

In Situ Location of a Starch Granule Protein in Durum Wheat Endosperm by Immunocytochemistry¹

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ABSTRACT

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Starch granule protein (SGP) was extracted with 0.1N NaCl from starch isolated from durum wheat *Triticum turgidum* cv. Vic flour (53% extraction). Ultrafiltered and lyophilized SGP was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A 32-kDa SGP was identified, excised from the gel, and used as an antigen to produce polyclonal antibodies. Western blotting confirmed antibody production. The antibodies were used in a gold label transmission electron study

of wheat endosperm tissue seven days after anthesis. Significance was determined using stereological techniques and nonparametric one-way analysis of variance. The number of gold particles per μm^2 area for starch (8.92), cell wall (0.53), and cytoplasm (0.04) differed significantly ($P < 0.01$). Labeling occurred throughout the starch, indicating that the 32-kDa starch granule protein is inherent to starch and is not localized on the granule surface.

Starch granule protein (SGP) is divided into two classes based on extraction techniques. Protein extracted with NaCl is referred to as surface-SGP, whereas protein extracted with sodium dodecyl sulfate (SDS) is known as integral-SGP. The terms *surface* and *integral* suggest possible location of the SGP. Integral-SGP may be physically entrapped within the starch granule aggregates or may be covalently bound to, or strongly associated with, the amylose-amylopectin matrix.

An association between a 15-kDa starch granule polypeptide and wheat hardness has been suggested, and implies a link between starch granule proteins and the physical properties of starch granules (Greenwell and Schofield 1986). Also, starch granule proteins, theorized to be located on the surface of starch granules, have been implicated in influencing the enzymatic degradation of starch granules (Greenwell et al 1985). Proteins associated with starch granules may be enzymes involved with starch metabolism and may serve as amyloplast membrane-bound proteins involved with metabolite transport (Pitcher et al 1987, Imam 1989, Ardila and Tandecarz 1992, Geowar-Singh et al 1992).

Polypeptides have been extracted from purified wheat starch. Five polypeptides, ranging from 5 to 30 kDa, have been extracted from winter and durum wheat starches with sodium chloride. A group of five larger polypeptides, ranging from 59 to 149 kDa, has been extracted from wheat starch with SDS (Schofield and Greenwell 1987).

Lowy et al (1981) extracted a 30-kDa polypeptide from Maris Huntsman winter wheat and *T. durum* A-type and B-type wheat starch granules with 0.1M NaCl. The polypeptide could not be extracted from starch granules with 500 mM NaCl, 35 mM SDS in 100 mM TRIS-HCl buffer (pH 8.0), nor from starch granules isolated from germinated wheat. Skerritt et al (1990) extracted protein from Flanders British soft wheat starch with 1% (w/v) SDS in 10 mM dithiothreitol (DTT) at 40°C. Similarities among wheat starch granule proteins and proteins from other cereal starches may exist.

Agents explored as possible starch granule protein extractants include low pH buffers such as 0.01M glycine (pH 3), and 0.1 and 0.5M NaCl; nonionic detergents, such as Triton-X 100; anionic detergents, such as SDS; and chaotropic salts, such as

LiCl₂ (Lowy et al 1981). Disulfide reducing agents, such as DTT and β -mercaptoethanol, have been added to preparations to extract starch granule proteins (Lowy et al 1981, Gough et al 1985, Schofield and Greenwell 1987, Seguchi 1986, Seguchi and Yamada 1989).

Since the definition of SGP is arbitrary, comparison of the results of investigators using different techniques of starch isolation, protein extraction, and protein characterization is difficult (Lowy et al 1981, Seguchi and Yamada 1989, Skerritt et al 1990, Sulaiman and Morrison 1990). When used in tandem with electron microscopy, immunological techniques can be used to determine the histochemical location of proteins in cereals and seeds at the subcellular level (Roth 1983, Brewin et al 1987, Parker et al 1990). Stereological techniques allow the objective extrapolation of information from a number of random two-dimensional micrographs to a three-dimensional structure. The quantitative data derived can be subjected to statistical analysis without interjecting author bias (Steer 1991).

The objective of this research on an NaCl-extractable 32-kDa SGP from the durum wheat cultivar Vic was to investigate the in situ location to determine whether the protein is inherently associated with starch granules during endosperm development. To our knowledge, there are no previous reports of starch protein in this molecular weight range and from durum wheats from the United States.

MATERIALS AND METHODS

Starch Granule Protein Extraction

Durum wheat seed (*T. turgidum* L. var durum, cv. Vic) grown in Minot, ND, during 1990, was milled on a Buhler Mill (Buhler-Miag, Minneapolis). The durum flour was slurried with an equal volume of distilled water in a blender at low speed for 30 sec. The slurry was centrifuged at 500 × g for 15 min. The residue sludge layer on top of the white starch layer was removed and discarded. The residue was reslurried and centrifuged. The pellet from the second centrifugation was washed with distilled water. The water-wash was filtered through cheesecloth, and the filtrate was centrifuged at 500 × g for 15 min to recover the starch. The starch was purified by resuspension in distilled water, centrifuged at 500 × g for 15 min, and the prime starch layer was reslurried at least four times. The starch was air-dried under an exhaust hood.

SGP was extracted from the water-washed durum starch with 0.1M NaCl, according to the procedure of Lowy et al (1981). The 0.1M NaCl SGP supernatant was ultrafiltered (Amicon Ultrafiltration stirred cell device 8400 and Reservoir R54, W. R. Grace & Co., Beverly, MA) under 10–15 psi of nitrogen at room temperature. The supernatant was ultrafiltered using a 50-kDa

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molecular weight cut off (MWCO) membrane (Amicon XM 50). The resulting filtrate was concentrated using a 10-kDa MWCO membrane (Amicon PM 10). The 10-kDa MWCO retentate was desalted with four volumes of distilled H₂O and either used directly or lyophilized. The protein in the 10-kDa MWCO retentate is hereafter referred to as NaCl-SGP.

SDS-PAGE

NaCl-SGP from durum was characterized by SDS-PAGE according to the procedure of Laemmli (1970). Low-range molecular weight standards and prestained molecular weight standards used for gels for western blotting were obtained from Bio-Rad Laboratories (Hercules, CA). The SDS-PAGE gels not used for western blotting or antigen preparation were immediately stained for protein with 0.1% (w/v) Coomassie Blue R250 solution (Steck et al 1980) or silver stained (Bio-Rad silver stain plus kit).

Polyclonal Antibody Preparation

Antigen was prepared by cutting strips from several gels. The strips were stained as stated and aligned with the unstained gel. A 32-kDa SGP band was located and excised from the unstained gel. The cut polyacrylamide gel strips containing the protein band were lyophilized, ground, suspended with Freund's Complete Adjuvant (Sigma Chemical Co., St. Louis, MO) and injected into adult female rabbits. Control serum was obtained from the rabbits before immunization.

Isoelectric Focusing

A freeze-dried NaCl-SGP sample was separated in 5% polyacrylamide gels containing 0.6% ampholine pH 3–10, and 1.2% of both ampholine pH 5–7 and pH 4–6. The solutions in the electrodes were anode 20 mM H₃PO₄ and cathode 40 mM NaOH.

Gel-Filtration Chromatography

A freeze-dried NaCl-SGP pooled sample from the ultrafiltrate preparation was dissolved in 1 ml of 50 mM Tris (pH 8.8) containing 100 mM NaCl, filtered through a 0.45- μ screen and loaded into a FLPC (Pharmacia LKB Biotechnology, Piscataway, NY) equipped with a gel-filtration chromatography column (Superdex 75 HR 10/30, 2.6 X 60 cm, Pharmacia LKB Biotechnology). Mobile phase (50 mM Tris, pH 8.8, containing 100 mM NaCl) flow rate was 1 ml/min. Fractions of 1 ml per tube were collected, dialyzed 16 hr at 4°C against 50 mM Tris buffer (pH 8.8), and freeze-dried. Dried samples were reconstituted with electrophoresis sample buffer and tested for protein content with the Biuret method. The column was calibrated using apoprotein, cytochrome C, carbonic anhydrase, bovine serum albumin, and blue dextran markers from Sigma (6.5, 12.4, 29.66, and 2,000 kDa, respectively).

Immunoblotting

SDS-PAGE gels were subjected to western blotting. NaCl-SGP was transferred from the polyacrylamide gel to a 0.45- μ m nitrocellulose membrane using the method of Burnette (1984) with a Bio-Rad Transblot apparatus. The nitrocellulose membrane was stained for protein with Ponceau S.

Antibody production and polypeptide specificity were assessed by probing nitrocellulose sheets from western blotting with pre-immune and polyclonal antisera to 32-kDa SGP as Hawkes (1986) described. Blocking solution contained 3% nonfat dry milk and 0.03% Tween in phosphate-buffered saline (PBS). Primary incubation with preimmune sera or polyclonal antisera to 32-kDa SGP (1:150, v/v) was followed with biotinylated goat anti-rabbit IgG (1:150, v/v) (Sigma). Antibody was detected with an ExtrAvidin alkaline phosphatase staining kit, according to manufacturer's directions (Sigma).

Transmission Electron Microscopy

Wheat (*T. turgidum* cv. Vic) was grown in a greenhouse; individual heads were tagged at anthesis; and kernels were harvested at 7, 9, 15, 31, and 37 days after flowering (DAF). Kernels were dissected in 0.1 M Millonig's buffer (pH 7.3), and the endosperm was cut into 1-mm pieces. Endosperm pieces were prepared for

transmission electron microscopy as Bechtel and Pomeranz (1978) described. Samples were fixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, stained with uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin. Half of the samples received an additional dehydration step with acidified 2,2-dimethoxypropane before embedding.

Ultrathin sections (600–800 Å) were cut with a diamond knife, mounted on nickel grids for immediate immunocytochemistry, as Smart and Amrhein (1987) described. Blocking solution contained 3% nonfat dry milk and 0.05% Tween-20 in PBS. Grids were incubated on preimmune sera or polyclonal antisera to 32-kDa SGP (1:500, v/v), followed with 10-nm gold-conjugated, goat/anti-rabbit immunoglobulin G (1:50, v/v) (Sigma). Sections used for stereological examination were not stained with lead citrate to avoid staining artifacts. Durum endosperm sections were

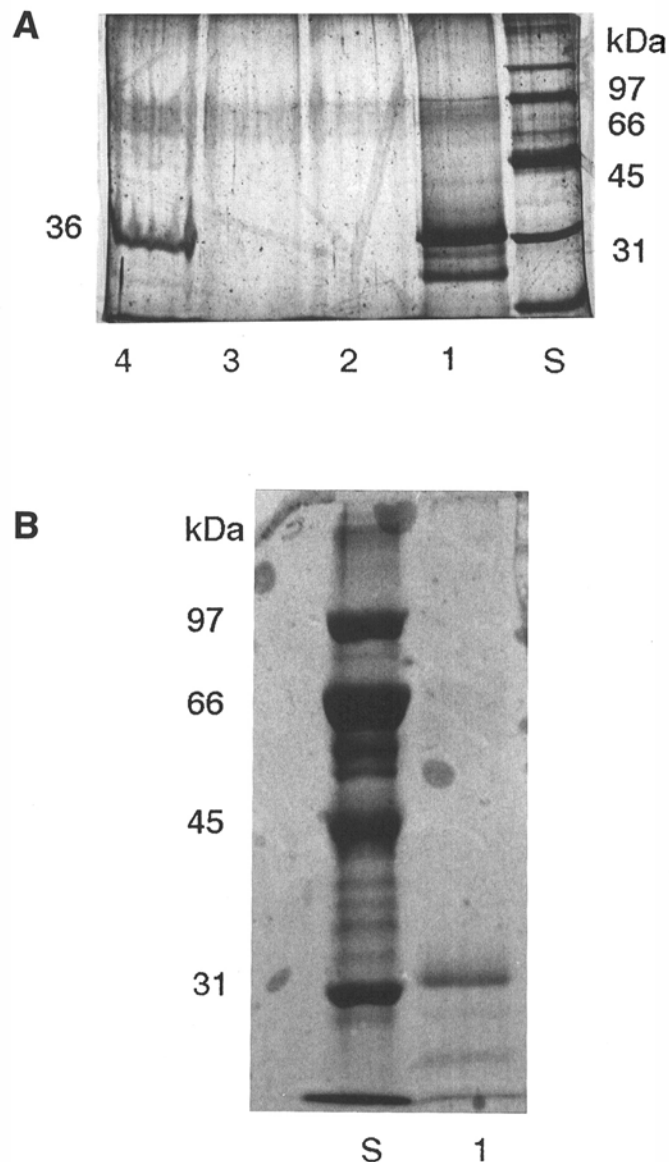


Fig. 1. A, Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of starch granule protein extracted with 0.1 M NaCl (lane 1) from durum wheat and gel-filtration chromatography peaks 1, 2, and 3 (lanes 2, 3, and 4, respectively). Molecular weight standards (S) are phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. The 36-kDa band was selected for use as an antigen. B, 32-kDa polypeptide eluted from a section of gel and re-electrophoresed.

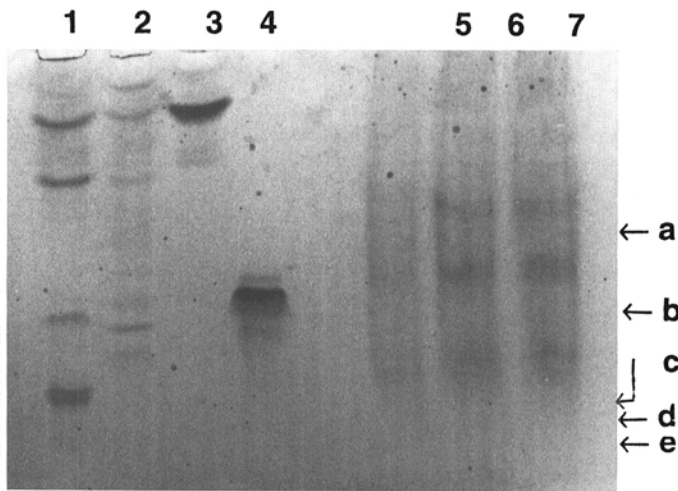


Fig. 2. Polypeptide bands (a–e) revealed by isoelectric focusing pattern of starch granule protein extracted from durum wheat with 0.1M NaCl. Extract 12, 7, and 5 μ l (lanes 1, 2, and 3), standards (lane 4–7): pH 4.6–8.8 (lane 4), myoglobin (lane 5), carbonic anhydrase II from bovine erythrocytes (lane 6), and β -lactoglobulin A (lane 7).

examined with a transmission electron microscope (JEM-100S, JEOL, Tokyo, Japan) at 6,000 \times magnification with an accelerating voltage of 60 kV.

A total of 42 micrographs were probed for three profile areas: starch, cell wall, and cytoplasm, using a point-counting grid as Weibel and Bolender (1973) described. The ratio of gold label particles per 1 μ m² area for each of the three profiles was analyzed with one-way analysis of variance.

RESULTS AND DISCUSSION

Starch Granule Protein Extraction

Durum wheat seed stock (*T. turgidum* L. var durum cv. Vic) was the most widely grown cultivar in North Dakota in the early to mid-1980's and is used as a standard for quality for new varieties released. Sodium chloride-extractable SGP demonstrated genetic variability (Greenwell and Schofield 1986, Morris et al 1992). From 46.7 kg of tempered durum, 25.0 kg of flour was obtained (53% extraction) after milling. SGP has been investigated using starch granules isolated from the flours of winter wheat and durum wheat (Lowy et al 1981), western white wheat (Seguchi and Yamada 1989), British and Australian soft wheats (Skerritt et al 1990).

The recovery of starch, 47% of durum flour, was lower than other published recovery rates. Berry et al (1971) had a starch recovery rate from durum semolina of 54%. Adkins and Greenwood (1966) had a starch recovery rate of 59% from steeped and ground bread wheat. However, for this investigation starch purity, not recovery, was critical.

The protein content of the water-washed and NaCl-extracted starch were 3.6 and 3.4 mg per gram of dried starch, respectively, as determined by the micro-Kjeldahl method (% N \times 5.7). Wheat starch has been estimated to contain 0.2% protein, of which 10% can be extracted with dilute NaCl (Schofield and Greenwell 1987). Other methods of protein determination, such as amino acid analysis, could provide quantitative determination of SGP.

SDS-PAGE

The SDS-PAGE gel of the NaCl-SGP extract depicts several polypeptide bands (Fig. 1a, lane 1). The 32-kDa band was selected as an antigen. The protein extracted from the gel strips was recovered by resuspending the strips in SDS-PAGE sample buffer and reloaded in a SDS-PAGE. One major band of \sim 32 kDa protein standard plus two minor bands between 30 and 21 kDa were observed (Fig. 1b).

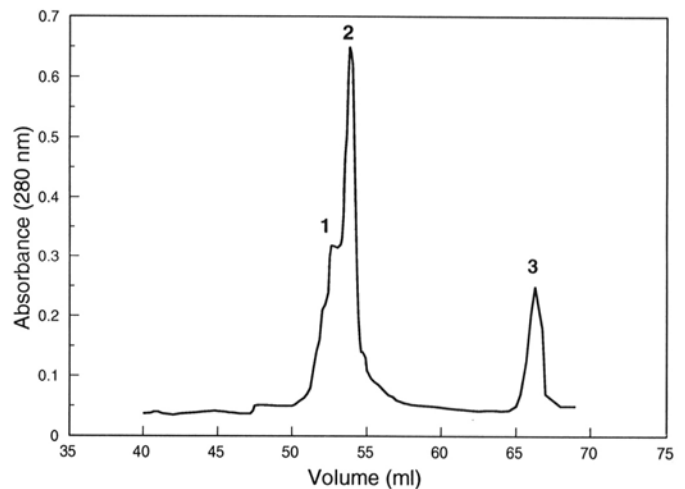


Fig. 3. Gel-filtration chromatography profile of starch granule protein extracted from durum wheat with 0.1M NaCl.

For this investigation, electrophoresis was used as a preparative tool in antigen separation and identification. Comparative analytical electrophoresis of SGP extracted with different solvents and conditions would assist in the optimization and standardization of SGP extraction and classification. However, before characterization of SGP can be accomplished, a standard method of starch isolation and a measurable definition of starch purity must be established.

Isoelectric focusing of the NaCl-SGP preparation revealed the presence of at least five bands. Two main broad bands at \sim pH 5–6 and at least three minor bands with a pI of \sim pH 4–5 (Fig. 2). The two main bands were recognized by the rabbit polyclonal antibodies (data not shown).

Gel-Filtration Chromatography

A chromatogram profile of the NaCl-SGP sample from the Superdex 75 HR 10/30 column is shown in Figure 3. Three major peaks were obtained; peak 3 contained the polypeptide band recognized by the rabbit's polyclonal antibodies (Fig. 3 and 4AB). An interaction of the NaCl-SGP with the packing material was observed causing a delay in the elution of the proteins from the column. The estimated molecular weight of gel filtration was lower than that of the SDS-PAGE.

Protein stain of SDS gel electrophoresis of the freeze-dried material collected from the three peaks revealed one major and several minor polypeptide bands in peak 3 (Fig. 4B). Higher molecular weight polypeptides were observed in peaks 1 and 2 that slightly crossreacted with the rabbit's polyclonal antibodies (Fig. 4b).

Immunoanalysis

A western blot of 0.1M NaCl-SGP extract separated by SDS-PAGE is shown in Figure 4. The nitrocellulose was probed with preimmune and polyclonal antisera to 32-kDa SGP to determine whether indigenous antibodies were present in the sera. Antigen-antibody reaction was detected in the western blot probed with polyclonal antisera to 32-kDa SGP, but not preimmune sera (Fig. 4a). The western blot probed with antisera to 32 kDa NaCl-SGP show slight cross-reactivity with a higher molecular weight NaCl-SGP (\sim 60 kDa) in Fig 4b.

Immunocytochemistry

Microscopic examination of sections of endosperm tissue older than seven DAF were characterized by tears and holes primarily associated with starch granules. Either entire starch granules or portions of starch granules were lost because there is no fixative reagent for starch so far. Therefore, seven DAF endosperm was selected for stereological analysis. Earlier in endosperm development, starch granules are smaller in size. The volume of starch

to cytoplasm is smaller than in mature endosperm. This may have had a stabilizing effect on the starch granules and prevented starch from washing out of the section during microtoming as was noted in the older tissue.

Background labeling of the sections was decreased by increasing the sera dilution, and especially by adding the detergent, Tween, to the blocking solution until sections probed with preimmune sera showed essentially no labeling of the tissue. Sections probed with polyclonal antisera to 32-kDa SGP contained extensive label associated with the starch granules (Fig. 5A). Generalized intragranule labeling of starch occurred throughout the granule structure. The generalized label pattern of the starch granules suggests that the polypeptide may be an enzyme of starch metabolism. The developing granule may entomb the protein. Schofield and Greenwell (1987) reported striated intragranule labeling in starch granules probed with antibodies against a 59-kDa SDS-SGP. Labeling was also noted with the cell wall and cytoplasm, but not for specific cellular organelles other than starch granules.

The amyloplast membrane and the interstitial space between the starch granule and the membrane were not specifically labeled (Fig. 5B). Membrane-bound translocators may assist in the transport of substrates across the plastid membrane required for starch synthesis (Keeling et al 1988). However, the assumption that the 32-kDa NaCl-SGP was a membrane-bound protein of amyloplast membrane origin was not supported by this investigation. However, the visual lack of labeling of amyloplast membrane probed with polyclonal antisera to 32-kDa NaCl-SGP does not negate the possibility that the other SGP may be of amyloplast-membrane origin. The metabolic similarities between amyloplast and chloroplasts raises the possibility of probing leaf tissue with the polyclonal antisera to 32-kDa NaCl-SGP to determine whether chloroplast starch would label in a manner similar to that of amyloplast starch.

Stereological analysis demonstrated that, of the total micrographed label, 95% was associated with the starch, compared to 4% with the cytoplasm and 1% with the cell wall (Table I). Profile ratios were subjected to analysis by nonparametric one-way analysis of variance (Table II). Labeling among all profile groups differed significantly at $P < 0.01$.

The limited labeling of the cytoplasm profile suggested that the 32-kDa NaCl-SGP was not an artifact of starch isolation. If this were the case, labeling of the cytoplasm would have been expected. Instead, the lack of cytoplasm labeling suggested that the protein was inherently associated with the starch granule. However, the limited cytoplasm labeling might be a consequence of limited synthesis and deposition of the antigen in the cytoplasm of wheat endosperm at seven DAF.

The generalized label pattern of the starch granules suggests that the polypeptide may be an enzyme of starch metabolism. The developing granule may entomb the protein. The protein reported here did not show the described striated intragranule type of labeling as did the starch granules probed with antibodies against a 59-kDa SDS-SGP reported by Schofield and Greenwell (1987).

CONCLUSIONS

Polyclonal antibodies raised against a polypeptide band of ~32-kDa 0.1M NaCl-SGP significantly labeled starch granules in developing endosperm. General intragranule labeling of the starch suggested the protein is inherently associated with starch and is not localized only on the granule surface. This research provides justification for the investigation of SGP as a class of wheat protein.

Future research concerning the optimization and standardization of SGP extraction, quantitation, and identification will facilitate the comparison of SGP in different wheat varieties, classes, and species, and in different cereals. The elucidation of the effects of SGP on endosperm development, starch metabolism, and starch physical and chemical properties will lead to a better understanding of starch functionality in food systems.

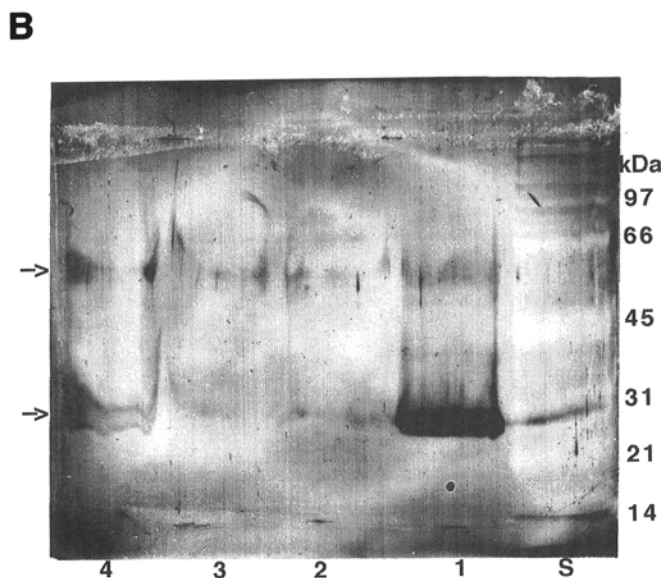
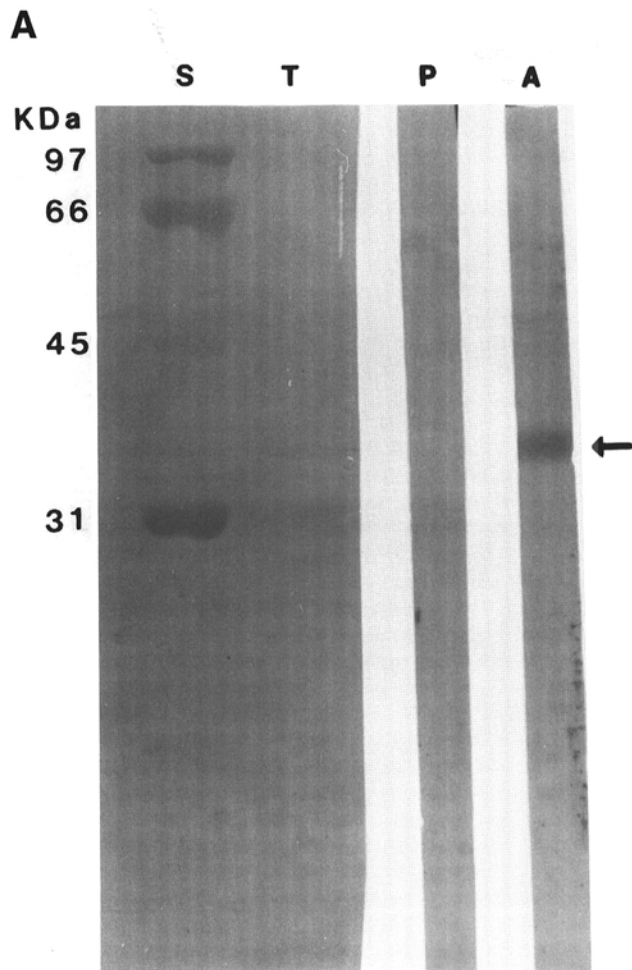


Fig. 4. Western blot of starch granule protein extracted from durum wheat with 0.1M NaCl by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Transferred molecular weight standards (S) are phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 31 kDa. **A**, Transferred extract (T) was stained with Ponceau S. Nitrocellulose strips probed with preimmune sera (P) and polyclonal antisera to 32-kDa NaCl-SGP (A) were detected with biotinylated goat/antirabbit IgG and ExtrAvidin alkaline phosphatase. **B**, Transferred gel-filtration chromatography profile loaded to the column (lane 1) and peaks 1, 2, and 3 (lanes 2, 3, and 4, respectively). Antigen location noted with arrow.

ACKNOWLEDGMENTS

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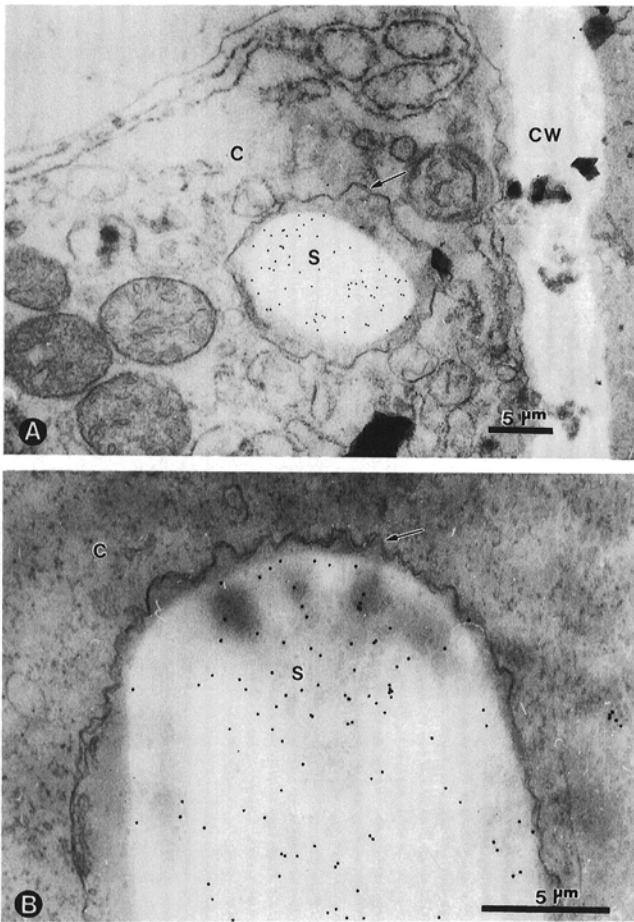


Fig. 5. Transmission electron micrographs of durum endosperm at seven days after flowering probed with polyclonal antisera to 32-kDa NaCl-extracted starch granule protein and detected with goat/antirabbit IgG gold conjugate. **A**, Endosperm profiles were designated cytoplasm (C), ch (S), and cell wall (CW). **B**, Starch granule (S) and cytoplasm (C) separated by amyloplast membrane bilayer indicated by arrow. Bar = 5 µm.

TABLE I
Cytoplasm, Starch, and Cell Wall Profiles of Durum Wheat Endosperm Probed with Polyclonal Antisera to 32-kDa NaCl-SGP^{a,b}

Profile	Area (µm ²)	% Total	Number of Gold Particles	% Total
Starch	4,390	10.8	32,144	94.5
Cytoplasm	35,323	87.0	1,418	4.2
Cell Wall	919	2.2	455	1.3

^aProfiles defined: cytoplasm (exclusive of starch and cell wall), starch, and cell wall.

^bNaCl-SGP = sodium chloride extracted starch granule protein.

TABLE II
Labeling Ratio of Gold Label Particles to µm² Area of Durum Wheat Endosperm Probed with Polyclonal Antisera to 32-kDa NaCl-SGP^{a,b}

Profile	Number of Gold Particles per µm ² Area			
	Mean ^c	SD ^d	Minimum	Maximum
Starch	8.92	6.69	0.00	36.67
Cell wall	0.53	0.54	0.00	02.24
Cytoplasm	0.04	0.04	0.00	00.24

^aProfiles defined: cytoplasm (exclusive of starch and cell wall), starch, and cell wall.

^bNaCl-SGP = Sodium chloride extracted starch granule protein.

^cMeans differed statistically ($P < 0.01$) as determined by nonparametric one-way analysis of variance.

^dStandard deviation.

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