values less than −4.0. In a few cases, where we wished to check on the identity of specific bands, particularly where the band in the SDS-PAGE 1D pattern was the only major band in that particular HPLC fraction, the HPLC fraction was subjected to direct N-terminal amino acid sequencing using the Procise 492 sequencer (Applied Biosystems).

Scanning Electron Microscopy (SEM). SEM was carried out by F. Hayes, Department of Materials Science, University of California, Davis, with an FEI Phillips XL30 SFEG SEM instrument with Secondary Electron Imaging. Starch samples were dusted onto double-coated carbon conductive tabs (Pelco Product number 16084-1, Pelco, Inc., Redding, CA) mounted onto aluminum stubs (Pelco Product number 16111), and the excess was removed by tapping the tabs against the benchtop. The sample was then placed into the Polaron BioRad Sputter Coater (BioRad, Hercules, CA) and gold sputter-coated for 2 min. Sample tabs were introduced to the SEM sample chamber and viewed with a secondary electron detector at 5.0 kV beam voltage at different magnifications. Images were saved as tiff files at approximately 350 kB.

Baking Tests. Baking tests were carried out using a gluten-free white bread recipe that we deemed typical of those used by celiac patients and standard U.S. kitchen measurements and equipment. All ingredients except Keestar 328 wheat starch were purchased from local food markets. For each formulation, 2 teaspoons (7 g) of bread machine yeast (Fleischmann's, Chesterfield, MO) was combined with 1 teaspoon of sugar (4.3 g) and 1/4 cup (59.1 mL) of warm water and allowed to ferment until frothy. The yeast mixture was then added to one egg whisked with 3/4 cup (177.4 mL) of water, 2 tablespoons (28.3 g) of melted butter, and 1/2 teaspoon (2.5 mL) of vinegar. Dry ingredients included 2 cups (330 g) of white rice flour (Bob's Red Mill, Milwaukie, OR), 1/3 cup (26 g) of dry milk, 2 teaspoons (6 g) of xanthan gum (Bob's Red Mill), 2 tablespoons (26 g) of sugar, and 3/4 teaspoon (6 g) of salt. In some of the formulations, a portion or all of the rice flour was replaced with either tapioca starch (1 cup = 115 g) or Keestar 328 starch (1 cup = 139 g). Dry ingredients were thoroughly mixed in a 5 quart bowl of a KitchenAid Professional Mixer model #KV25G0X (St. Joseph, MI) at the lowest setting before being combined with wet ingredients. The resulting batter was then mixed at setting 6 for 3 min. Next, 300 g of batter was spooned into greased 5 3/4" × 3 1/4" × 2 1/4" mini-loaf pans, covered, and allowed to rise for 45 min. Loaves were baked in a preheated oven at 350 °F (177 °C) for 30 min. After they were baked, loaves were allowed to cool for 5 min before being removed from pans. Volumes, colors, and crumb structures of the resulting loaves were compared visually. Loaf volumes were determined by a seed displacement method using dried green lentils. Each loaf was placed in a 2 L beaker, the beaker was filled to capacity with green lentils, and the volume of the lentils surrounding each loaf was measured. The loaf volume was determined by subtracting the volume of lentils surrounding each loaf from the total volume of the container.

Prolamin ELISA Testing (R-5 Antibody). Samples of the four commercial starches were sent to two different laboratories (Food Allergy Research & Resource Program Laboratory, University of Nebraska, and ELISA Technologies, Inc., Gainsville, FL) for estimation of the amount of gluten proteins in the starches by means of the R-5 antibody ELISA method (*10*). Testing at the Nebraska laboratory was with an ELISA kit from R-Biopharm (Marshall, MI), while the testing at ELISA Technologies was with the Transia Plate Prolamins ELISA assay (Diffchamb SA PR0320, Lyon, France). The cocktail extraction method was used.

RESULTS

Nitrogen and Phosphorus of Commercial Starches. Results from analysis of the commercial starch samples for nitrogen, phosphorus, and moisture are given in **Table 1**. Phosphorus analysis was included to account for nitrogen present in the form of lysophospholipids in calculations of protein percentages (*16*). Corrected percent protein for the commercial starch samples ranged from 0.112% for Keestar 328 to 0.198% for Whetstar 4. Aytex and Sanostar had intermediate, but similar, protein contents of 0.139 and 0.133%, respectively. Single starch lots were tested as received. Systematic comparison of different lots was not carried out.

Extraction of Surface and Internal Proteins from Starch Granules. Surface protein extracts separated by SDS-PAGE displayed a wide range of protein sizes, but only three major bands were evident in the internal protein extract. Proteins in these bands were identified by MS/MS as starch synthase I and II, starch branching enzyme, and granule bound starch synthase (**Figure 1**).

SEM of Commercial Starches. Scanning electron micrographs showed that Aytex P starch contained populations of both the large A type granules and the smaller B type granules (**Figure 2**). Keestar 328, which is sold as an A granule starch, contains some B granules. However, as compared to Aytex P starch, Keestar 328 starch contains a reduced number of B type

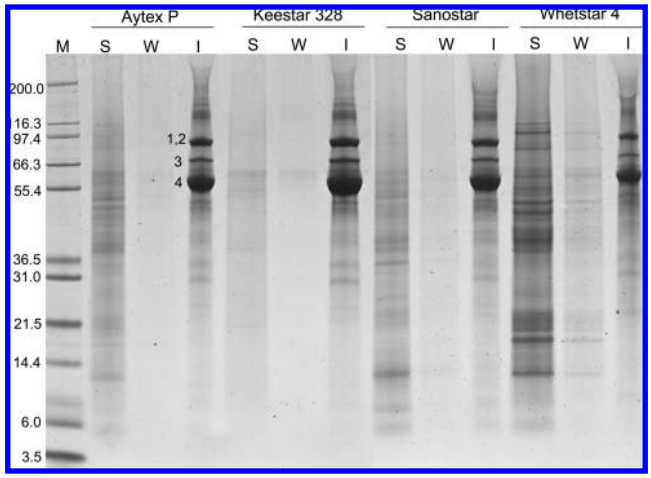

Figure 1. Surface and internal proteins extracted from Aytex P, Keestar 328, Sanostar, and Whetstar 4 starches. M, molecular markers; S, surface proteins extracted with 2% SDS/DTT; W, proteins extracted in first H_2O wash; and I, internal proteins extracted with 2% SDS/DTT after gelatinization. Protein from 17 mg of starch was loaded for each lane. Numbered protein bands in the Aytex internal starch protein extract were identified as 1, starch synthase II, gi|8953573|, gi|5825480|, and gi|7529653|; 2, starch branching enzyme, gi|1885344| and gi|1620662|; 3, starch synthase I, gi|5880466|; and 4, granule bound starch synthase 1, gi|4760582|, gi|4588609|, and gi|136765|. Accession numbers are for the closest matches in the NCBI-nr database.

granules. To determine if SDS might disrupt granule structure, Keestar 328 starch was examined before and after extraction with 2% SDS followed by extensive water washing. No obvious changes were noted in the granule surface as a result of SDS extraction, supporting the likelihood that we were extracting only surface-associated proteins.

2DE of Extracted Proteins. The 2D patterns [isoelectric focusing (IEF) vs SDS-PAGE] of the four commercial starches are shown in **Figure 3**. In **Figure 3A**, we have indicated in boxes the approximate areas expected to include the main types of proteins found in total extracts of wheat endosperm. We based these areas on considerable experience with identification of proteins in 2D gels (*17, 18*) and with the positions of purified wheat proteins in such gels (D. D. Kasarda and A. E. Adalsteins; unpublished results).

The gel patterns differed for the proteins extracted from these four starches. The Whetstar 4 pattern appeared similar to a total extract of flour or endosperm in that it contained high molecular weight (HMW) glutenin subunits, low molecular weight (LMW) glutenin subunits, gliadins, and albumins/globulins, although the albumin/globulins of lower molecular mass (<25 kDa) were more intense than would generally be expected for a total protein extract of wheat endosperm. Sanostar contained comparatively fewer proteins in the regions of the gels containing HMW glutenin subunits, LMW glutenin subunits, and gliadins and a qualitatively different population of albumins and globulins. Aytex P, like Sanostar, also contains comparatively fewer proteins in the regions of HMW glutenin subunits, LMW glutenin subunits, and gliadins. The Keestar 328 and Aytex P starch protein patterns were similar, except that Keestar 328 had lower protein content and, consequently, a fainter pattern. In general, Sanostar, Aytex P, and Keestar 328 were similar above molecular masses of about 25000 Da, although in the lower range, Sanostar had considerably more intense staining for albumin/globulin proteins. The pattern intensities from gel to gel did not correspond exactly with protein content as measured by nitrogen analysis (**Table 1**).

Because of the low protein content of Keestar 328 starch, the pattern of proteins extracted from this starch was more difficult to discern than those from the other starches. Thus, the contrast of the gel image in **Figure 3D** was enhanced so that the overall protein pattern could be more readily observed.

MS/MS Analysis of Starch Proteins. To identify starch surface proteins on a global scale, we utilized a proteomics approach that combined HPLC fractionation, SDS-PAGE separation, and MS/MS protein identification. Proteins were extracted from each of the four commercial starches and separated by HPLC as shown in **Figure 4**, which represents the absorption traces (210 nm) for equal volumes of solution obtained by extracting equal weights of each of the four starches. The proteins in the numbered peaks for each starch were then separated by SDS-PAGE (**Figure 5**), and proteins in the gel bands were identified by MS/MS. **Table 2** is a summary list of all proteins identified by MS/MS or N-terminal sequencing.

These proteins are identified by NCBI accession numbers that represent best matches. The proteins are grouped in classes in **Table 2**. For example, class 13 corresponds to γ-gliadins, while class 18 corresponds to LMW glutenin subunits. Numbers on the bands in the 1D gels of **Figure 5** indicate bands that gave significant identifications, the numbers key to the various classes listed in **Table 2**. When more than two proteins were identified for a given band, only the two most significant identifications were indicated on the figures because of space limitations. However, none of the unreported identifications were unique; they are all represented in **Table 2**. Most identifications in **Table 2** fit, within expected limitations, with the appropriate molecular masses of the identified proteins. However, we occasionally had identifications, for example, of HMW glutenin subunits, which have masses greater than 70 kDa, at very low mass ranges (<20 kDa) and, conversely, identifications of low mass proteins, such as α-amylase inhibitors, in unexpectedly high mass ranges (>70 kDa). These effects might result from some protein breakdown during starch processing and from trailing of proteins in the applied sample as they move down the polyacrylamide gel. The mass spectrometric analysis is capable of very high sensitivity, and the latter effect might not be evident otherwise.

Many of the same protein types were identified for all four starches. Most of the proteins matched sequences found in wheat, a few in barley, rye, maize, or rice, and a few in various other species, which is to be expected considering the incomplete nature of the DNA sequence information available for wheat. Of the 54 protein classes assigned in **Table 2**, 12 were identified in all four starches: α-amylase inhibitors, chitinase, γ-gliadins, histones, HMW glutenin subunits, LMW glutenin subunits, embryonic storage protein, puroindolines/grain softness proteins, purothionins, starch synthases, triticin, and tritin. There were 25 types that were identified in at least two of the starches. Although we were not able to quantify our results, it appeared likely, on a semiquantitative basis, that the four starches had more glutenin (both HMW and LMW glutenin subunits were identified in all starches) than gliadin on the granule surface. It is conceivable that results are skewed by, for example, a tendency to identify LMW glutenin subunits preferentially to α- and γ-gliadins. It is recognized that some proteins have peptide sequences that are more likely to provide good signals during MS analysis than others (*19*), and it has been our experience that the α- and γ-gliadins provide few tryptic peptides amenable to identification by MS/MS. Histones were present on all four starches but were most strongly evident in Whetstar 4, which also was highest in total protein on the basis

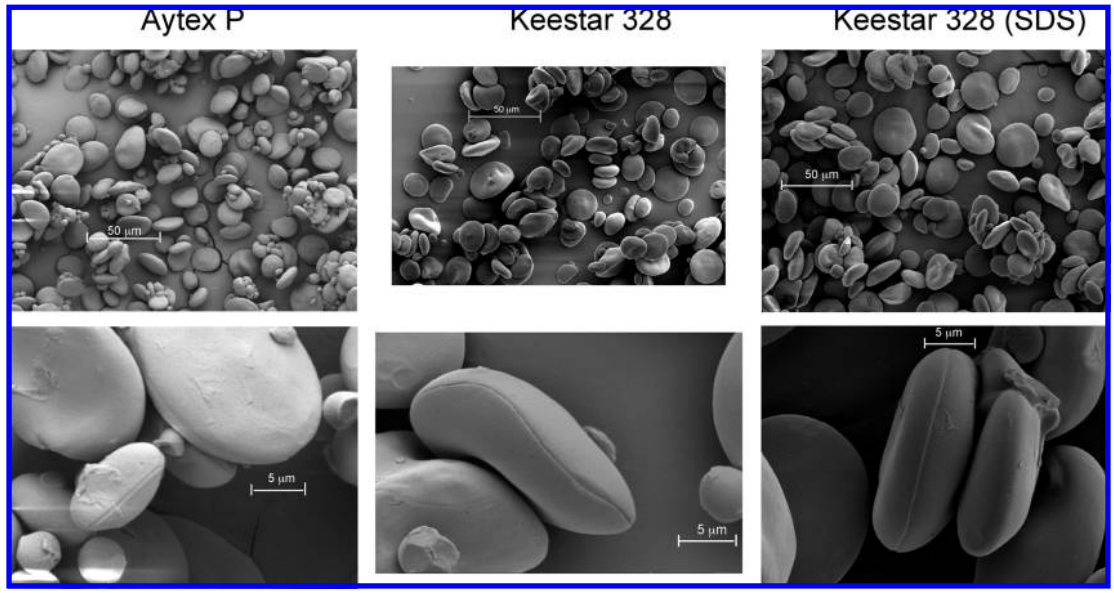

Figure 2. SEM of the Aytex P and Keestar 328 starches. Upper micrographs at low magnification show granule size distribution and lower micrographs at higher magnification show starch granule surface characteristics. Keestar 328 is shown as received (middle panels) and after extraction with 2% SDS solution, in the right-hand panels. Micrograph sizes were adjusted in the figure so that scale bars were of identical length for the top (50 μm) and bottom (5 μm) series.

of nitrogen analyses and gluten proteins on the basis of electrophoretic patterns. We did not identify ω-gliadins in any of the starches by MS/MS, although they were clearly evident in electrophoretic patterns of Whetstar 4 (**Figure 3**) as defined by the coordinate ranges 8.1−9.2 (y-axis) and 1.0−4.9 (x-axis). The ω-gliadins are fairly large (35−50 kDa) and have very low levels of lysine and arginine in their compositions, making them less susceptible to tryptic cleavage. Nevertheless, we interpret our 2D gels (**Figure 3**) as indicating that ω-gliadins are underrepresented in the proteins of the purified starches, with the exception of Whetstar 4, relative to other gluten proteins. Purothionin (*20*) was not identified in the MS/MS analyses but was identified by N-terminal sequencing of appropriate HPLC fractions containing a major protein in the 6 kDa range. We are not sure why purothionin was not identified as it appeared clearly in the SDS-PAGE patterns (**Figure 5**). Purothionin has not been identified as being associated with native wheat starch, but Bloch et al. (*21*) carried out in vitro experiments to demonstrate that purothionin does bind to proteinase K-treated wheat starch.

R-5 ELISA Tests. The gluten estimations (gliadins × 2) by two different laboratories are given in **Table 3**. The methods used are assumed to measure only gliadins, and it is further assumed that the amounts of glutenin and gliadin are approximately equal and close to 50%; hence, multiplication of the ELISA result by a factor of 2 yields the amount of gluten. Whetstar 4 gave fairly high values as might be expected from the nitrogen analyses of **Table 1** and the glutenlike 2D pattern of **Figure 3A**. Notable are the very low values for Keestar 328, which are consistent with data from nitrogen analysis, 1-DE, 2-DE, and HPLC/SDS-PAGE analyses (**Table 1** and **Figures 1**, **3**, and **4**). The low levels of gluten protein in Keestar 328 detected by the R5 analyses place it in the range being proposed by the Codex Alimentarius for intrinsically gluten-free foods (<20 ppm).

Baking Tests of Keestar 328 Starch. After noting the low protein levels of Keestar 328 starch and the highly favorable R5 ELISA results, preliminary baking tests were performed to determine whether Keestar 328 might be suitable for use in gluten-free yeast breads. Two formulations were used as gluten-free bread standards, one made with only white rice flour and the other made with equal parts of white rice flour and tapioca starch. The white rice flour formulation produced a dense loaf with a 500 mL volume and a fine cakelike crumb structure (**Figure 6A**), while the loaf made with rice flour and tapioca starch had a volume of 450 mL and a gummy texture, particularly along the base of the loaf (**Figure 6B**). Substitution of the tapioca starch with Keestar 328 wheat starch resulted in a loaf with a volume of 525 mL and a more breadlike structure than either of the gluten-free standard loaves (**Figure 6C**). When the ratio of Keestar wheat starch to rice flour was increased to 3:1, the volume of the resulting loaf was 650 mL, 30% greater than that of the rice flour loaf (**Figure 6D**). Two other formulations were tested, one in which the ratio of Keestar 328 starch to rice flour was 7 to 1 and another where the wheat starch replaced all of the rice flour. The resulting loaves had even greater loaf volumes, 675 and 700 mL, but the loaves were unable to retain their shapes (not shown).

DISCUSSION

Diversity of the Surface Proteins of Wheat Starch. Starch synthase is known to be associated with starch granules. Almost all other surface-associated proteins of starch were derived from the cytoplasm (sucrose synthase) or from subcellular organelles other than the amyloplast, such as the endoplasmic reticulum (storage proteins) and nuclei (histones) (*22, 23*). The majority of the starch surface proteins were storage proteins and proteins associated with protecting the grain from biotic and abiotic stresses. Storage proteins included both gluten (HMW and LMW glutenins, plus gliadins) and nongluten proteins (albumins and globulins). Stress/defense proteins included thaumatin, α-amylase, and α-amylase/subtilisin inhibitors, chitinase, pathogenesis-related protein, serpin, tritin, xylanase inhibitor, peroxidase, and peroxiredoxin. Other proteins have roles in protein synthesis, such as cyclophilin, elongation factor, heat shock protein, and ribosomal proteins or in carbohydrate metabolism, such as aldehyde reductase, β-amylase, enolase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and reversibly glycosylated peptide. Some of the proteins identified have not

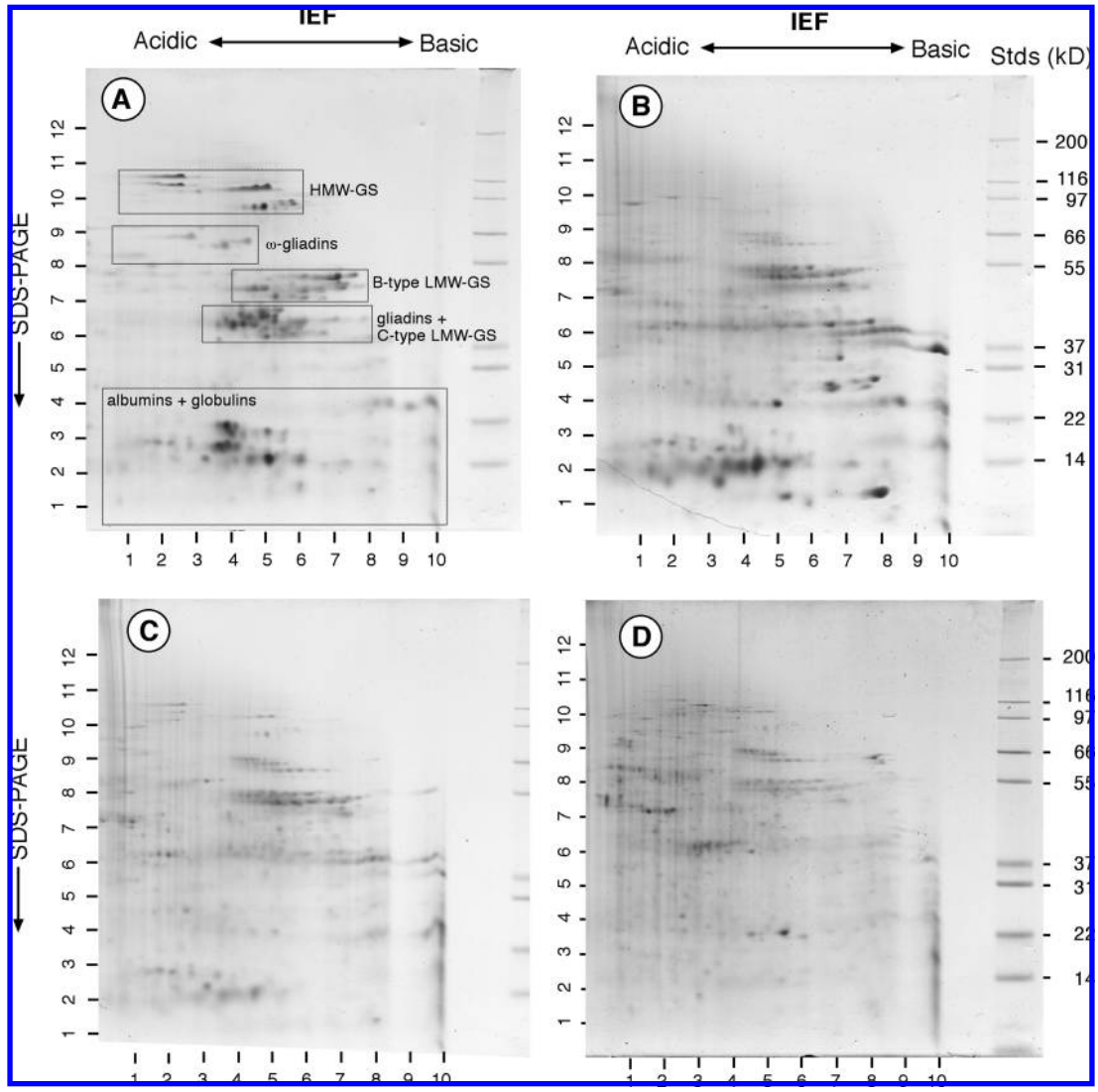

Figure 3. 2DE (IEF vs SDS-PAGE) patterns of proteins from (**A**) Whetstar 4, (**B**) Sanostar, (**C**) Aytex P, and (**D**) Keestar 328. The molecular masses of the protein standards are indicated on the right side panels of the figure. All patterns have scales applied that can be used to refer to specific groups of proteins by way of *x* and *y* coordinates. The coordinate scales were adjusted to be identical for all four panels by using the MW standards and the vertical streak, indicating the basic end of the IEF (first dimension) gel.

been recognized as being starch surface-associated in past work; most notable were all four classes of histones, purothionins, tritin, and HMW and LMW glutenin subunits. Many stress-related proteins are basic in character and a tendency for starch to bind positively charged proteins, such as the puroindolines (*24*), may be involved in the considerable number of stress-related proteins observed.

2D Gel Patterns. Although all of the 2DE patterns of the four commercial starches bear some apparent similarities, there are notable differences, especially between Whetstar 4 and the other three starches. For example, the LMW glutenin subunits that can be seen in **Figure 3A** (area bounded by *y*-coordinates 7.0−7.7 and *x*-coordinates 4.0−8.0) appear different from the equivalent cluster in the starches of **Figure 3B−D**. However, the identifications of protein bands in that molecular mass range are similar for all starches, often LMW glutenin subunits, but other types were present, as well (**Figure 5**). The apparent differences in the 2D patterns (**Figure 3**) may result from intensity differences (e. g., Whetstar has more protein, especially more gluten protein), differences in the mixture with other types of proteins (such as starch synthases, triticin, and other globulins) in the patterns, and slight deamidation of proteins resulting from the methods used to produce the starch—perhaps in the drying process.

Protein and Gluten Contents of Various Starches. When we corrected for the nitrogen-containing lipids of starch granules (*16*), the remaining nitrogen corresponded to about 1120 ppm protein (**Table 1**) for the Keestar 328 starch, which would be sufficient to cause problems for celiac patients if all of the nitrogen was derived from gluten proteins. However, much of that protein is internal protein, presumably starch synthases (**Figure 1**). Rahman et al. (*9*) found that internal proteins are sensitive to the concentration of SDS used to extract the proteins from gelatinized starch—with 10% SDS extracting much more of the internal proteins than 1% SDS. It appears likely that surface proteins make up only a small part of the total starch protein and that gluten proteins encompass only a small fraction of the surface-associated proteins.

The ELISA test based on the R5 monoclonal antibody (*10, 25*) has been well-accepted during the past few years for determining the gluten content of various food products. In 2005, the R5 ELISA test was endorsed as a type I method (highest level, defining method) by the Codex Committee for Methods of

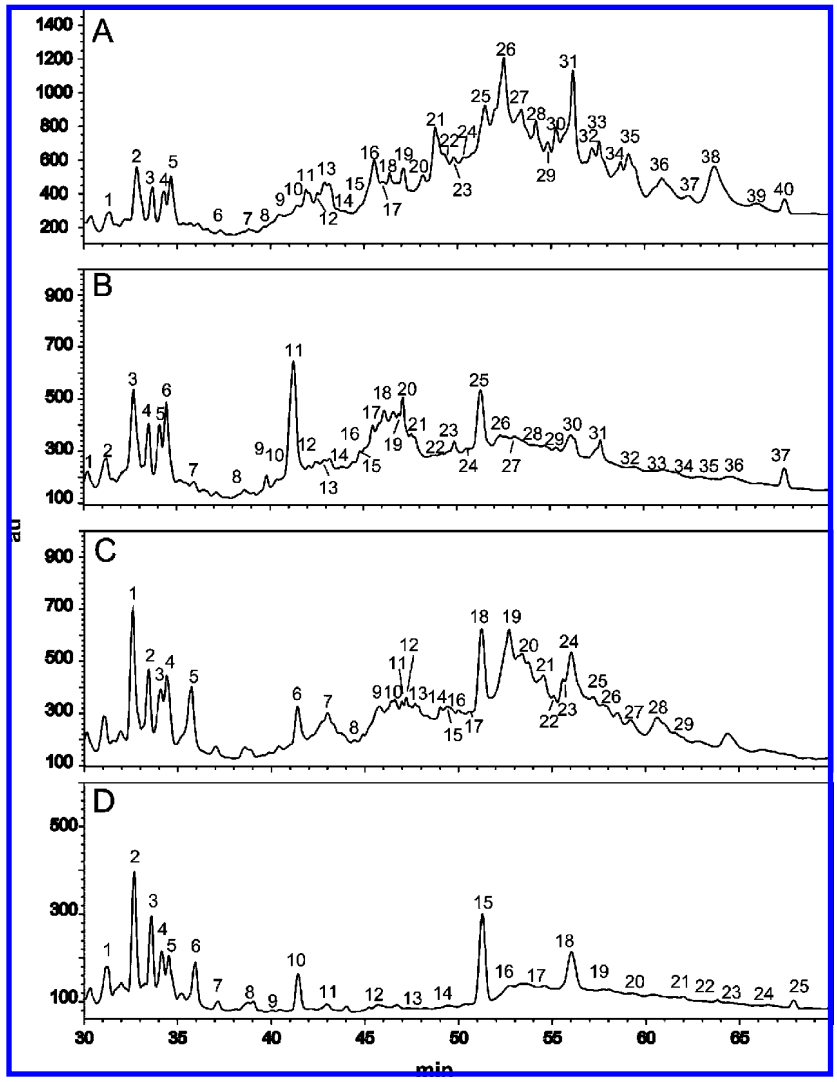

Figure 4. HPLC traces of proteins extracted from 3.5 g of Whetstar 4 (**A**), Sanostar (**B**), Aytex P (**C**), and Keestar 328 (**D**). Numbers on the traces indicate fractions that were subjected to further analysis by SDS-PAGE. Equal volumes were loaded for the patterns of **A**−**D**. The monitoring wavelength was 215 nm.

Analysis and Sampling. The R5 test is based on an antibody that reacts most strongly with the sequence QQPFP and closely related sequences. Because this sequence is widely distributed among the proteins of the various gliadin families, the R5 test seems more likely to reflect gluten content than the other popular test based on an anti-ω-gliadin antibody (*5*). The ω-gliadins are largely unstructured and have no disulfide bonds. These proteins may be more prone to being washed from the starch and to vary in amount for different processing methods. Although we cannot precisely quantify the relative proportions, the commercial starches, with the possible exception of Whetstar 4, appear to have more absorbed glutenin than gliadin on the basis of SDS-PAGE and MS identifications (**Figure 5**) and the frequencies with which glutenin and gliadin proteins appeared in our identifications (**Table 2** and **Figure 5**).

Binding of glutenins to starch has not been reported previously. The R5 antibody does not react strongly with glutenin proteins, perhaps because the key epitopes, even if present, are nonrepeating (*25*). Consequently, if glutenin is more strongly bound to the starch than gliadin, the gluten content determined by R5 may be low. In our studies, the R5 antibody was assumed to react only with gliadin, and multiplication by a factor of 2 was used to estimate gluten amount. This is reasonable for products in which the gluten is unfractionated, but less so for products such as isolated starch granules, and consequently, we cannot say with certainty how much gluten is present. We point out that even if the glutenin were as much as 4-fold in excess of gliadin, the Keestar 328 starch would still fall within the 20 ppm range that is likely to be assigned by Codex Alimentarius as an acceptable level for intrinsically gluten-free products and be well within the acceptable range of 100 ppm for wheat starches to be used for products intended for use by celiac patients. If an antibody that was specific for a sequence characteristic of the LMW GS could be developed, we think it would be desirable to use a mixture of such an antibody and the R5 antibody for starch analyses.

Keestar 328 Starch. Our results from R5 testing indicate that the least amount of gluten (5−8 ppm) was found in the A granule-enriched starch Keestar 328. Gel electrophoresis also revealed that this starch has very low levels of protein.

In the Draft Revised Standard of the Codex Alimentarius (Codex document CL 2007/43-NFSDU; ALINORM 08/31/26), a proposal has been made to limit naturally gluten-free products to 20 ppm gluten as determined by the R5 test and products such as wheat starch for celiac patients to 0−100 ppm. The 20 ppm level has presumably been chosen because naturally gluten-free products sometimes show evidence of gluten when subjected to ELISA testing (*26*). Even if glutenin was much greater

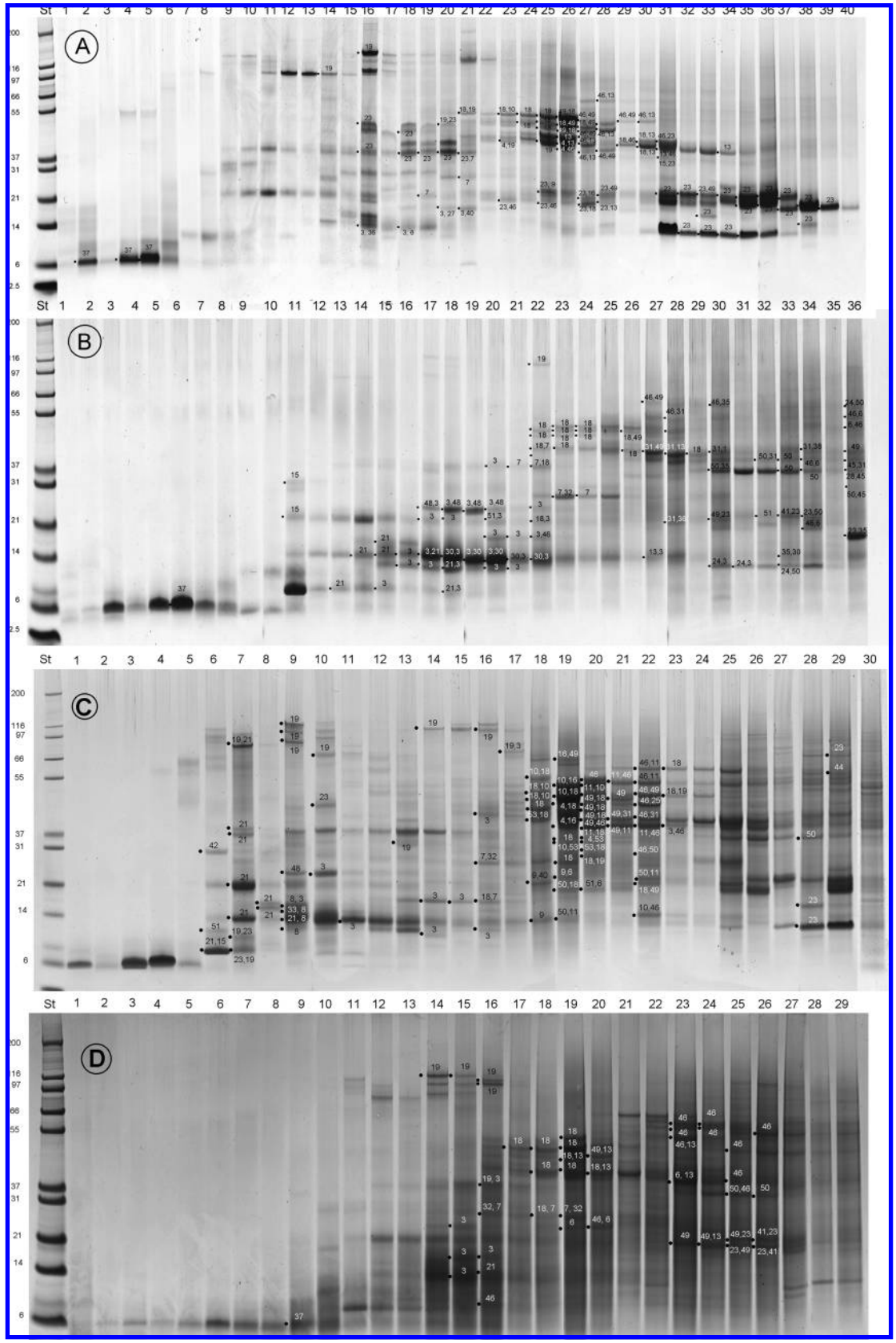

Figure 5. 1D SDS-PAGE patterns of HPLC fractions of proteins extracted from starch of (**A**) Whetstar 4, (**B**) Sanostar, (**C**) Aytex P, and (**D**) Keestar 328. Numbers on bands indicate one or more significant protein identifications and are keyed to **Table 2**. Lanes in panels **A**−**C** correspond to HPLC fractions shown in panels **A**−**C** of **Figure 4**, respectively. In panel **D**, lanes corresponded to 29 fractions obtained from an additional experiment in which proteins extracted from 20 g of Keestar 328 were separated by HPLC (data not shown).

in amount than gliadin in Keestar 328 (assuming a value for gliadin of 3.25 ppm), the product would still be likely to fall within the range considered safe for wheat starch. The question of whether or not patients can tolerate small amounts of gluten

Table 2. List of Proteins Identified in Commercial Starches

1. aldehyde reductase: a. gil543632| (Bromus?)
2. α-amylase/subtilisin inhibitor: a. gil123975|; b. gil225172|
3. α-amylase inhibitors: a. gil54778503|; b. gil54778515|; c. gil123963|; d. gil123966|; e (durum) gil39578552|; f. gil123957|
4. α-gliadin/(α/β)-gliadin: a. gil82587|; b. gil7442122|; c. gil1256261|; d. gil100783|; e. gil7209257|; f. gil1256791|
5. ankyrin: a. (rice) gil34896854|
6. β-amylase: a. gil6729696|; b. gil1771782|; c. (barley) gil6729696|; d. (maize) gil4321978|; e. (barley) gil10953875|; f. gil32400764|
7. chitinase/chitinase a: a. (rye) gil741317|; b. gil131095|; c. (rye) gil25528901|
8. CM16 protein: a. gil21709|
9. cyclophilin: a. gil14334173|; b. gil13925737|
10. elongation factor 1-α: a. (Nicotiana) gil1169476|; b. gil32400867|; c. (barley) gil949878|; d. (maize) gil2282584|; e. (barley) gil461988|; f. (rice) gil2662343|
11. embryonic storage protein: a. gil170696|
12. enolase: a. (rice) gil780372|; b. (rice) gil33113259|
13. γ-gliadin: a. gil1063270|; b. gil82595|; c. gil15148391|; d. gil72331|; e. gil15148385|; f. gil7230478|; gil34329279|
14. globulin 11s: a. gil472867|
15. globulin, 25 kDa: a. (durum) gil40849983|
16. globulin BEG 1: a. (barley) gil421978|
17. glucose and ribitol dehydrogenase homologue: a. gil7431022|
18. glutenin, LMW: a. gil4741697|; b. gil44885910|; c. gil17425188|; d. gil1857652|; e. gil17425190|; f. gil17425168|; g. gil17425166|; h. gil51870700|; i. gil17425164|; j. gil47607142|; k. (durum) gil4741697|; l. gil45477537|; m. gil31415653|; n. (durum) gil9931204|; o. (*Aegilops* × *Triticum*) gil50404489|; p. gil9967353|
19. glutenin, HMW: a. gil21751|; b. gil82602|; c. gil22090|; d. gil29150726|; e. (rye) gil14329753|; f. g. gil543543|; h. gil543542|; i. gil31790174|; j. gil39599016|; k. gil170743|; l. gil1917607|
20. glyceraldehyde-3-phosphatase dehydrogenase: a. (barley) gil18978|; b. (maize) gil1184774|
21. grain softness protein/puroindoline: a. gil607198|; b. gil663263|; c. gil607202|; d. gil9957230|; e. gil607702|; f. gil46358507|
22. heat shock protein: a. (maize) gil22335|
23. histone: a. gil7439660|; b. gil2641211|; c. gil14916992|; d. gil121982|; e. gil30024110|; f gil121974|; g. (rice) gil30017573|; h. (*Arabidopsis*) gil21592838|; i. gil536888|; j. gil1325968|; k. gil536888|; l. gil12206|; m. gil21801|; n. gil13898832|; o. gil531056|; p. (maize) gil1708107|; q. (maize) gil399854|
24. histone: a. gil224293|; b. gil12248031|
25. HSP-70 molecular chaperone: a (barley) gil7441877|
26. lipid transfer protein: a. gil10835497|
27. low temperature-responsive RNA-binding protein: a. gil1229138|
28. malate dehydrogenase (Mesembryanthemum): a. gil12229778|
29. nucleosome/chromatin assembly factor C: a. (maize) gil14550114|
30. pathogenesis-related protein: a. gil6002595|
31. peroxidase 1: a. gil22001285|
32. peroxiredoxin: a. gil12247762|
33. proteinase inhibitor, WCI: a. gil20798981|
34. protein disulfide isomerase: a. gil48093453|; b. gil32400792|
35. protein synthesis inhibitor (rye): a. gil7442154|; b. (barley) gil132580|
36. puroindoline: a. gil408873|; b. (*Aegilops*) gil13235060|
37. purothionin: a. gil4007850|; b. gil2213880|
38. reversibly glycosylated peptide: a. gil4158232|
39. ribosomal protein: a. (*Arabidopsis*) gil20259193|
40. ribosomal protein: a. (rice) gil39578552|; b. (*Arabidopsis*) gil21592338|; c. (*Oryza*) gil50911805|; d. (*Arabidopsis*) gil21592414|; e. gil32401381|; f. (*Brassica*) gil28436071|; g. gil22204120|
41. secretory protein: a. gil56690088|
42. seed globulin, 19 kDa: a. gil32400820|
43. seed storage globulin: a. (oats) gil226510|
44. selenium binding protein: a. (rice) gil13560275|
45. serpin: a. gil871551|
46. starch synthase: a. gil4760580|; b. |4588609|; c. gil4588607|; d. gil4760584|; e. gil3493049|; f. gil16751511|; g. gil4760582|; h. gil6624283|; i. gil25815183|; j. gil11037536|
47. sucrose synthase: a. gi:75221424
48. thaumatin-like protein: a. gil20257409|
49. triticin or triticin component: a. gil7548844|
50. tritin: a. gil479388|
51. unknown proteins: a. (rice) gil1658313|; b. (*T. aestivum*) gil1323750|; c. gil32400760|; d. (rice) gil51978958|; e. gil21813|
52. UOS1 protein: a. (rice) gil53792761|
53. xylanase: a. gil51247633|
54. xylanase inhibitor: a. gi) gil247309|

Table 3. Gluten Content of Starches by R5 ELISA Testing

	gluten (ppm)	
starch	University of Nebraska	ELISA Technologies
Keestar 328	8	5
Sanostar	41	45
Aytex P	71	75
Whetstar 4	212	363

in their diets (and how much) continues to be controversial, in part because it is very difficult to conduct appropriate studies. A recent study by Catassi et al. (*27*) was carefully done and provides the most current information regarding gluten thresholds. In a double-blind, placebo-controlled trial in which 49 celiac patients ingested capsules containing 0, 10, or 50 mg of gluten per day for 90 days, Catassi et al. (*27*) concluded that most celiac patients can tolerate a total daily intake up to about 50 mg per day of gluten.

Aytex P has an average of 73 ppm gluten according to R5 ELISA testing, which corresponds to 0.073 mg gluten per g of starch; Sanostar, an average of 43 ppm gluten, or 0.043 mg gluten per g of starch; and Keestar 328, an average of 7 ppm gluten, or 0.007 mg gluten per g of starch. To consume 50 mg

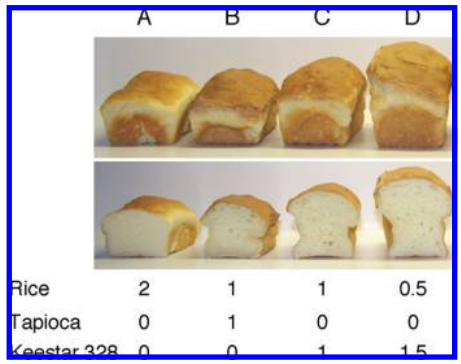

Figure 6. Comparison of loaf volumes and crumb structures of breads produced with various combinations of rice flour, tapioca, and Keestar 328 starch. (**A**) Two cups of rice flour, (**B**) 1 cup of rice flour and 1 cup of tapioca starch, (**C**) 1 cup of rice flour and 1 cup of Keestar 328 wheat starch, and (**D**) 0.5 cup of rice flour and 1.5 cups of Keestar 328 wheat starch.

of gluten per day in the form of Keestar 328 wheat starch, a celiac patient would have to consume more than 7 kg per day.

A variety of gluten-free products have been produced using flours from rice, garbanzo and fava beans, soy, sorghum, quinoa, amaranth, teff, and buckwheat and starches from tapioca, potato, and corn. While other flours and starches may produce an acceptable product for those unable to consume gluten, most are missing the unique flavor and texture of wheat-based bread. In addition, some of these ingredients impart unique sweet or vegetal flavors to the finished product. Because the European experience supports better flavor for wheat starch-based products, we tested the performance of the wheat starch with the lowest gluten content in a gluten-free white bread recipe typically used by celiac patients. Preliminary studies suggest that Keestar 328 may be a good substitute for a significant portion of the flour and starch in gluten-free breads. Further adjustments in other ingredients, in particular the amount and type of hydrocolloid used as a gluten replacement and the amount of water, may result in breads that are more similar to those made from wheat flour but yet can be tolerated by most celiac patients.

Although the Keestar 328 starch is readily available in the United States from its manufacturer, ADM, it has not been sold for food use. ADM does not currently carry out microbiological testing for bacterial contamination on Keestar 328, given that it was not intended for food use, and they have informed us that modifications to the process for making Keestar 328 would be necessary to make a product suitable for food use. However, if the company could be persuaded to make such modifications, it would result in a suitable starch with extremely low levels of gluten being readily available in North America. In the meantime, Sanostar and Aytex starches are apparently safe for use by celiac patients on the basis of Codex standards, and our findings are not in contradiction to this conclusion. In general, however, it would make sense to seek the lowest levels available, given the difficulty in establishing safe levels of gluten intake in celiac disease, and we feel it is worthwhile to indicate the potential of Keestar 328.

Lowering Protein Levels in Wheat Starch. Can the gluten content of wheat starch be lowered below the levels found in Keestar 328? We speculate that this may be possible. Keestar 328 is an enhanced A granule starch that has been fractionated by a proprietary method not disclosed to us by its manufacturer. The reason for the low levels of protein in the A granule starch might lie in the diminished surface area of A granules as compared with equivalent weights of B granules (*28*) or in the more elaborate washing during processing used to achieve enhanced A granule proportions. As can be seen in **Figure 2**, Keestar 328 does show an increased proportion of A granules but still contains many B granules. Further fractionation is probably achievable to enhance the proportion of A granules, while at the same time washing more proteins from the starch surface. In addition, some of our preliminary work (results not shown) indicated that a laboratory-prepared starch from soft wheat flour was lower in protein content than starches prepared from hard wheat flours. Soft wheat flours usually have low protein percentages, whereas hard wheat flours usually have considerably higher protein percentages. It would be desirable to investigate the possibility that starches washed from soft wheat cultivars might tend to have lower protein contents than starches from higher protein hard wheat flours. If these various combined speculations should turn out to be correct, an A granule-enhanced, soft wheat starch might have extremely low gluten content. Some of our preliminary investigations indicated that extraction of starches from a well-developed dough as opposed to a batter also lowered the protein content of the starch (results not shown), and this observation might also be investigated.

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