

Heat-Induced Fragmentation of the Maize Waxy Protein During Protein Extraction from Starch Granules

Helen He Mu,¹ Chen Mu-Forster,² Monica Bohonko,¹ and Bruce P. Wasserman^{1,3}

ABSTRACT

Cereal Chem. 75(4):480-483

The starch granule of maize contains a characteristic set of tightly bound polypeptides. Granule-associated polypeptides are typically extracted from starch granules by heating starch granule suspensions at 90–100°C in a detergent such as SDS. Solubilized proteins are recovered by centrifugation and analyzed by gel electrophoresis. Previously identified tightly bound granule intrinsic proteins consist of the 85-kDa starch-branching enzyme IIb, the 76-kDa starch synthase I, and the 60-kDa waxy (Wx) protein, also known as granule-bound starch synthase I. However, SDS extracts from starch granules of maize also contain a cluster of proteins ranging in mass between 47 and 32 kDa. In this study, we analyzed this group of granule-associated proteins and found that each was recognized by the Wx antibody.

A 15 amino acid N-terminal sequence from the 47-kDa polypeptide was identical to the predicted N-terminus of the Wx protein. Further analysis revealed that each immunoreactive polypeptide between 47 and 32 kDa was a heat-induced fragmentation product of the Wx protein. Conditions for the extraction of granule proteins were evaluated. Our results demonstrate that granule proteins are effectively released by mild extraction (10-min incubation at 72°C). Relative to the Wx protein, starch synthase I and starch branching enzyme IIb were less susceptible to thermal fragmentation. These results demonstrate that the 85-, 76-, and 60-kDa polypeptides are authentic granule-intrinsic proteins, and that the majority of polypeptides between 47 and 32 kDa are artifacts of high-temperature granule extraction procedures.

Starch granules from maize endosperm are composed of ≈98–99% starch, 0.3–0.6% protein, and 0.1–0.6% lipid. Granule-associated proteins are important physiologically because of their functions in granule formation. These proteins are also important for industrial application because they influence starch purity and functionality (Appelqvist and Debet 1997).

Starch granule-associated proteins are classified as either surface proteins or intrinsic proteins. Surface proteins are defined as polypeptides at the granule surface, and granule-intrinsic proteins are embedded within the starch matrix. Operationally, surface proteins are defined as polypeptides that are susceptible to proteolytic degradation or to removal by washing with 2% SDS at subgelatinization temperatures (Denyer et al 1993, Rahman et al 1995, Mu-Forster et al 1996). The surface proteins in maize are predominantly zeins, which appear to result from the adhesion of protein bodies after amyloplast envelope rupture (Mu-Forster and Wasserman 1998). The surface-localized zeins are readily removed using the protease thermolysin. Because intrinsic proteins are embedded within the granules, they only become susceptible to proteolysis upon disruption of the starch matrix during gelatinization.

Maize (Mu-Forster et al 1996) and wheat (Rahman et al 1995) each contain a characteristic group of granule-associated polypeptides. In maize, three common granule-associated polypeptides, each of which is involved in starch biosynthesis, have been observed. These are the 60-kDa waxy (Wx) proteins, also known as granule-bound starch synthase I (GBSSI), the 76-kDa bound form of starch synthase I (SSI), and the 85-kDa bound form of starch branching enzyme IIb (SBEIIb). While the Wx protein is exclusively associated with the starch matrix, SSI and SBEIIb also exist as soluble forms in the amyloplast stroma (Mu-Forster et al 1996).

The analysis of granule-associated proteins has become a standard analytical tool used to characterize Wx mutants of various starch-bearing crops (Echt and Schwartz 1981, Shure et al 1983, Nakamura et al 1995). Granule-intrinsic proteins are typically isolated by boiling starch granules in SDS, which is added to solubilize starch-associated proteins (Denyer et al 1993, Rahman et al 1995, Mu-Forster et al 1996). Solubilized proteins are recovered by centrifu-

gation and analyzed by gel electrophoresis. The length of the boiling step used to extract the Wx protein has varied, with durations of 1–2 min (Echt and Schwartz 1981, Shure et al 1983, Nakamura et al 1995), 5 min (Echt and Schwartz 1981), and 10 min (Mu et al 1994, Mu-Forster et al 1996) reported.

To gain further insight into starch granule structure and function, our laboratory has been interested in the characterization of granule-associated proteins from maize endosperm. In addition to the Wx protein, SSI, and SBEIIb, our laboratory (Mu-Forster et al 1996) and others (Yamamori and Endo 1996) have routinely observed three major low molecular weight intrinsic polypeptides of 47, 33, and 32 kDa. We sought to identify these polypeptides to determine whether they have a function in starch biosynthesis. Our approach was to purify each of these polypeptides and to identify them by amino acid sequence analysis followed by cloning and sequence analysis of each corresponding full-length cDNA. During the course of this study, we observed that the N-terminus of the 47-kDa polypeptide exhibited complete identity to the N-terminus of the Wx protein. This led us to investigate the origin of the granule associated 47-kDa polypeptide as well as the closely migrating polypeptide doublet band of 33–32 kDa. We hypothesized that these low molecular weight polypeptides were induced by the heating step used to extract granule proteins. In this study, we demonstrated that the granule-associated polypeptides of 47, 33, and 32 kDa are thermal fragmentation products of the Wx protein. Conditions leading to the thermal fragmentation of granule-associated proteins are described.

MATERIALS AND METHODS

Kernels of maize (*Zea mays*, inbred line B73) were collected from ears of greenhouse-grown plants at 18–21 days after pollination, frozen in liquid N₂, and stored at –80°C. Antibodies to SSI, SBEIIb, and Wx protein were previously described (Mu et al 1994, Mu-Forster et al 1996, Mu-Forster and Wasserman 1998). Thermolysin (protease type X from *Bacillus thermoproteolyticus*; EC 3.4.24.4) was obtained from Sigma Chemical Co., St. Louis, MO.

Starch granules were isolated by low-speed centrifugation as previously described (Mu et al 1994, Mu-Forster et al 1996).

Granule surface polypeptides, principally zeins, were removed by digestion of starch granule suspensions with thermolysin. Thermolysin digestion mixtures contained 50 mg (dwb) of isolated starch granules, 100 μg of thermolysin and 5 mM CaCl₂ in 1 mL volume. Hydrolysis was conducted at 55°C for 4 hr, and reactions were terminated by addition of ethylenediaminetetraacetic acid (EDTA)

¹ Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, 65 Dudley Rd., New Brunswick NJ 08901-8520. Phone: 732/932-9611 ext. 220. Fax: 732/932-6776.

² Present address: 800 N. Lindbergh Blvd. St. Louis, MO 63198.

³ Corresponding author. E-mail: wasserman@aesop.rutgers.edu

to 20 mM (Cline et al 1984, Xu and Chitnis 1995). Starch granules were recovered by centrifugation at $13,000 \times g$ for 5 min. Residual thermolysin was removed by five successive washings with water. Proteins were extracted as described below. Controls contained buffer in place of thermolysin (Mu-Forster and Wasserman 1998).

Granule-associated proteins were recovered by suspending 50 mg (dwb) of starch granules with 1 mL of extraction buffer (60.5 mM Tris/HCl, pH 6.9, supplemented with 3% SDS, 5% β -mercaptoethanol, and 10% glycerol). Granule suspensions were then incubated at either 72°C or boiling, as indicated under each experiment. Based on the results shown in Figs. 1 and 2, recommended extraction conditions for minimization of heat-induced fragmentation are 72°C for 10 min. Extraction mixtures were then cooled to room temperature, and annealed starch was sedimented by centrifugation at $13,000 \times g$ for 15 min. Extracted proteins were analyzed by SDS-PAGE using 12% gels (Porzio and Pearson 1976). Each lane was loaded with 50 μ L of the granule extract. Polypeptides were visualized by immunoblotting or by double-staining with Coomassie blue and silver (Integrated Separation Systems, Hyde Park, MA).

For immunoblotting (Harlow and Lane 1988), proteins were electrophoretically transferred from SDS gels to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in 0.1% SDS, 100 mM glycine, and 10 mM Tris/HCl, pH 8.0 (Towbin et al 1979, Harlow and Lane 1988). The membranes were soaked for at least 1 hr in 0.15M NaCl and 10 mM Tris/HCl, pH 7.4, supplemented with 0.1% Tween-20 and 1% bovine serum antibody (TBST). The membranes were then washed once with TBST for 15 min and twice for 5 min. Antiserum was then added at 1:100,000 dilution, and membranes were incubated for 1 hr with gentle shaking. After three more washes with TBST, blots were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) at 1:5,000 dilution for 1 hr. Blots were then washed three times with TBST and visualized using enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL).

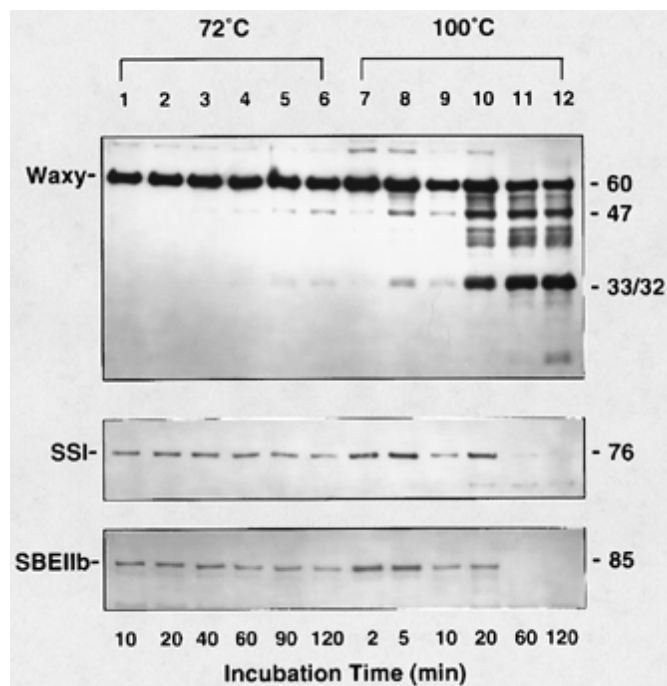


Fig. 1. Starch granule suspensions extracted at 72°C (lanes 1–6) or 100°C (lanes 7–12). Solubilized proteins separated by SDS-PAGE. Durations of each extraction are indicated. Immunoblots were probed using antibodies recognizing the Waxy protein (1:200,000 dilution), SSI (1:200,000 dilution), and SBEIIb (1:100,000 dilution) as indicated.

RESULTS

Analysis of Starch Granule Proteins

Granule-intrinsic proteins are typically released from the starch matrix by gelatinization of starch granules in 3% SDS. In our previous studies (Mu-Forster et al 1996, Mu-Forster and Wasserman 1998) protein extraction was routinely performed by heating the starch granules by boiling for 10 min. Granule extracts from maize starch prepared in this manner yield a characteristic pattern of polypeptides (Mu-Forster et al 1996). The three prominent polypeptides at 85, 76, and 60 kDa represent SBEIIb, SSI, and Wx protein (Fig. 1). Granule extracts from maize prepared by us (Mu et al 1994, Mu-Forster et al 1996) and other laboratories (Yamamori and Endo 1996) have shown that maize granule extracts also commonly yield strongly staining bands at 47, 33, and 32 kDa. The polypeptides running <30 kDa are zeins that are located at the starch granule surface (Mu-Forster and Wasserman 1998). It has been demonstrated that these hydrophobic surface proteins are readily removed from the starch granule surface by treatment with thermolysin at subgelatinization temperatures.

Our approach for establishing the identity of additional granule-intrinsic proteins was to electropurify selected polypeptides from excised gel bands and obtain N-terminal amino acid sequence information. Due to its relative abundance, the 47-kDa polypeptide was the first protein analyzed. The 15 amino acid sequence (Ala-Ser-Ala-Gly-Met-Asn-Val-Val-Phe-Val-Gly-Ala-Glu-Met-Ala) was obtained. This was unexpected because analysis of this sequence revealed

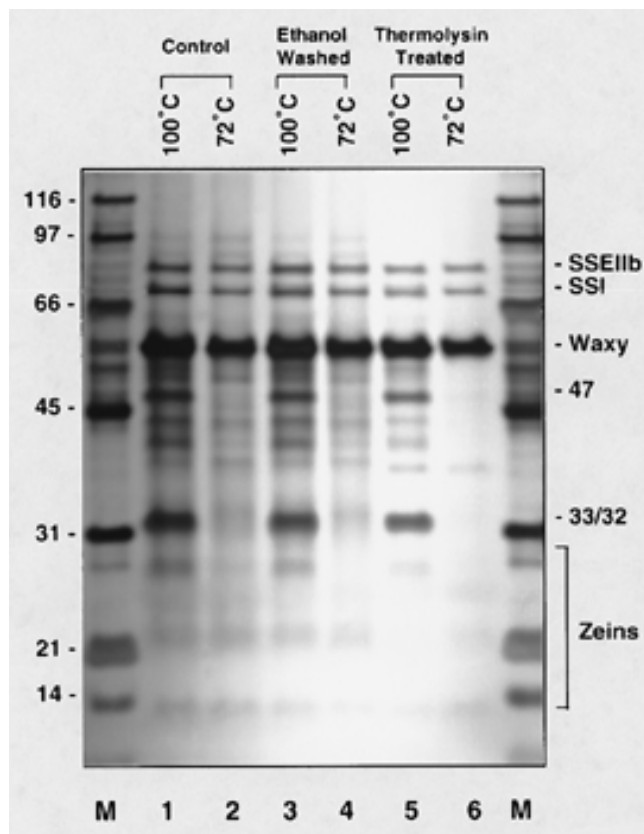


Fig. 2. Starch granules untreated (lanes 1 and 2), ethanol-washed (lanes 3 and 4), or thermolysin-digested (lanes 5 and 6). Starch granule suspensions extracted at 100°C (lanes 1, 3, and 5) or 72°C (lanes 2, 4, and 6). Three sets of samples were incubated in parallel for 4 hr at 55°C. Two sets were incubated in buffer alone and the third set contained thermolysin. After this incubation, one of the buffer-incubated sets and the thermolysin-treated set (lanes 5 and 6) were subjected to aqueous washing. The second buffer-incubated set (lanes 3 and 4) was washed three times with 70% ethanol. After these treatments, solubilized proteins were separated by SDS-PAGE, and stained with Coomassie Blue and silver. M = molecular mass markers.

complete sequence identity with the N-terminus of the Wx protein (Klosgen et al 1986). In addition, we noted earlier that the 47-kDa polypeptide, as well as those at 33–32 kDa, were clearly recognized by the Wx antibody.

Thermal Fragmentation of Starch Granule Proteins

The finding of a common N-terminus in both the 47-kDa polypeptide and the Wx protein prompted us to investigate whether the 47-kDa polypeptide was induced by heating during the extraction step. To further investigate the relationship between the 47-kDa fragment and the Wx protein, immunoblots using antibodies to the Wx protein were obtained (Fig. 1). The 47-kDa polypeptide was clearly recognized by the Wx antibody. In addition, polypeptides of 50, 49, 42, 39, 37, 33, and 32 kDa were clearly detected when starch granules were extracted by boiling for 5 min or longer.

Because starch granule extractions are routinely conducted at high temperature, it seemed possible that the polypeptides recognized by the Wx antibody were thermal fragmentation products. Therefore, a time course experiment in which the temperature and duration of extraction was varied was conducted. The extraction temperature of 72°C was chosen because this is slightly above the gelatinization temperature of common maize starch. The time course study confirmed that the Wx protein is indeed highly susceptible to time-dependent thermal fragmentation. At 72°C, the effect of heating became evident after 40 min (Fig 1, top panel, lane 3). The 47-, 33-, and 32-kDa fragments were the first new fragments to appear. At 100°C, fragmentation of the Wx protein was detected during incubations as brief as 2 min. By 20 min, significant fragmentation was clearly detected, with at least seven fragmentation products observed. At 100°C, the appearance of these fragmentation products correlated with the loss of the 60-kDa parent Wx protein (Fig. 1, top panel, lanes 10–12).

The susceptibility of SSI and SBEIIb to thermal fragmentation was also investigated. At 72°C, the 76-kDa SSI was only minimally degraded. However, evidence of fragmentation became apparent after 1.5 hr. When extracts were prepared by boiling, complete disappearance of the 76-kDa polypeptide occurred between 20 min and 1 hr (Fig. 1, middle panel, lanes 10 and 11). Moreover, a faint putative breakdown product of ≈68 kDa was formed after 20 min.

SBEIIb exhibited greater stability against thermal fragmentation at 72°C than either SSI or Wx protein (Fig. 1, bottom panel). After 2 hr at 72°C, no obvious loss of SBEIIb was observed. However, at 100°C, it behaved similarly to SSI with regard to its tendency to form thermal fragmentation products. These results indicate that of the three major starch granule-associated polypeptides studied, the Wx protein is the most prone to thermal fragmentation.

Granule-Intrinsic Polypeptide Composition

Based on the above findings, we hypothesized that combined elimination of both granule surface proteins and heat-induced fragmentation products would yield a starch consisting of only three primary intrinsic polypeptides—SBEIIb, SSI, and Wx protein. Therefore, laboratory-isolated starch was subjected to thermolysin digestion to remove surface proteins (Mu-Forster and Wasserman 1998) and proteins were then extracted at 72°C (Fig. 2).

Figure 2 shows protein extracts from either untreated starch granules (lanes 1 and 2), ethanol-washed starch granules (lanes 3 and 4), or thermolysin-digested starch granules (lanes 5 and 6). For each sample, granule extracts were prepared by heating at either 100 or 72°C for 10 min.

Figure 2 (lanes 5 and 6) demonstrates that thermolysin digestion effectively depleted the bulk of the low molecular weight surface polypeptides. In extracts prepared at 100°C, thermal fragmentation products are clearly evident. However, extraction at 72°C prevented the formation of these products and resulted in a solubilized protein fraction consisting predominantly of just three polypeptides: SBEIIb, SSI, and Wx protein. Thermolysin treatment was considerably more effective than ethanol washing (Fig. 2, lanes 5 and 6 vs. lanes 3

and 4). Moreover, apparent yields of SBEIIb, SSI, and Wx protein were not significantly reduced by lowering extraction temperatures from 100 to 72°C. Thus, thermolysin treatment in conjunction with a 10-min extraction at 72°C yielded an extract composed principally of SBEIIb, SSI, and Wx protein. Consequently, these three polypeptides represent the primary intrinsic polypeptides of starch granules from maize endosperm.

DISCUSSION

Starch granule-bound proteins have been of great interest due to their importance in the starch synthesis (Martin and Smith 1995, Wasserman et al 1995, Smith et al 1997) and starch granule functionality (Appelqvist and Debet 1997). It was previously shown that maize starch granules contain a broad range of tightly associated proteins, with masses ranging between 85 and 10 kDa (Mu et al 1994, Mu-Forster et al 1996, Yamamori and Endo 1996). However, it had not been possible to distinguish surface polypeptides from intrinsic polypeptides. Furthermore, the formation of Wx protein fragmentation products had been observed (Rahman et al 1995), but the origin of these products was unknown. The need to classify these granule-associated proteins prompted us to investigate the identity of the granule-intrinsic proteins of maize and the nature of their association with the starch granule.

Before this study, the identities of only three prominent granule-intrinsic proteins, SBEIIb, SSI and Wx, were known (Mu-Forster et al 1996). In addition, the identity of the surface-associated polypeptides between 30 and 10 kDa as zeins had been established (Mu-Forster and Wasserman 1998). However, the identity of the tightly associated granule polypeptides between 50 and 30 kDa remained to be explored. We therefore adopted a multistep approach to investigate the origin and identity of these proteins. First, thermolysin treatment proved essential for effective removal of the surface-localized polypeptides. Second, the importance of minimizing granule extraction temperatures was taken into consideration. Accordingly, by demonstrating that the 47-, 33-, and 32-kDa proteins were thermal breakdown products of the 60-kDa Wx protein, this study provided a strategy for eliminating artifacts during granule extraction and for pinpointing the authentic starch granule-intrinsic proteins.

Of the three predominant granule-intrinsic polypeptides from maize, the Wx protein was most prone to thermal fragmentation. Relative to SSI and SBEIIb, why is Wx protein more susceptible? One possible mechanism for thermal fragmentation is deamidation of glutamyl and asparagyl residues (Scotchler and Robinson 1974). It is noted that a 20°C increase in temperature can result in a fivefold reduction of polypeptide half-life due to deamidation reactions (Scotchler and Robinson 1974). The 47-kDa fragment did not appear until 40 min during extraction at 72°C but was evident after 2 min at 100°C, which is generally consistent with this notion.

A second explanation could involve differential binding affinities of the three polypeptides with starch granules. The Wx proteins are entirely granule-bound, while 76-kDa SSI and 85-kDa SBEIIb are distributed between soluble fractions and the starch granule (Mu-Forster et al 1996). Calculations show that ≈90% of total endosperm SSI is granule-bound, whereas 50% of total endosperm SBEIIb is granule-bound (Mu-Forster et al 1996). It could be speculated that maize Wx protein has more starch-binding domains and a greater binding affinity for starch than either SBEIIb or SSI proteins. If the Wx protein binds to the starch at multiple domains, granule expansion during severe heating could place added physical stress on this polypeptide during granule gelatinization, leading to fragmentation at multiple sites. It will, therefore, be important to determine whether the mechanism by which the Wx protein interacts with the starch matrix is unique relative to SBEIIb and SSI. Identification of the starch-binding domains of each of the three granule-associated polypeptides should provide the means to better understand binding interactions between starches and proteins and the effects of such interactions on starch functionality.

ACKNOWLEDGMENTS

This research was supported in part by the USDA National Research Initiative (91-37304-6579 and 95-02531), ExSeed Genetics and the New Jersey Agricultural Experiment Station with State and Hatch Act Funds.

LITERATURE CITED

- Appelqvist, I. A. M., and Debet, M. R. M. 1997. Starch-biopolymer interactions—A review. *Food Rev. Int.* 13:163-224.
- Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. 1984. Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. *Plant Physiol.* 75:675-678.
- Denyer, K., Sidebottom, C., Hylton, C. M., and Smith, A. M. 1993. Soluble isoforms of starch synthase and starch-branching enzyme also occur within starch granules in developing pea embryos. *Plant J.* 4:191-198.
- Echt, S., and Schwartz, D. 1981. Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. *Genetics* 99:275-284.
- Harlow, E., and Lane, D. 1988. *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory: New York.
- Klosgen, R. B., Gierl, A., Schwartz-Sommer, Z., and Saedler, H. 1986. Molecular analysis of the waxy locus of maize. *Mol. Gen. Genet.* 203:237-244.
- Martin, C., and Smith, A. M. 1995. Starch biosynthesis. *Plant Cell* 7:971-985.
- Mu, C., Harn, C., Ko, Y. T., Singletary, G. W., Keeling, P. L., and Wasserman, B. P. 1994. Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv73) endosperm. *Plant J.* 6:151-159.
- Mu-Forster, C., Huang, R., Powers, J. R., Harriman, R. W., Knight, M., Singletary, G. W., Keeling, P. L., and Wasserman, B. P. 1996. Physical association of starch biosynthetic enzymes with starch granules of maize endosperm. Granule-associated forms of starch synthase I and starch branching enzyme II. *Plant Physiol.* 111:821-829.
- Mu-Forster, C., and Wasserman, B. P. 1998. Surface localization of zein storage proteins in starch granules from maize endosperm. Proteolytic removal by thermolysin and in vitro crosslinking of granule-associated polypeptides. *Plant Physiol.* 116:1563-1571.
- Nakamura, T., Yamamori, M., Hirano, H., Hidaka, S., and Nagamine, T. 1995. Production of waxy (amylose free) wheats. *Mol. Gen. Genet.* 248:253-259.
- Porzio, M. A., and Pearson, A. M. 1976. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* 490:27-34.
- Rahman, S., Kosar Hashemi, B., Samuel, M. S., Hill, A., Abbott, D. C., Skeritt, J. H., Preiss, J., Appels, R., and Morell, M. K. 1995. The major proteins of wheat endosperm starch granules. *Aust. J. Plant Physiol.* 22:793-803.
- Scotchler, J. W., and Robinson, A. B. 1974. Deamidation of glutaminy residues: Dependence on pH, temperature and ionic strength. *Anal. Biochem.* 59:319-322.
- Shure, M., Wessler, S., and Fedoroff, N. 1983. Molecular identification and isolation of the waxy locus in maize. *Cell* 35:225-233.
- Smith, A. M., Denyer, K., and Martin, C. 1997. The synthesis of the starch granule. *Ann. Rev. Plant Physiol. Mol. Biol.* 48:65-87.
- Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
- Wasserman, B. P., Harn, C., Mu-Forster, C., and Huang, R. 1995. Progress toward genetically modified starches. *Cereal Foods World* 40:810-817.
- Xu, Q., and Chitnis, P. R. 1995. Organization of photosystem. I. polypeptides. *Plant Physiol.* 108:1067-1075.
- Yamamori, M., and Endo, T. R. 1996. Variation of starch granule proteins and chromosome mapping of their coding genes in common wheat. *Theor. Appl. Gen.* 93:275-281.

[Received December 1, 1997. Accepted March 18, 1998.]