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Extraction and Proteome Analysis of Starch Granule-Associated Proteins in Mature Wheat Kernel (*Triticum aestivum* L.)

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Abstract: Starch consists of the two glucose polymers, amylose and amylopectin, and is deposited as semicrystalline granules inside plastids. The starch granule proteome is particularly challenging to study due to the amount of interfering compounds (sugars, storage proteins), the very low starch granule-associated protein content and also the dynamic range of abundant proteins. Here we present the protocol for extraction and 2-DE of wheat starch granule-associated proteins whose most important steps are: (i) washing and sonication to remove interfering compounds (storage proteins) from the surface of the granules, (ii) scanning electron microscopy (SEM) observations to monitor purification and granules swelling, (iii) appropriate protein extraction and solubilization to obtain enough proteins for Coomassie blue staining and proteomic analysis. Our objective was to minimize the amount of contamination by storage proteins and to preserve the structure of the starch and of starch-associated proteins and to maximize the number of polypeptides that can be resolved. For quantitative proteomic analysis of proteins associated with wheat starch granules, we developed a two-step protein extraction protocol including TCA/acetone precipitation and phenol extraction. With this protocol, proteins were extracted from wheat starch granules and solubilized and satisfactory blue-stained 2-DE protein maps were obtained. The majority of the spots associated with starch granules were identified by peptide mass fingerprinting and MS/MS and functionally classified into carbohydrate metabolism and stress defense.

Keywords: protein extraction method • proteome • starch • granules • wheat kernel

Introduction

Starch granules are the major carbohydrate storage organites for many types of plant cells. Starch is the major constituent of the wheat endosperm and acts as the main storage compound.[1−3] It is composed of amylose, a linear chain of α(1-4)D-glucan polymer and amylopectin, a branched chain of a α(1-4) and (1-6) D-glucan polymer. These components are synthesized in amyloplasts in the form of distinct granules. Large type A granules (diameters greater than 10 μm) are initiated early in development, smaller type B granules (diameters between 5 and 10 μm) are initiated during mid-development, and much smaller type C granules (diameters less than 5 μm) are initiated late in development.[4]

The glucan biosynthetic system in plants is complex due to the multiple forms of each enzyme conserved during evolution and coexpressed at the same location and at the same time as starch accumulation. Studies in many plant species have shown that starch biosynthetic enzymes exist as both soluble proteins in the stroma and as internal granule-associated proteins.[5,6]

Amylose is synthesized by granule bound starch synthase (GBSS) from ADP-glucose activated by ADP-glucose pyrophosphorylase (AGPase) while amylopectin is synthesized by the coordinated actions of AGPase, starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (DBE).[7,8]

In cereal starch biosynthesis, amylopectin synthesis requires multiple types of SS (SSI, SSII, SSIII, and SSIV), BE (BEI and BEII), and DBE. In addition, several types of these enzymes have multiple isoforms, the number of which is plant species specific. Of these enzymes, each isoform of SS, BE, and DBE plays a distinct role in amylopectin biosynthesis.[8,9] Recently functional interactions among starch biosynthetic enzymes have been demonstrated.[10,11]

Wheat genome is composed of three genomes, namely A, B, and D, each coding for enzymes isoforms involved in starch synthesis. To understand the mechanism associated with starch deposition in the wheat kernel, it is of prime importance to know which enzymes are present in this major wheat kernel compound and to develop a proteomic approach allowing qualitative and quantitative studies. However, the proteome of starch granules in mature wheat endosperm has been far less studied than amyloplasts.[12−14] Two major difficulties may explain why starch granule proteome was less studied: (i) in the native endosperm, starch granules are entrapped in protein matrix making difficult the isolation of pure starch granules without any aggregated storage proteins and (ii) numerous compounds, particularly sugars, may interfere during protein

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extraction from starch granules. So as the sample preparation is a critical step, the purpose of the present note is to report on an extraction protocol specifically developed to allow proteome analysis of starch granules type A and B together or separately. Indeed, as the biosynthesis of A- and B-type starch granules in wheat endosperm is developmentally regulated, we consider important to be able to compare the composition of each type in term of proteins. This article provides also an overview of the starch granule proteome revealed through 2-DE profiles.

Material and Methods

Material. The hexaploid French wheat cultivar Recital was used in this study. It was grown in the field at the INRA Plant Breeding Station of Clermont-Ferrand, France, in 2007, under normal conditions with full fungicide protection.

Starch Granule Purification. Starch granules were obtained with some modifications of the standard procedure to improve the purity.[15] The seeds were manually crushed then soaked overnight in 1 mL of water at 4 °C. After centrifugation, 500 μL of water were added to the pellet. The slurry was layered on 1 mL of 80% (w/v) CsCl and centrifuged at 3500× *g* for 5 min. Pellets containing the starch granules were then washed three times with 1 mL of washing buffer (55 mM Tris-HCl pH 6.8, 2.3% (w/v) SDS, 1% (w/v) dithiothreitol (DTT), 10% (v/v) glycerol) for 30 min at 20 °C. At the beginning of each washing step, granules were disrupted using sonication with an ultrasonic processor (Vibracell, VC50, Bioblock Scientific, Illkirch, France) at 20 W power for a 20 s pulse before continuous mixing. The granules were washed three times more for an hour each. The starch granule pellet was also washed three times for 5 min with cold water, once with cold acetone and finally air-dried. Each washing step was followed by centrifugation at 3500× *g* for 5 min. All washing and centrifugation were carried out at room temperature to avoid SDS precipitation.

Fractionation of the A- and B-Type Starch Granules. The starch granules were separated into large size (A-type: diameter > 10 μm) and small size (B-type: diameter < 10 μm) using differing granule sedimentation rates in Percoll solutions (70 and 100%, v/v).[16] Briefly, a starch suspension (0.1 g/mL) was laid on a 70% (v/v) Percoll solution and centrifuged; the supernatant contained B-type starch granules. The pellet containing A-type starch granules was purified by repeating the separation three times in a 100% Percoll solution.

The starch granule pellet was washed three times with water, once with cold acetone and finally air-dried. Each washing step was followed by centrifugation at 3500× *g* for 5 min as previously described.

Scanning Electron Microscopy (SEM). The kernel was first cut longitudinally and the endosperm cut to be 2 mm thin and then sprinkled on a double-side adhesive tape, mounted on aluminum electron microscope stubs, coated with gold in an Emscope SC 500 (Elexience, Verrieres le Buisson, France) and observed using a SEM apparatus (Philips SEM 505, FEI Eindhoven, Holland). Digital images were made with a Microvision system (Microvision Instruments, Evry, France). Quality of granules purification was also monitored by control with SEM observation in the same way as kernel.

Protein Extraction: TCA/Acetone Precipitation and Phenol Extraction. A first attempt to purify wheat starch granules proteome was previously described.[15] This procedure was significantly improved to analyze, using a proteomic approach, proteins strongly attached to and/or present within the starch granules. Starch granules were first washed using SDS buffer (62.5 mM Tris-HCl, pH 8.7, 2% (w/v) SDS, 10 mM DTT). The pH was increased to 8.7 instead of 6.8 generally used in order to reduce the endogenous acid hydrolysis. The granules: buffer ratio was 50 mg/mL. The mix was sonicated at 20 W for 20 s then heated for 10 min at 100 °C under constant agitation to allow the starch granules to release their content. The slurry was cooled on ice for 5 min and then centrifuged at 16,000 g at 4 °C, for 15 min. The supernatant (S1) was collected and the pellet was treated as starch granules were, using washing step with SDS-buffer (same ratio), sonicated and centrifuged. The supernatant (S2) was collected. The two supernatants (S1 and S2) were pooled and the pellet was washed once and centrifuged at only 3500× *g* to avoid sedimentation of some proteins at higher speed. The supernatant (S3) was also collected. The three supernatants (S1, S2, and S3) were pooled and proteins precipitated with one volume of 30% (w/v) TCA in acetone. The resulting pellet was washed twice with ice cold (−20 °C) 80% (v/v) acetone and dried under a gentle stream of air.

At this step, the high level of viscous polysaccharides (amylose) still present in the pellet prevent all the proteins to be easely solubilized, so a second step of purification was required. Phenol soluble compounds were extracted from the pellet as described previously.[17,18] The TCA/acetone pellet was suspended in 500 μL dense SDS buffer (0.1 M Tris-HCl buffer, pH 8.0, 30% (w/v) sucrose, 2% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol) and 500 μL of Tris-saturated phenol (pH 8.0, Sigma, St. Louis, MO). The mixture was vortexed for 5 min and then centrifuged at 17 000× *g* for 5 min at 20 °C. The upper phenol phase was pipetted into a new tube and 5 volumes of cold 0.1 M ammonium acetate in methanol were added to the phenol phase and the mixture was stored at −20 °C overnight. Precipitated proteins were recovered at 10 000× *g* for 5 min, and then washed with cold methanolic ammonium acetate twice and cold 80% acetone twice. The final protein pellet was dried.

One and Two-Dimensional Electrophoresis of Starch Granule Proteins, Gel Staining, and Image Analysis. To check the different supernatants during the washing steps, a fraction of each supernatant was precipitated with cold acetone and the pellet was solubilized in one-dimensional sample buffer (80 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 1% (w/v) DTT, 40% (v/v) glycerol) and analyzed by SDS-PAGE (T: 14%, C: 2.1%). One dimensional electrophoresis of proteins associated to starch granules was performed as previously described[19] with a lower concentration of polyacrylamide (T, 10%; C, 0.44%).

For two-dimensional electrophoresis analysis, the resulting dried pellet containing proteins was dissolved in two-dimensional buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM DTT, 1% (v/v) IPG buffer 4−7, 6−11 or 3−11, 0.4% (v/v) protease inhibitor) and solubilization of proteins was performed during 2 h at room temperature with constant agitation. Sonication at 20 W for 20 s was applied to the mix in order to fully resuspend the pellet. The protein concentration was assayed according to Bradford assay using reagents from Sigma (St. Louis, MO) with Bovine Serum Albumin as standard.[20] To get a good individual protein resolution (without streaking or spot overlapping), 150 μg of protein extract was chosen for protein loading. Extracted proteins were dissolved in 250 μL of two-dimensional buffer to passively rehydrate immobilized pH gradient strips (Immobiline Dry Strips, GE Healthcare, Uppsala, Sweden), pH 4−7, 13 cm. To improve basic proteins

identification, basic immobilized pH gradient strips (Immobiline Dry Strips, pH 6−11, 13 cm) were also used. To obtain the global proteome map proteins samples were applied in 340 μL of 2 DE rehydratation solution by reswelling 18 cm Immobiline DryStrip pH 3−11 NL, 18 cm. Isoelectric focusing was achieved on Ettan IpGphor 2 unit (GE Healthcare, Uppsala, Sweden) with a stepwise protocol to 60 kVh. After isoelectrofocalisation, the strips were incubated for 15 min in equilibration buffer (6 M urea, 7.5 mM Tris-HCl pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS) with DTT 1% (w/v) and another 15 min in the same equilibration buffer with iodoacetamide 2.5% (w/v) and bromophenol blue. 2-DE gels were continuous gels (T: 10%, C: 0.44 or T: 12.5%, C: 0.97% or T: 14%, C: 2.1%). Separation was carried out at 10 mA/gel for 30 min and subsequently at 35 mA/gel until bromophenol blue front had reached the end of the gel.

Gel staining was performed using Coomassie Brillant Blue G250 staining.[21] Mass spectrometry compatible silver staining was performed.[22]

Phosphoprotein staining was achieved using Pro-Q Diamond phosphoprotein stain (Invitrogen Life Sciences, Carlsbad, CA) with ethanol instead of methanol.[23] During the staining procedure gels containers were wrapped in foil to avoid light exposure.

Gels were scanned using GS 800 calibrated densitometer (Biorad, Hercules, CA) and Pharos (Biorad, Hercules, CA). The images were analyzed with the Samespots software v6.2 (Non linear Dynamics, Newcastle, UK).

The obtained protein profiles were the result of duplicated protein extractions used in at least two 2-DE replicates for each extract.

Mass Spectrometry and Protein Identification. The spots were excised from the gel, destained and hydrolyzed as described earlier with sequencing-grade modified trypsin (V5111; Promega, Madison, WI).[24] Briefly, 15 μL of trypsin (10 ng/μL in 25 mM NH_4HCO_3) was added to each dried spot for in-gel digestion.

Positive ion MALDI mass spectra analyses were performed by the platform "PFEM-Plateau Protéomique" located at the INRA Center of Clermont-Ferrand, France.

Analyses were recorded in the reflectron mode of MALDI-TOF MS (Voyager DE-Pro, Perseptive BioSystems, Farmingham, MA) using Voyager software for data collection and analysis.

The MS was calibrated with a standard peptide solution (ProteomiX 3, LaserBio Laboratories, Sophia-Antipolis, France). Internal calibration of samples was achieved using trypsin autolysis peptides.

Monoisotopic peptide masses were assigned and used from *Viridiplantae* nrNCBI (release 06/2009, 1 201 934 seq), Swiss-Prot (release 06/2009) or from a local restricted version of *poaceae* uniprot database searches with the MASCOT 2.2 software (Matrix Science). Matches to protein sequences from the *Viridiplantae* taxon were considered acceptable if at least four peptide masses from the PMF matched and a significant score was obtained from MASCOT. Scores are significant if they are above the 95% significance threshold ($p < 0.05$). The maximum fragment ion mass tolerance was set up at 25 ppm, one missed cleavage was allowed, and possible modification of cysteines by carbamidomethylation as well as oxidation of methionine were considered.

MS/MS analyses were conducted by the platform "Biopolymers-Interaction-Structural Biology" located at the INRA Center of Angers-Nantes, France.

LC−MS/MS analyses were performed through the use of a Switchos-Ultimate II capillary LC system (LC Packings/Dionex, Amsterdam, The Netherlands) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75-μm x 15-cm, LC Packings) with a linear gradient from 2% to 40% (v/v) acetonitrile in 50 min, followed by an increase to 50% acetonitrile within 10 min, at a flow rate of 200 nL min^{-1}. Mass-data acquisitions were piloted by the Mass Lynx software (Micromass/Waters) using a data dependent acquisition mode (the MS data were recorded for 1 s on the mass-to-charge (m/z) range 400−1,500, after which time the three most intense ions (doubly, triply, or four times charged) were selected and fragmented in the collision cell for MS/MS measurement.

Raw data were processed by means of the Protein Lynx Global Server (PLGS) v. 2.1 software (Micromass/Waters). Protein identification was performed by comparing the collected MS and MS/MS data with the Uniprot sequence databanks (release 15.2 of 05/05/09, Swiss-Prot, release 57.2 of 05/05/09) or the TIGR Gene Indices databank (*Triticum aestivum* release of 15/07/08). Databank searches were performed through the use of the Mascot server v. 2.2 program (Matrix Science). The mass tolerance was set at 150 ppm for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode) and one missed cut per peptide was allowed. Results from the different databank searches were compared in order to achieve final protein identification.

Proteins were considered to be identified if at least two non redundant peptides were found to match a single reference in the databases. A cutoff was applied onto peptides individual ion scores according to the significance threshold of the MASCOT program ($p < 0.05$).

In order to confirm some identifications or when databank searches did not result in protein identification which met our validation criteria, unassigned MS/MS spectra were submitted to a *denovo* sequencing using the functionality implemented in PLGS. The results were fed into the program OVNIp (http://www.appli.nantes.inra.fr:8180/OVNIp/), which enhances the confidence in protein identification by increasing the sequence coverage through alignment of the *denovo* stretches onto identified proteins. A manual inspection and validation was performed.

Results and Discussion

To perform quantitative proteomic studies of starch associated proteins from wheat seeds, the two main prerequisites were:

(i) purified starch granules have to be isolated

(ii) proteins have to be extracted without interfering compounds.

1. Starch Granule Purification. Starch granules were isolated from mature seeds or alternatively from flour obtained with a Brabender junior meal. Surface-associated proteins were removed by extensive washing in aqueous buffer containing 2.3% SDS and 1% DTT. Sonication was also performed at the beginning of the washing step in order to get complete separation of matrix protein with starch granules. SDS-PAGE (Figure 1) was performed to reveal extracted proteins present in the supernatant at each washing step. A complex protein pattern was obtained, and six washes (Figure 1, lanes 1 to 6) were required to remove all contamination. We considered that

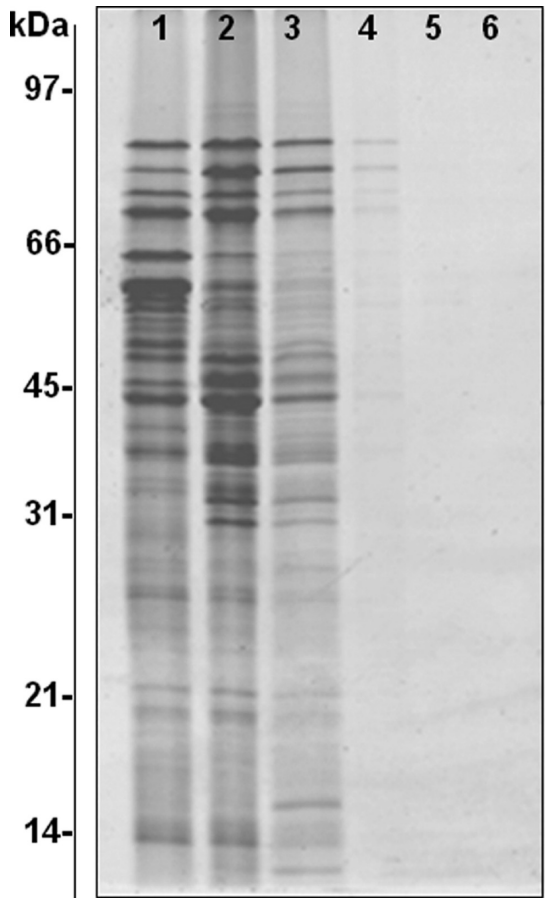

Figure 1. Silver-stained SDS-PAGE (T, 14%; C, 0.44%) patterns (1−6) of proteins present in supernatant after successive washing steps (1−6).

the absence of significant amounts of proteins in the last supernatant (Figure 1, lane 6) confirmed that the majority of surface proteins had been removed. Purity of the starch fraction was also monitored using SEM. Microscopic images showed no proteic or cell debris contaminating the granules (Figure 2). Moreover, the effect of SDS extraction and sonication did not alter the visual appearance of starch granules observed in SEM.

2. Extraction of Starch Granule-Associated Proteins. Sample preparation is crucial step for high quality resolution of proteins in 2-DE. Existence of nonprotein contaminants is well-known to affect protein migration.[25] Conventional removal of nonprotein contaminants uses organic solvent (e.g., acetone or 10% (w/v) TCA in acetone).[26] To extract starch granule-bound proteins, the purified starch was suspended in SDS buffer, sonicated for granules dispersion and heated 10 min in boiling water to gelatinize the starch. The starch granule gelatinization was checked using SEM (data not shown). Then the starch solution was cooled on ice and centrifuged, the supernatant was kept and the pellet suspended twice in SDS buffer, boiled and centrifuged (Figure 3). The pooled supernatants were precipitated in a 30% TCA/acetone solution. Then our protocol as compared to the initial one[15] included the additional step in which proteins from the pellet obtained by precipitation was extracted with a mixture of phenol and dense SDS buffer. These proteins were then precipitated with methanol and ammonium acetate, followed by resolubilization in IEF buffer.

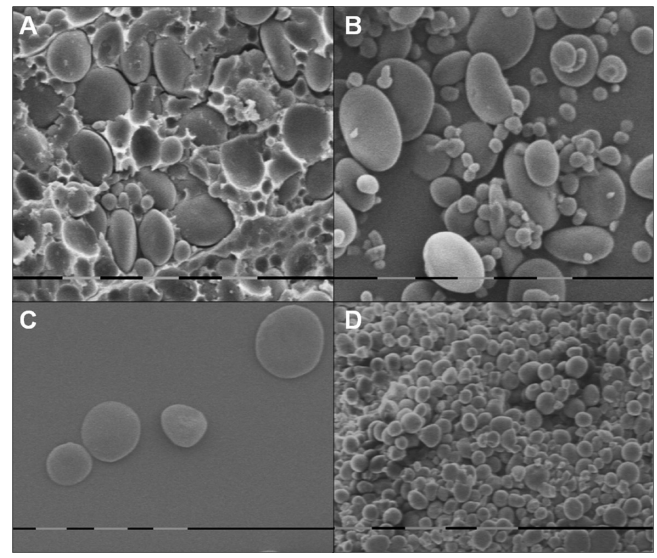

Figure 2. Scanning Electron Microscopy of (A) floury endosperm, (B) isolated A and B granules, (C) purified A-type, and (D) B-type starch granules. Scale bar: 10 μm.

This protocol (Figure 3) consists in the use of two steps of precipitation/extraction. Phenol dissolves both proteins (including membrane proteins) and lipids, leaving carbohydrates in the aqueous phase. Consequently the proteins in the phenol phase were purified and concentrated together followed by methanol precipitation. The notable advantage of phenol extraction is that it minimizes protein degradation due to endogenous proteolysis activities during extraction.[27,28] Another advantage is the good compatibility of this plant protein extraction with mass spectrometry analysis.[29]

Despite the number of washing steps used in this protocol, the average final protein yield was around 350 μg proteins per gramme of granules. This was close to the amount obtained for maize amyloplasts.[30] So this approach increased the extraction yield as well as the protein concentration in electrophoresis granules extracts from mature wheat seeds compared to the original process (data not shown).

3. Separation of A and B-Type Starch Granules. Scanning Electron Microscopy. Isolated starch granules were fractionated into A- and B-types.[16] The fractions highly enriched in A- or B-type granules were observed by SEM (Figure 2). No apparent contamination between the two populations was observed. A-granules were lenticular whereas B-granules were spherical, as previously described in wheat.[31]

2-DE Separation. A more comprehensive description of the granule-bound proteome and quantification was achieved by 2-DE. 2-DE gels were used to characterize proteins associated with starch granules and silver staining enabled us to observe less abundant proteins between 30 and 60 kDa.

Protein map showed a wide distribution of spots in the p*I* range from 3 to 11 and a mass range between 15 and 140 kDa (Figure 4A). These results confirmed that our extraction protocol for starch associated proteins allowed qualitative and quantitative analysis.

Image analysis of the 2-DE profiles showed a total of 150 Coomassie-stained spots that could be used for quantitative analysis. Depending on the p*I*-MW standard, the spots had apparent p*I*s varying between 4.1 and 9.8 (Figure 4). When 2-DE separation was performed in the pH 4−7 and 6−11 ranges, a total of 250 spots were visible on silver-stained gels (Figure 4B).

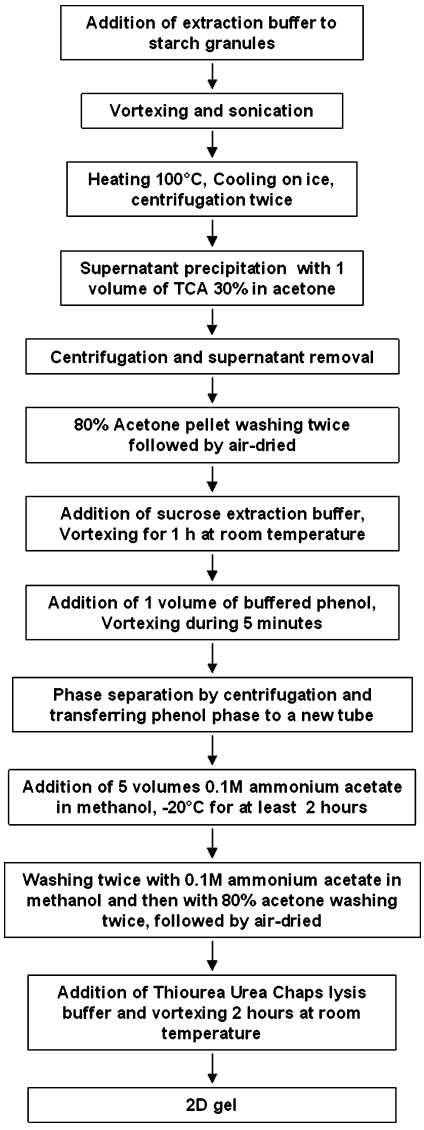

Figure 3. Scheme showing the main steps of starch granule associated proteins extraction by the TCA/acetone/phenol protocol.

The pH 4.0−7.0 and 6.0−11.0 ranges gave significantly better separations than the broad pH 3.0−11.0 range and were thus selected to identify some spots.

4. Protein Identification. In many cases, proteins were successfully identified by MALDI-TOF or MS/MS with matches to predicted proteins from members of the *Poaceae*. A total of 85 spots were identified including different proteins, which were grouped into functional categories (Table 1). The majority (74 out of 85 identified) of proteins matched wheat proteins of carbohydrate metabolism and were more especially involved in starch synthesis.

Starch Metabolism. The granule bound starch synthase (GBSS)—the key enzyme responsible for amylose synthesis—appeared as many spots (spots 1−8, 28, 29) (Table 1). Three series of five to eight charge variants spots at about 60 kDa dominated the gel image (Figure 4) and likely represent the mature protein with minor differences in molecular weight corresponding to the three loci encoding this GBSS protein. Some of the low molecular spots (spots 15−17, 19, 20, 57, 66, 67, 71, 73−78) matched GBSS and could be the result of fragmentation that occurred during the extraction process but GBSS and other granule-associated proteins have been reported to be refractory to proteolysis treatment of the granule.[32,33]

Spots assigned as GBSS through peptide mass fingerprinting (Table 1) were classified in 4 major zones in the two-dimensional gel (Figure 4B). The distribution of peptides used to identify the GBSS was shown in Figure 5. Zone 1 was composed of spots (1−8, 28, 29) giving peptides throughout mature GBSS. Three spots (1−3) had specific peptides like the peptide 92−112 corresponding to their slightly higher molecular mass.

Zone 2 was composed of 5 spots (19, 20, 57, 71, 73) corresponding to a short C-terminal truncation (at position 503 or 530). Zone 3 was composed of 2 spots (66, 67), the peptides masses found clearly indicated a N-terminal truncation of the mature protein. Zone 4 corresponded to 8 spots, 5 of them (74−78) having a large deletion from the C-terminal side and all the glycosyl transferase 1 domain missing. Two others spots (16, 17) corresponded to a large deletion from the N terminus: in this case glycosyl transferase 5 domain (or starch synthase catalytic domain) was absent. Spot 15 was hypothesized to be a mixture of two truncated proteins having both C or N truncation resulting of lower molecular masses.

In addition to the GBSS, starch granules also contained other starch biosynthetic enzymes. Within the group of proteins related to starch branching enzymes, the proteins related to starch synthase I (spots 10 to 14) and II (spots 32, 34, 36, 37 and 38) were well-defined with different isoforms. These isoforms could result from post-translational modification.

Spot 53 was identified as β-amylase. Beta-amylases are exoamylases that release maltose from the nonreducing ends of glucans or dextrins by cleavage of α(1-4) linkages. Beta-amylase is considered to be one of the key enzymes catalyzing starch breakdown, but because the enzyme was shown to be located outside the plastid, its role in starch breakdown remains unclear. The presence of β-amylase inside the starch granules indicates that this enzyme is needed to directly break down the starch polymer. It has been shown that β-amylase is involved in starch hydrolysis in plastids from fruit cells.[34] Immunochemical[35] and biochemical[36] studies have demonstrated that β-amylase is deposited on starch granules in the subaleurone layer during caryopsis desiccation.[37]

Phosphorylation. The multiplicity of spots identified as the same protein (GBSS) but differing in molecular mass and/or p*I* pointed to post-translational modifications. One of the most commonly occurring was phosphorylation. Starch phosphorylation is considered to be an integral part of the starch-biosynthetic pathway.[38] Pro-Q Diamond allowed phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins to be detected directly on SDS-PAGE and 2-DE.[39] The analysis of the starch granule proteome (Figure 6) with the phosphorylation specific dye Pro-Q Diamond showed that at least GBSS and SSI and SSII were phosphorylated in their internal granule-associated form. The positive signal was more intense in the more acidic spots corresponding to the mature protein consistent with the p*I* decrease induced by the presence of a phosphate group (Figure 6). Phosphorylation of BEIIb, SSIIa, and starch phosphorylase in wheat amyloplasts has already been revealed by radioactive labeling using ^{32}P ATP.[40] Some authors recently described phosphorylation of enzymes involved in starch metabolism (GBSS, BEIIb, and starch phosphorylase) in wheat and maize.[10,30]

The low molecular weight spots (Zones 2, 3 and 4, Figure 4B) corresponding to GBSS did not exhibit a positive phos-

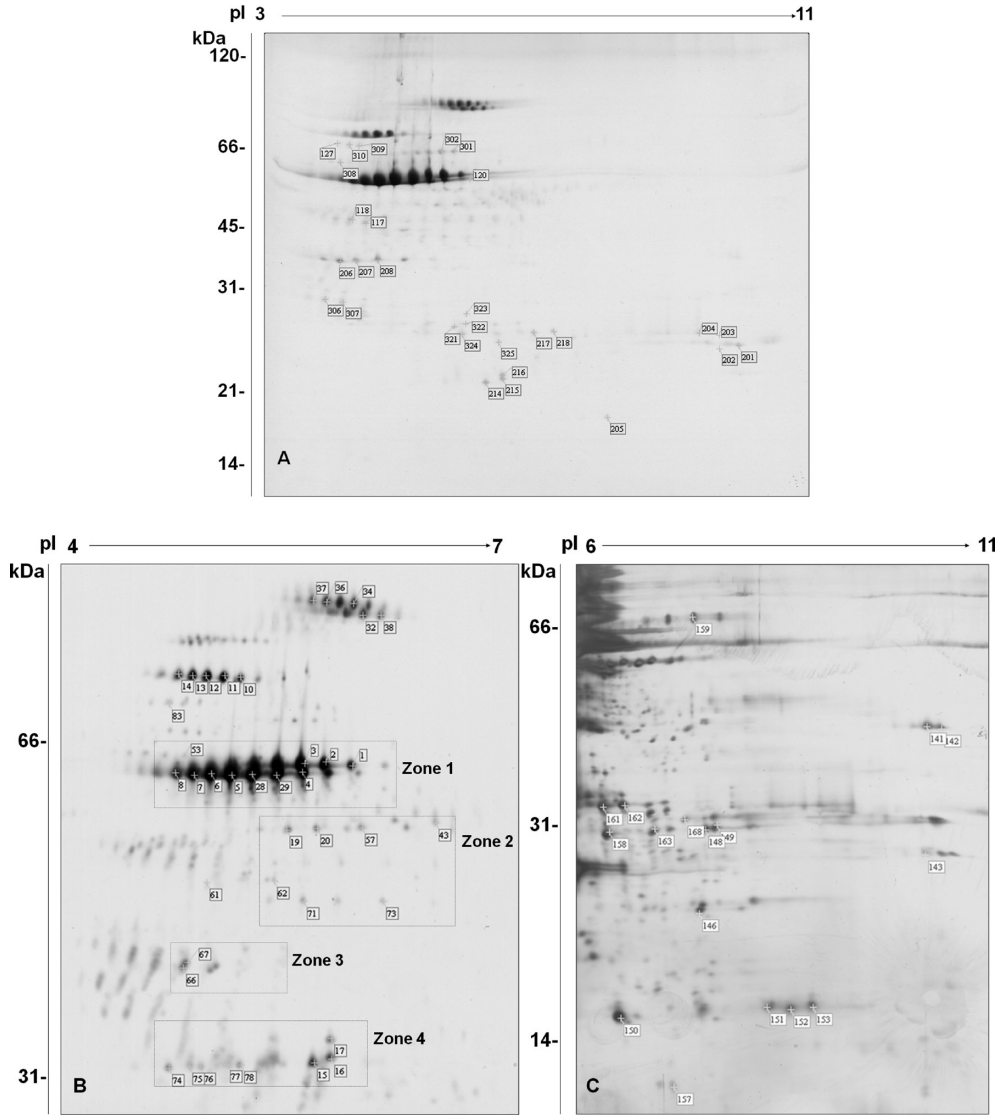

Figure 4. 2-DE gels of starch associated granule proteins. (A) Coomassie blue-stained IPG (pH 3−11) × SDS-PAGE (T, 12.5%; C, 0.97%) Silver-stained gels of starch associated granule proteins. (B) Silver-stained IPG (pH 4−7) × SDS-PAGE (T, 10%; C, 0.44%). (C) Silver-stained IPG (pH 6−11) × SDS-PAGE (T, 14%; C, 2.1%). Identified spots are annotated in the gel by the number that appears in Table 1.

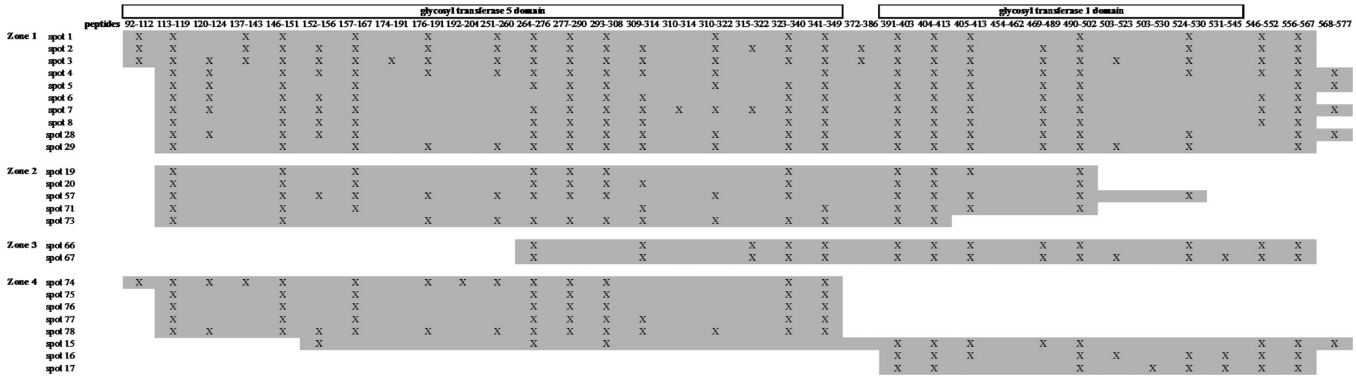

Figure 5. Distribution of GBSS peptides (location of each peptide in the primary sequence).

phorylation signal. Fragmentation during the extraction process has already been described in maize.[41] Absence of signal with Pro Q Diamond stain for the fragmented species can be due to missing phosphorylation sites within the fragmented proteins; it can be either that the dynamic range of the Pro-Q Diamond stain precludes the simultaneous detection of high and low abundance phosphorylated proteins. No clear correlation was found between the proteins exhibing phosphorylation and the spots ability to be phosphorylated, either when considering the presence of serine or threonine (the two amino

Table 1. Identification of Starch Granule-Associated Proteins by Mass Spectrometry (A: MALDI -Tof and B: MS/MS)

A: MALDI-TOF									
								theoretical[e]	
spot[a]	databank	entry	protein name	Mascot score	coverage (%)[b]	number of peptides[c]	E value[d]	MW	p*I*
			carbohydrate (starch) metabolism						
1	Swiss-Prot	Q9SXK4	Starch Synthase (GBSSI)	178	36	21(26)	1.20×10^{-13}	67254	7.1
2	Swiss-Prot	Q9SXK4	Starch Synthase (GBSSI)	257	43	27(20)	1.50×10^{-21}	67254	7.1
3	Swiss-Prot	Q9SXK4	Starch Synthase (GBSSI)	300	47	29(20)	7.80×10^{-26}	67254	7.1
4	Swiss-Prot	Q8W2G8	Mutant granule bound starch synthase I (Fragment)	269	48	26(20)	9.80×10^{-23}	59580	5.6
5	Swiss-Prot	Q9FUU6	Granule bound starch synthase I	246	44	26(19)	2.00×10^{-20}	67025	6.81
6	Swiss-Prot	Q9FUU6	Granule bound starch synthase I	243	44	27(19)	3.90×10^{-20}	67025	6.81
7	uniprot_poaceae	Q9FUU6	Granule bound starch synthase I	329	49	31(14)	9.80×10^{-29}	67025	6.81
8	Swiss-Prot	Q9FUU6	Granule bound starch synthase I	202	40	23(25)	4.90×10^{-16}	67025	6.81
10	Swiss-Prot	Q43654	Starch Synthase 1, chloroplast precursor	201	36	24(26)	6.20×10^{-16}	71587	5.83
11	Swiss-Prot	Q43654	Starch Synthase 1, chloroplast precursor	186	34	20(29)	2.00×10^{-14}	71587	5.83
12	uniprot_poaceae	Q43654	Starch Synthase 1, chloroplast precursor	203	36	25(24)	3.90×10^{-16}	71587	5.83
13	Swiss-Prot	Q43654	Starch Synthase 1, chloroplast precursor	169	30	23(27)	9.80×10^{-13}	71587	5.83
14	uniprot_poaceae	Q43654	Starch Synthase 1, chloroplast precursor	173	33	23(27)	3.90×10^{-13}	71587	5.83
15	Swiss-Prot	Q9FUU6	Granule bound starch synthase I	121	27	17(30)	6.20×10^{-8}	67025	6.81
16	Swiss-Prot	O81583	Granule-bound starch synthase (Fragment)	81	38	12(38)	5.80×10^{-4}	28160	5.58
17	Swiss-Prot	O81583	Granule-bound starch synthase (Fragment)	74	38	10(38)	3.40×10^{-3}	28160	5.58
19	Swiss-Prot	Q9SXK3	Starch Synthase (GBSSI)	124	31	17(30)	3.10×10^{-8}	67044	7.06
20	Swiss-Prot	Q9SXK3	starch synthase (GBSSI)	126	32	16(30)	2.00×10^{-8}	67044	7.06
28	Swiss-Prot	Q9SXK3	Starch Synthase (GBSSI)	275	46	28(17)	2.50×10^{-23}	67044	7.08
29	Swiss-Prot	Q9SYU0	Granule-bound starch synthase	220	45	24(21)	7.80×10^{-18}	63968	8.84
32	uniprot_poaceae	Q9LEE2	Starch Synthase IIa-3 precursor (Starch synthase II- B)	356	38	35(15)	2.00×10^{-31}	87140	6.27
34	uniprot_poaceae	Q9M466	Starch Synthase IIa-1 precursor	405	47	39(11)	2.50×10^{-36}	87291	6.05
36	viridiplantae	gi|82943644	Starch Synthase II-A	332	41	33(17)	4.00×10^{-28}	87623	6.14
37	viridiplantae	gi|82943644	Starch Synthase II-A	230	29	27(23)	6.30×10^{-18}	87623	6.14
38	uniprot_poaceae	Q9LEE2	Starch Synthase IIa-3 precursor (Starch synthase II- B)	152	21	19(27)	4.90×10^{-11}	87140	6.27
53	uniprot_poaceae	Q7X9M2	Beta amylase (Fragment)	76	31	9(41)	1.80×10^{-3}	31100	8.6
57	uniprot_poaceae	Q9SYU0	Granule-bound starch synthase	138	31	19(27)	1.20×10^{-9}	63968	8.84
66	viridiplantae	gi|4588609	Granule-bound starch synthase precursor	117	26	18(28)	1.30×10^{-6}	63968	8.84
67	viridiplantae	gi|4588609	Granule-bound starch synthase precursor	142	32	20(26)	4.00×10^{-9}	63968	8.84
71	uniprot_poaceae	Q8LLD5	Granule-bound starch synthase	77	17	13(33)	1.70×10^{-3}	66504	6.59
73	uniprot_poaceae	Q9XEN9	Granule-bound starch synthase	108	27	15(31)	1.20×10^{-6}	63388	7.86
74	uniprot_poaceae	Q9SXK3	Starch Synthase (GBSSI)	103	27	15(31)	3.90×10^{-6}	67044	7.08
75	viridiplantae	gi|4760582	Starch Synthase (GBSSI)	80	23	13(33)	6.20×10^{-3}	67044	7.08
76	uniprot_poaceae	Q9SXK3	Starch Synthase (GBSSI)	80	23	13(33)	6.30×10^{-3}	67044	7.08
77	viridiplantae	gi|4760582	Starch Synthase (GBSSI)	106	27	16(30)	1.60×10^{-5}	67044	7.08
78	viridiplantae	gi|4588609	Granule-bound starch synthase precursor	158	33	21(25)	1.00×10^{-10}	63968	8.84
			nutrient reservoir activity						
43	uniprot_poaceae	Q08837	Triticin (Fragment)	118	24	14(32)	1.20×10^{-7}	57318	9.37
			stress response						
61	uniprot_poaceae	P93693	Serpin	123	43	13(37)	3.90×10^{-8}	43120	5.44
62	viridiplantae	gi|5734504	emb|CAB527091| serpin	96	37	11(39)	1.40×10^{-4}	42969	5.62

Table 1. Continued

B: MS/MS									
								theoretical[e]	
spot[a]	databank	entry	protein name	Mascot score	coverage (%)[b]	number of peptides[c]	E value[d]	MW	p*I*
			carbohydrate (hexose) metabolism						
141	TIGR wheat	TA83217_4565_7	Aldolase epimerase	269	10	3	5.39×10^{-27}	39717	9.36
142	TIGR wheat	TA83217_4565_7	Aldolase epimerase	264	10	3	5.27×10^{-24}	39717	9.36
159	uniprot_viridiplantae	Q8RWR5	Beta-D-glucan exohydrolase	80	1	1	1.14×10^{-5}	67714	6.86
			carbohydrate (starch) metabolism						
117	uniprot_viridiplantae	Q5NKR2	Granule-bound starch synthase	131	3	2	4.11×10^{-14}	67071	7.5
118	uniprot_viridiplantae	Q8LLD5	Granule-bound starch synthase	221	7	5(3)	1.53×10^{-31}	66503	6.59
120	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	331	8	5	3.234×10^{-37}	66968	7.49
127	uniprot_viridiplantae	Q9LEC0	Starch synthase I-1	224	6	4(3)	7.476×10^{-22}	71586	5.76
148	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	375	11	5	4.496×10^{-36}	66968	7.49
149	uniprot_viridiplantae	B3V9H7	Waxy	268	6	3	1.537×10^{-24}	67224	7.5
150	TIGR wheat	TC341160_15	Granule-bound starch synthase	314	8	5(4)	3.122×10^{-29}	67083	6.96
151	uniprot_viridiplantae	B3V9H7	Waxy	120	4	2	7.546×10^{-7}	67224	7.5
152	uniprot_viridiplantae	B3V9H7	Waxy	198	6	3	1.002×10^{-14}	67224	7.5
153	uniprot_viridiplantae	B3V9H7	waxy	279	6	3	6.176×10^{-27}	67224	7.5
157	uniprot_viridiplantae	Q2WGB1	Starch synthase II	130	2	2	4.849×10^{-10}	87289	6.14
158	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	715	16	10(8)	3.4×10^{-78}	67024	6.81
161	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	365	8	4	2.362×10^{-31}	66968	7.49
162	uniprot_viridiplantae	B3V9H7	Waxy	732	17	9(8)	1.1×10^{-77}	67224	7.5
163	uniprot_viridiplantae	Q5NKR2	Granule-bound starch synthase	421	11	5	1.23×10^{-38}	67071	7.5
206	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	1335	32	19(17)	6.5×10^{-167}	66326	6.81
207	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	848	23	11	6.5×10^{-123}	66326	6.81
208	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	991	27	13	6.5×10^{-127}	66270	7.49
214	uniprot_viridiplantae	Q8LLD5	Granule-bound starch synthase	437	13	6	6.76×10^{-42}	65804	6.59
215	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	618	14	10(7)	1.6×10^{-79}	66270	7.49
216	uniprot_viridiplantae	B3V9H7	Waxy	830	18	13(10)	8.2×10^{-89}	66526	7.5
217	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	842	25	11	6.5×10^{-100}	66270	7.49
218	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	349	9	4	2.957×10^{-43}	66270	7.49
301	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	148	8	3	6.127×10^{-20}	66326	6.81
302	uniprot_viridiplantae	B3V9H7	Waxy	105	5	2	2.132×10^{-16}	66526	7.5
306	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	492	16	7	3.3×10^{-56}	66326	6.81
307	uniprot_viridiplantae	Q8LLD5	Granule-bound starch synthase	526	15	8(7)	1.30×10^{-61}	65804	6.59
308	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	158	7	3	2.1×10^{-19}	66326	6.81
309	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	421	14	6	6.5×10^{-60}	66326	6.81
310	uniprot_viridiplantae	Q9LEC0	Starch synthase I-1	270	9	4	1.557×10^{-27}	71004	5.76
321	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	901	19	10	6.5×10^{-121}	66326	6.81
322	uniprot_viridiplantae	Q9S7N5	Granule-bound starch synthase	814	20	12(11)	6.5×10^{-98}	66270	7.49
323	uniprot_viridiplantae	B3V9H7	Waxy	828	20	11(10)	1.3×10^{-90}	66526	7.5
324	uniprot_viridiplantae	Q9SLS8	Starch synthase	632	24	9	5.2×10^{-83}	66283	7.49
325	uniprot_viridiplantae	Q9FUU6	Granule-bound starch synthase	462	13	6	1.6×10^{-63}	66326	6.81
			stress defense response						
143	uniprot_viridiplantae	Q9FRV0	Basic endochitinase c	306	18	4(3)	1.73×10^{-25}	28682	8.82
146	uniprot_viridiplantae	P16347	Endogenous alpha-amylase/subtilisin inhibitor	311	29	4	4.34×10^{-28}	19848	6.77

Table 1. Continued

			B: MS/MS						
								theoretical[e]	
spot[a]	databank	entry	protein name	Mascot score	coverage (%)[b]	number of peptides[c]	E value[d]	MW	pI
168	TIGR wheat	CK205966_9	Thaumatin like cytokinin-binding protein	84	5	1	1.07×10^{-2}	25666	8.97
201	uniprot_viridiplantae	Q9FRV0	Basic endochitinase c	541	23	6	4.10×10^{-59}	28302	8.82
202	uniprot_viridiplantae	Q9FRV0	Basic endochitinase c	226	18	4(3)	8.15×10^{-27}	28302	8.82
203	uniprot_viridiplantae	Q4Z8L7	Chitinase	214	21	3	6.21×10^{-23}	28260	8.66
204	uniprot_viridiplantae	Q8L5C6	Xylanase inhibitor	189	12	3	6.31×10^{-17}	33275	8.66
205	uniprot_viridiplantae	P16347	Endogenous alpha-amylase/subtilisin inhibitor	282	29	5(4)	4.50×10^{-38}	19848	6.77

[a] Spots numbers correspond to those in Figure 4. [b] Percentage of sequence coverage based on the peptides identified. [c] A: number of matched peptides (number of unmatched peptides); B: number of total peptides (number of unique peptides). [d] E value. [e] Theorical MW(Da) and p*I* values were obtained from MASCOT search results.

acids having the more frequent occurrence of phosphorylation) or when predicting phosphorylation status with Netphos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/).

SSIIa was also found to be phosphorylated in wheat amyloplast extract.[40] Trimeric complexes could be formed between SSI, SSII, and either BEIIa or BEIIb and some protein−protein interactions have been shown to be phosphorylation dependent.[38,42]

Other Proteins. In the present study, the spot 43 was identified as triticin. Its pattern remained unaltered and reproducible. It was also reported in wheat amyloplasts.[43,44]

Additional proteins were revealed when the starch associated proteins were separated on a basic gradient (Figure 4C). Some of these basic proteins were identified as two aldolase epimerases (spots 141, 142) and one β-D-glucan exohydrolase (spot 159). These proteins are involved in carbohydrate (hexose) metabolism and more particularly in glycolysis.

Two spots (61 and 62) were identified as serpins. These proteins are related to be involved in stress defense. The presence of these proteins in starch granules is quite surprising. However, in barley, these proteins have also been described to be peripheral proteins tightly bound to the surface of granules.[33] These authors explain that cellular compartmentalization is already dissolved in the mature starchy endosperm as part of programmed cell death allowing contact between starch granules and the rest of the cell content.[43]

5. Proteome Comparison of A-Type and B-Type Mature Starch Granules. When determined by SDS-PAGE (Figure 7), proteins isolated using the phenol/dense protocol showed high quality protein band resolution with little contamination by polysaccharides (good resolution and low background after silver staining, no smearing and accumulated material in the wells). Some authors demonstrated the importance of sonication in ensuring that polysaccharides remain solubilized.[44] SDS-PAGE showed a much weaker electrophoretic profile of proteins below 60 kDa as previously described.[45] The band corresponding to SBE (140 kDa) was visible in both the A- and B-type extracts. This is in contradiction with previous results, which showed that starch-branching enzymes were preferentially associated with A-type starch granules.[46] Our hypothesis is that these proteins are so tightly linked to the B-type granules that we could extract them only if the second step of granule swelling was performed (Figure 3).

For the 2 DE gels comparison, four replicates (two biological $\times$ two technical) were carried out for each type of starch granule. Image analysis was performed for each proteome associated with a starch granule type. According to analysis of variance ($p < 0.05$), differences between A and B concerned only some basic proteins (spots 201 to 205, Table 2). These five spots had a significantly higher percentage of normalized volume in A-type extract than in B-type. Neither qualitative (presence/absence) nor quantitative differences were observed concerning the many other spots and in particular those relevant for carbohydrate (starch or hexose) metabolism.

Some basic spots were also identified as chitinase-activity related proteins (spots 201, 202, 203 and also 143). Chitinases are enzymes that catalyze the hydrolysis of the β-1,4-*N*-acetyl-D-glucosamine linkages in chitin polymers. Several chitinases are constitutively expressed. Basic endochitinase C from rye was shown to act in defense against fungal pathogens containing chitin and was localized on the starchy endosperm of the seed.[47] A member (spot 204) of the xylanase inhibitor family was also identified. Wheat grains contain three classes of

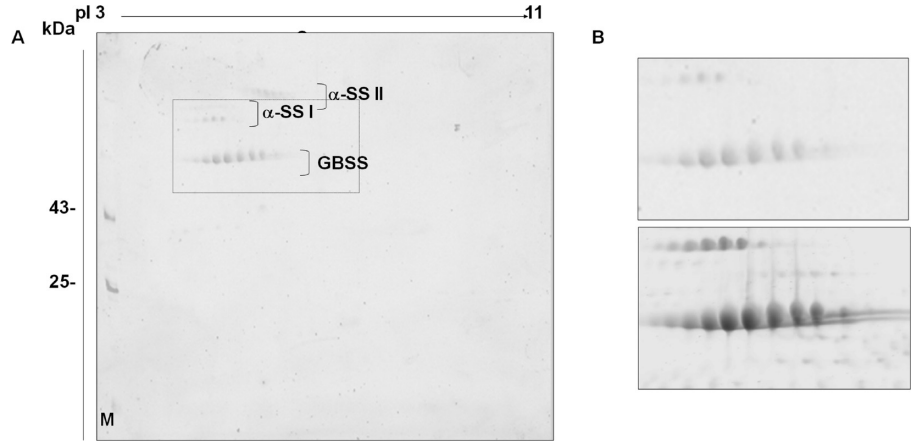

Figure 6. 2-DE gel image of starch granule proteome revealed by Pro-Q Diamond in gel stain (A). M: Mixtures of phosphorylated control proteins were used as internal controls for detection of phosphoproteins by Pro-Q Diamond. Enlarged region (GBSS zone) Pro-Q staining (B up) and Coomassie blue staining (B down).

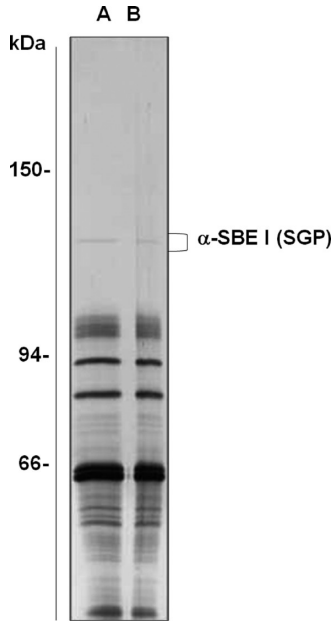

Figure 7. SDS-PAGE profile of starch granules associated proteins extracted from purified (A) type-A and (B) type-B granules.

Table 2. Quantitive Variations for the 5 Spots Differing between A-Type and B-Type Granules Proteome (p value < 0.01 and q value < 0.05)

spot[a]	protein name	av. vol. ratio[b]	variation A vs B %
201	basic endochitinase C	1.9	+46.45
202	basic endochitinase C	1.6	+36.62
203	Chitinase	1.9	+48.44
204	xylanase inhibitor	1.9	+47.7
205	Endogenous alpha-amylase/ subtilisin inhibitor	2.3	+61.51

[a] Spots numbers correspond to those of figure 4. [b] Average volume ratio based on the normalized spot volume provided by Samespots software analysis.

xylanase inhibitors, that is, *Triticum aestivum* xylanase inhibitor, xylanase inhibiting protein and thaumatin-like xylanase inhibitor. Spot 204 belonged to the xylanase inhibiting protein class described as an endo-1-4, β-D-xylanase inhibitor.[48,49] This xylanase inhibitor also displays inhibitory activity toward barley alpha-amylases. Binding to xylanases or amylases appears to be necessary for inhibition activity.[50]

Some xylanase inhibitors belong to the thaumatin family. These proteins are known to act in protecting seeds from diseases. In our extracts, spot 168 was identified as a thaumatin-like cytokine-binding protein.

Spot 205 and also spot 146 were identified as α-amylase inhibitor. The endosperm proteome has been reported to contain abundant levels (<11% of total proteins) of α-amylase inhibitors and α-amylase/trypsin inhibitors, which are thought to protect the starch reserves in the endosperm from degradation.[51] These proteins can inhibit endogenous and exogenous α-amylase, an enzyme that plays an important role in carbohydrate metabolism.[52]

The presence of such basic proteins could be explained by remnants of lipid membrane between starch granules and the protein matrix of wheat endosperm.[53] Lipid−protein interactions could be involved in the adsorption of the basic proteins onto the surface of starch granules. Many stress related proteins are basic and the ability of starch to bind positively charged proteins has been shown in the large number of stress events occurring during grain formation.[54] Some authors studied starch associated proteins from commercial wheat starches,[54] and found stress defense proteins including α-amylase subtilisin inhibitor, chitinase and serpin.

Concluding Remarks

In conclusion, an important area of proteomics is to study changes in protein expression in organites like amyloplasts or starch granules during wheat grain development using 2-DE gel and image analysis.

Although the extraction method is more time-consuming, we consider that the benefits of visualizing a substantially larger number of proteins far outweight the small additional time investment in sample preparation. Consequently, we developed an efficient protocol for protein extraction including notably:

(i) Thorough washing and sonication steps to remove interfering compounds (e.g., storage proteins) from the surface of granules,

(ii) Extraction with sonication steps, TCA acetone precipitation and phenol extraction,

(iii) Adaptation of the IEF gradient and of the acrylamide concentration in the second-dimensional SDS-PAGE.

The resulting high quality of 2-DE gels enabled us to identify starch granule-associated proteins by MS and to establish a proteome map. Our results showed relatively abundant variations for only some spots between A-type and B-type granules. The majority of spots submitted to MS analysis were identified. A total of 87% of these spots were related to carbohydrate (hexose or starch) metabolism and about 12% were involved in stress defense mechanisms. Other proteins remain to be identified using mass spectrometry tools.

This extraction protocol is expected to have a wide application and to be very valuable in our ongoing studies of:

(i) the proteomic changes that occur during the final stages of grain development and maturation, particularly in response to environmental stresses;

(ii) the description of wheat lines carrying different null waxy genes and qualitative and quantitative differences in their granule proteome;

(iii) the characterization of some ancient wheats and related species.

Characterization of differential phosphorylation state of proteins involved in starch metabolism underlined the need to continue analysis of functional meaning of post-transcriptionally modified proteins. A comprehensive analysis of the transcript levels of genes encoding starch-synthesis enzymes together with the improvement in proteomic analysis is now also crucial to improve our understanding of the regulatory mechanism of starch biosynthesis.

Abbreviations: GBSS, granule bound starch synthase; SS, starch synthase; SBE, starch branching enzyme; DBE, debranching enzyme; AGPase, ADP-glucose pyrophosphorylase; MS, mass spectrometry; 2-DE, two-dimensional electrophoresis.

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