Preparation of small-sized particles from vicilin (vegetal protein from Pisum sativum L.) by coacervation

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Molecular Characterization of Some Amylopectins

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ABSTRACT

Amylopectins from different sources were studied to gain information on their molecular structure. High-performance size-exclusion chromatography was used to assess the profiles of isoamylase-debranched, pullulanase-debranched, β-amylase-hydrolyzed, and α-amylase-hydrolyzed amylopectins. With the two debranching enzymes, two principal fractions were generated. However, amylopectin depolymerized with pullulanase left a higher amount of nondegraded polymer, indicating that the two enzymes follow a different mode of action on amylopectin structure. Profiles obtained after hydrolysis with β- or α-amylase in general showed that shorter chains were generated at higher enzyme concentrations. Static and dynamic light scattering were used to determine solution behavior, molecular weight, gyration radius, and hydrodynamic radius of amylopectins. These parameters suggested substantial structural differences within tested amylopectins.

Amylopectin is one of the largest molecules in nature; it is the principal component in the majority of starches and perhaps the most important in terms of their functional properties (Manners 1989). There are a number of models that describe amylopectin structure; all models suggested were made on the basis of enzymic degradation experiments combined with the analysis of the oligomeric products obtained (Thurn and Burchard 1985). The relationship between amylopectin structure and physicochemical and functional properties is an important factor under consideration in recent years. The debranching enzymes, isoamylase and pullulanase specifically, hydrolyze the α-(1→6)-D-glycosidic interchain linkages but have no action on the major α-(1→4)-D-glycosidic linkages (Ong et al 1994). β-Amylase acts only on the outer chains or on the outer part of the inner chains of amylopectin, leading to 55–58% of maltose and a residual β-limit dextrin. α-Amylase is an endoenzyme that hydrolyzes α-(1→4)-D-glycosidic linkages in a random manner; with amylopectin, α-amylase leaves a residual branched α-limit dextrin with a structure that is dependent on the source of the α-amylase (Duprat et al 1980, Guilbot and Mercier 1985). Thurn and Burchard (1985) postulated that the various structural elements of a macromolecule, such as polydispersity, overall molecular dimensions, hydrodynamic behavior and internal mobility, should exert a marked influence on its properties in solution. For this reason, they became interested in the structure and properties of native, nondegraded amylopectin, which can be determined by light scattering (LS).

There are two types of LS measurements, dynamic and static light-scattering (DLS and SLS, respectively). DLS is based on the scattering of light by diffusing particles. At any instant, the suspended particles will have a particular set of positions within the scattering volume. Particles scatter radiation to the detector, but the relative phases of scattered wavelets differ due to the differing incident phases that they experience at these positions and due to different particle-detector distances (Hallet 1994). In SLS, the intensity of scattered radiation is averaged over a fairly long time (≈2 sec), and this is in most cases long enough to smooth out all internal mobility (Burchard 1992). Measurements at different molar masses reveal an unexpected weak increase of the radius of gyration, which is interpreted by lateral (side-by-side) aggregation of chains. The conclusion could be confirmed by DLS according to which an increase in segment density occurs as the molar mass increases (Burchard 1993). Recent developments of DLS technique allow a determination of the relaxation time distribution over a large time range. If different components of the system have characteristic relaxation times that are not too close, they can be determined individually in one measurement (Ousalem et al 1993).

Recently, high-performance size-exclusion chromatography (HPSEC) has been used to elucidate the profiles of starch components (MacGregor and Morgan 1984, Kobayashi et al 1986). HPSEC is capable of fractionating amylose and amylopectin in starch on the basis of hydrodynamic volume and molecular weight (Wang and White 1994b). Most HPSEC work has been done on the structure of amylose (Takeda et al 1989, Roger and Colonna 1993) and on the chain-length distribution of amylopectin (Hizukuri 1985, 1986; Wang and White 1994a). HPSEC offers the advantage of a quick analysis.

The present study was undertaken to characterize the fine structure of different amylopectins, with and without enzymic treatments, using HPSEC and LS techniques.

MATERIALS AND METHODS

Amylopectin Isolation

Amaranth seeds (*Amaranthus hypochondriacus*) were provided by INIFAP, Chapingo, México. Starch (waxy type) was isolated using the mercuric chloride method. This method is usually performed by dipping a sample in mercuric chloride solution (0.01*M*) for 1 hr to inhibit the α-amylase activity. The sample is then milled in a commercial blender. The starch obtained at this point must be filtered and, if necessary, more mercuric chloride may be added during filtration (Paredes-López and Hernández-López 1992). Waxy and normal corn starches were a gift from Industrializadora de Maíz, S.A. de C.V. (Guadalajara, Jal., México). Amylopectins were isolated from amaranth, waxy, and normal corn starches using the methodology reported by Banks and Greenwood (1967). This technique involves the addition of butanol and thymol; these chemicals have the property of forming amylose and amylopectin complexes. Commercial corn and potato amylopectins were purchased from Sigma Chemical Co. (St. Louis, MO); no information was provided on the type of corn and potato from which these amylopectins were isolated. The purity of each amylopectin was tested using the iodine binding capacity (IBC) (Schoch 1964).

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HPSEC

Amylopectins were debranched with isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) with 40 U/mg of each enzyme (Hayashibara Shoji, Inc., Okayama, Japan), and also were hydrolyzed with α-amylase (EC 3.2.1.1) from porcine pancreas (250 U/mg) (E. Merck, Darmstadt, Germany) and with β-amylase (EC 3.2.1.2) type II-B from barley (53 U/mg) (Sigma). Commercial presentations of isoamylase and pullulanase enzymes were in buffers. α-Amylase (3 mg) and β-amylase (15 mg) were dissolved in 1 ml of 0.1*M* acetate buffer, pH 5.5. Amylopectins (8 mg) were dissolved in 1 ml of 0.1*M* acetate buffer and heated for 20 min at 100°C. After cooling, 5 or 15 µl of each enzymatic solution was

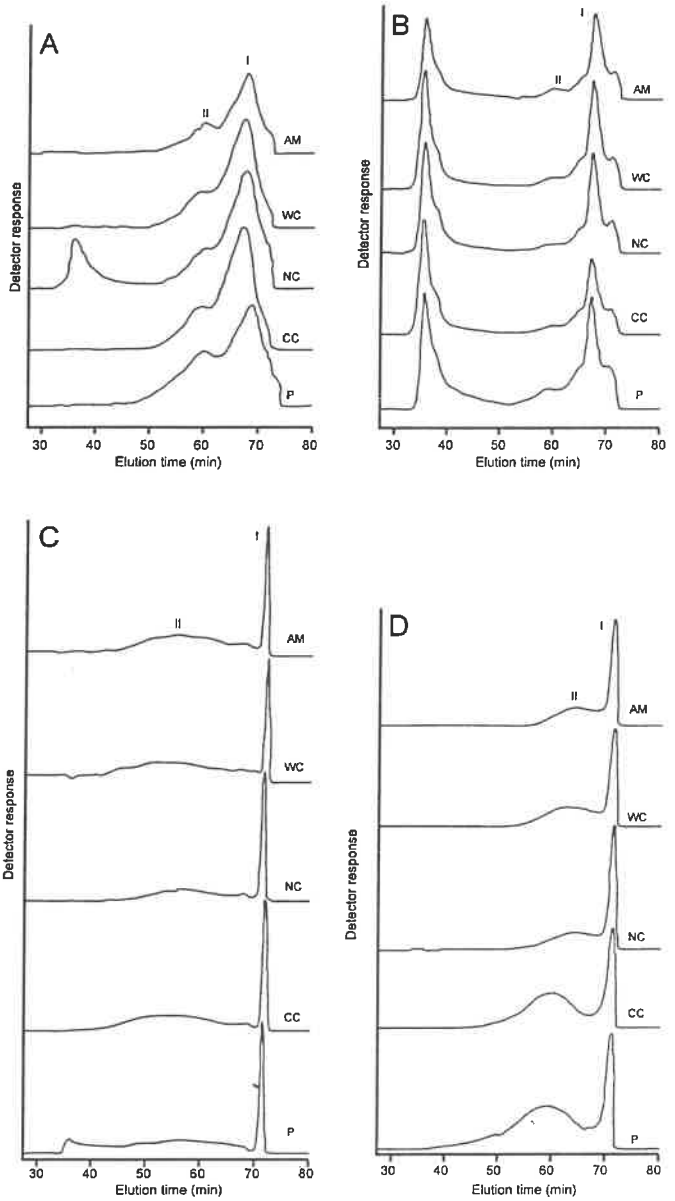

Fig. 1. High-performance size-exclusion chromatograms of amylopectins using 5 µl of enzyme. **A,** isoamylase-debranched; **B,** pullulanase-debranched; **C,** β-amylase-hydrolyzed; **D,** α-amylase-hydrolyzed. AM = amaranth; WC = waxy corn; NC, normal corn; CC = commercial corn; P = potato.

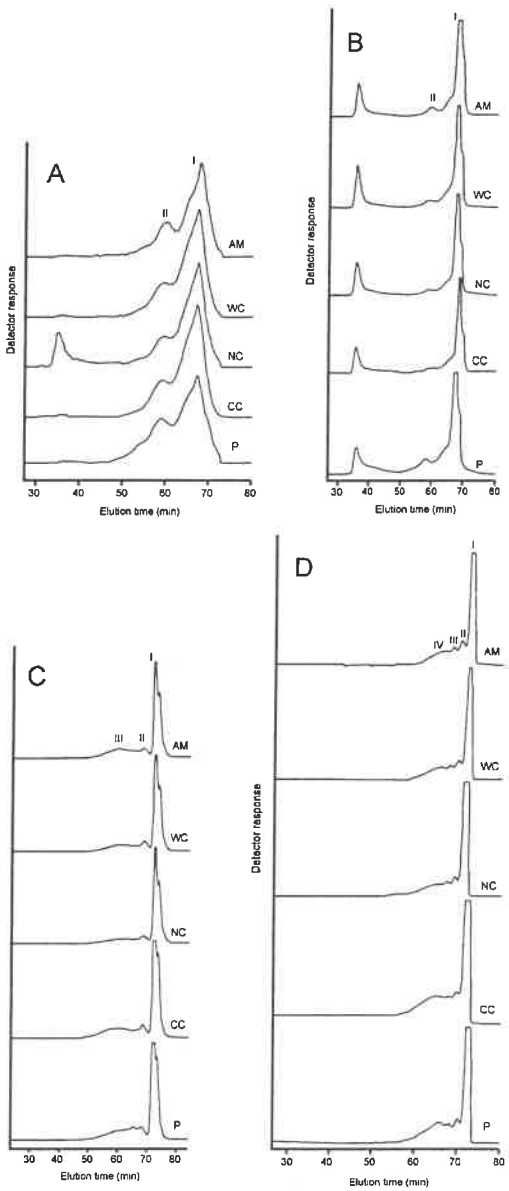

Fig. 2. High-performance size-exclusion chromatograms of amylopectins using 15 µl of enzyme. **A,** isoamylase-debranched; **B,** pullulanase-debranched; **C,** β-amylase-hydrolyzed; **D,** α-amylase-hydrolyzed. AM = amaranth; WC = waxy corn; NC = normal corn; CC = commercial corn; P = potato.

TABLE I
Degree of Polymerization of Isoamylase-Debranched, Pullulanase-Debranched, β-Amylase-Hydrolysis and α-Amylase-Hydrolysis Amylopectins

Sample	Isoamylase				Pullulanase				β-amylase					α-amylase					
	5 µl		15 µl		5 µl		15 µl		5 µl		15 µl			5 µl		15 µl			
	I	II	I	II	I	II	I	II	I	II	I	II	III	I	II	I	II	III	IV
Amaranth	11	47	11	47	10	48	10	49	1	74	1	11	50	1	31	1	2	11	23
Waxy corn	12	51	12	51	11	55	11	49	1	88	1	11	50	1	34	1	2	13	25
Normal corn	12	49	12	51	10	54	10	47	1	64	1	10	46	1	31	1	2	13	25
Commercial corn	11	51	11	52	10	52	10	49	1	92	1	10	50	1	36	1	2	14	30
Potato	11	53	11	52	10	57	10	49	1	58	1	11	25 40	1	44	1	2	15	25

added, and the solutions were incubated for 15 min at 37°C. Digested samples were heated for 20 min at 100°C to inactivate the enzymes. Insoluble material was removed by centrifugation (10,000 × *g* for 20 min) and the supernatant was filtered through a 0.45-µm filter. The chain distribution of debranched and hydrolyzed amylopectins was estimated by an HPSEC system that included a programmable HPLC-pump (Waters 590) (Waters, Milford, MA), autosampler (Water 717), degasser (Erma ERC-3312) (Erma Optical Works LTD, Japan), and a differential refractive index detector (Erma ERC-7510); a guard column (TSK gel SWXL guard column, 6 mm i.d. × 4 cm, TosoHass, Stuttgart, Germany), a column (TSK gel G3000 SWXL 7.8 mm i.d. × 30 cm, TosoHaas), and two columns (TSK gel G2000 SWXL 7.8 mm i.d. × 30 cm). Columns were maintained at 37°C and the detector at 40°C. Water used was taken from a Milli-RO-6-plus and Milli-Q-plus water purification system (Millipore, Beedford, MA). Eluent was 0.1*M* acetate buffer with 0.02% sodium azide, carefully degassed and filtered through durapore GV (0.45 µm) membranes from Millipore before use (flow rate of 0.5 ml/min). A 100-µl sample was injected into the HPSEC system. Software used for the acquisition, storage, and processing of data was the Apex chromatography workstation (Autochrom Inc., France).

TABLE II
Static and Dynamic Results on Total, Pullulanase-Debranched and β-Amylase-Hydrolyzed Amylopectins [a,b]

Sample	$M_w \times 10^{-6}$	RG(nm)	RH(nm)	RG/RH
Total				
(no enzymatic treatment)				
Amaranth	5.0	301	75	4.0
Waxy corn	53.0	242	58	4.2
Normal corn	13.0	113	59	1.9
Commercial corn	59.0	273	66	4.1
Potato	0.4	15	21	0.7
Pullulanase				
Amaranth	0.7	38	30	1.3
Waxy corn	0.5	58	52	1.1
Normal corn	1.0	67	15	4.5
Commercial corn	0.7	48	30	1.6
Potato	1.3	66	38	1.7
β-amylase				
Amaranth	0.4	51	36	1.4
Waxy corn	3.8	38	29	1.3
Normal corn	2.3	68	38	1.8
Commercial corn	0.7	29	31	0.9
Potato	0.1	40	21	1.9

[a] M_W = Molecular weight; RG = gyration radius; RH = hydrodynamic radius. M_W and RG were calculated with Zimm plot using static light scattering. RH was calculated using dynamic light scattering.

Light Scattering

Amylopectin solutions (1 mg/ml) were prepared in 10% (v/v) dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) in water. Amylopectins were weighed, DMSO solution was added, and then heated for 20 min at 100°C to dissolve the samples. After cooling, solutions were filtered through a 0.45-µm filter. Amylopectin solutions debranched with pullulanase and hydrolyzed with β-amylase were also prepared. Pullulanase (10 µl) or β-amylase (10 µl) was added to 3 ml of each solution (DMSO 10%). Samples were incubated 15 min at 37°C. Digested samples were heated for 20 min at 100°C to inactivate the enzyme. Insoluble material was removed by centrifugation (10,000 × *g* for 20 min), and the supernatant was filtered through a 0.45-µm filter. Scattering experiments were performed at 37°C in the 30–150° range in the homo-dyne mode with full photon-counting detection and a 128-channel K7025 Malvern correlator. Incident radiation at 514.5 nm was obtained from a 3W Ar Ion Spectra-Physics laser and vertically polarized. The refractive index increment (dn/dc) was 0.146 ml/g. All fitting and monitoring programs were written in BASIC and run on a HP9300 microcomputer. Optical alignment was checked over the angular range 30–150° using filtered benzene. For SLS experiments, photons were counted for 10 sec to obtain one point of the Zimm plot. In DLS, the programs ALV-5000 (acquisition) and GENDIST (treatment) were

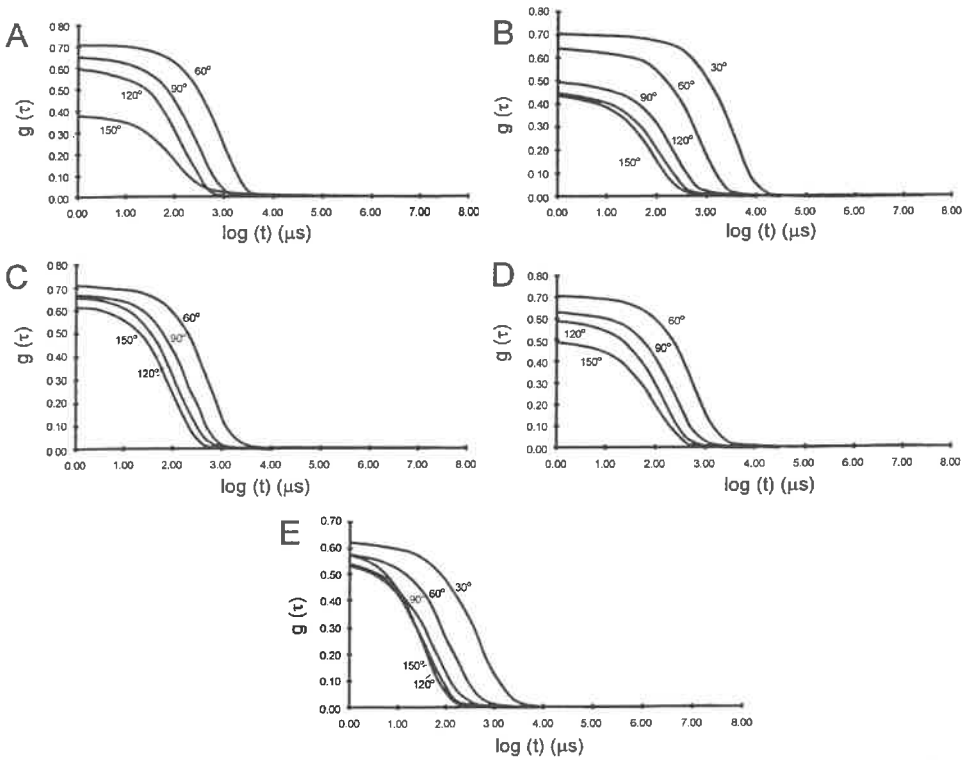

Fig. 3. Angular dependence of time correlation function of amylopectins without enzymatic treatment from dynamic light scattering. **A,** amaranth; **B,** waxy corn; **C,** normal corn; **D,** commercial corn; **E,** potato.

used. Measurements were made after 60-sec accumulation. Data were treated using cumulants (Roger and Colonna 1993)

RESULTS AND DISCUSSION

Purity of Amylopectin

Low IBC values (mg/100 mg of amylopectin) for amaranth (0.55), waxy corn (1.22), and commercial corn (0.39) suggested high purity in these amylopectins; high IBC values for normal corn (5.41) and potato (3.23) amylopectin could be due to amylose contamination.

HPSEC Studies

Isoamylase-debranched amylopectins with 5 µl (Fig. 1A) or 15 µl of enzyme (Fig. 2A) presented profiles with two principal populations: DP 11–12 and 47–53 (Table I) representing A and B chains in the cluster model, respectively (Robin et al 1974). Bradbury and Bello (1993) and Park and Rollings (1994) reported similar DP values. In normal corn amylopectin (Figs. 1A and 2A), one peak occurred at ≈35 min of elution time representing long chains of contaminating amylose not degraded by isoamylase. When the samples were debranched by 5 µl (Fig. 1B) and 15 µl (Fig. 2B) of pullulanase, two principal fractions, DP 10–11 and 47–57, were produced. However, the DP of fraction II with 5 µl of enzyme was higher, except for amaranth amylopectin (Table I), indicating that high enzyme concentrations hydrolyzed major amounts of α-(1→4) linkages, suggesting contaminating activity. All amylopectins treated with pullulanase showed a high level of nondegraded amylopectin at ≈35 min (Figs. 1B and 2B) that increased with decreasing enzyme amount, which indicated that pullulanase may act differently on amylopectin as compared to isoamylase. This is a very important factor to consider in studies on amylopectin.

The profiles of samples hydrolyzed with 5 and 15 µl of β-amylase solution are presented in Figures 1C and 2C. In both treatments of all amylopectins, a fraction (I) corresponding to glucose was obtained (Table I). Fraction II corresponds to the β-dextrin limit when 5 µl of enzyme solution was used. Potato and normal corn amylopectins exhibited lower DP values than did amaranth, waxy, and commercial corn amylopectins (Table I), probably due to the minor level of branches in the later amylopectins (Paredes-López et al 1994). Three populations were obtained for all amylopectins with 15 µl of enzyme: fraction I corresponded to glucose, fraction II to DP 10–11 and fraction III to DP 40–50 (Table I). Potato amylopectin fraction III had the lowest DP because it also presented a population of DP 25, resulting from either a different amylopectin structure or phosphate groups that block β-amylase. The other amylopectins did not show substantial differences in DP values.

When α-amylase was employed, two fractions were obtained with 5 µl (Fig. 1D, Table I). Fraction I corresponds to glucose and fraction II to DP 31–44 for all amylopectins. Four fractions were produced with 15 µl (Fig. 2D, Table I). Fractions I and II correspond to glucose and maltose, respectively; fraction III to DP 11–15; and fraction IV to DP 23–30. These values were lower than those of fraction II with 5 µl, indicating that high amounts of enzyme hydrolyzed major amounts of amylopectin, again suggesting contaminating activity.

Light Scattering Studies

Amylopectins without enzymic treatments were studied by SLS and DLS (Table II) to examine behaviors in solution, molecular weights (M_w), and structures. Amaranth (5×10^6) and potato (0.4×10^6) amylopectins presented the lowest M_w values. Waxy, normal, and commercial corn amylopectins gave M_w values between 13 and 59×10^6, in agreement with those between 17 and 88×10^6 reported by Aberle et al (1994) for different amylopectins. Gyration radius (RG) for amaranth showed the highest value,

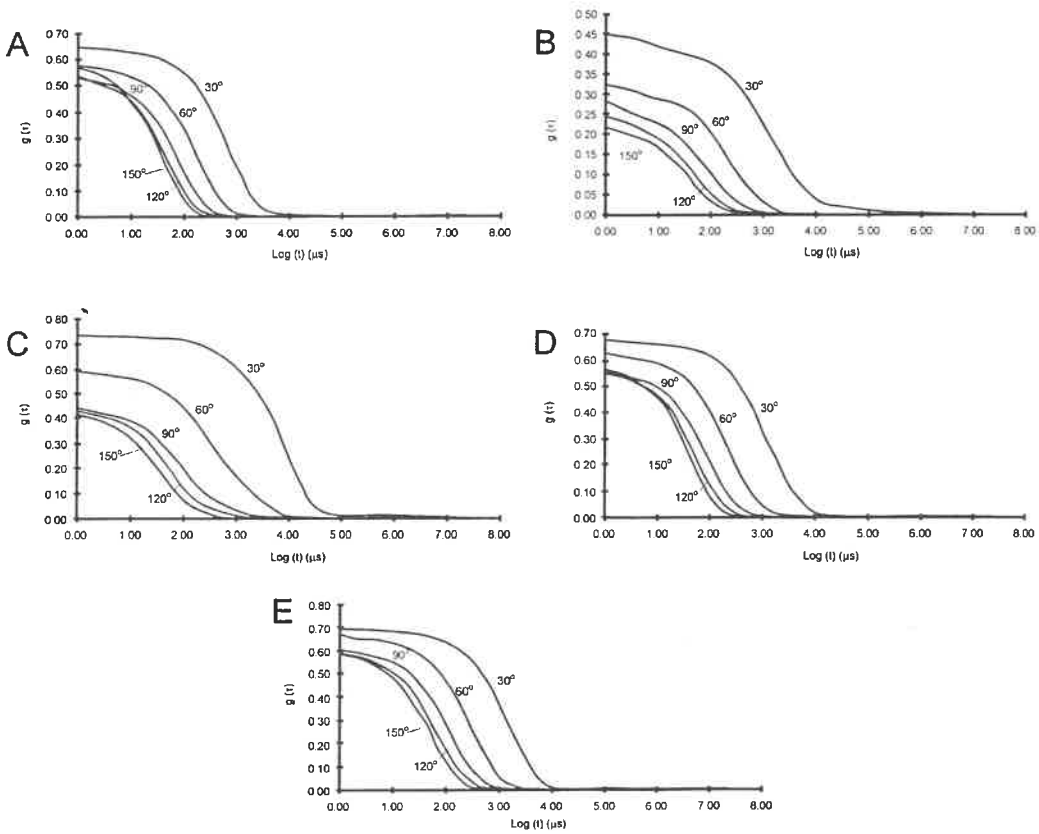

Fig. 4. Angular dependence of time correlation function of pullulanase-debranched amylopectins from dynamic light scattering. **A,** amaranth; **B,** waxy corn; **C,** normal corn; **D,** commercial corn; **E,** potato.

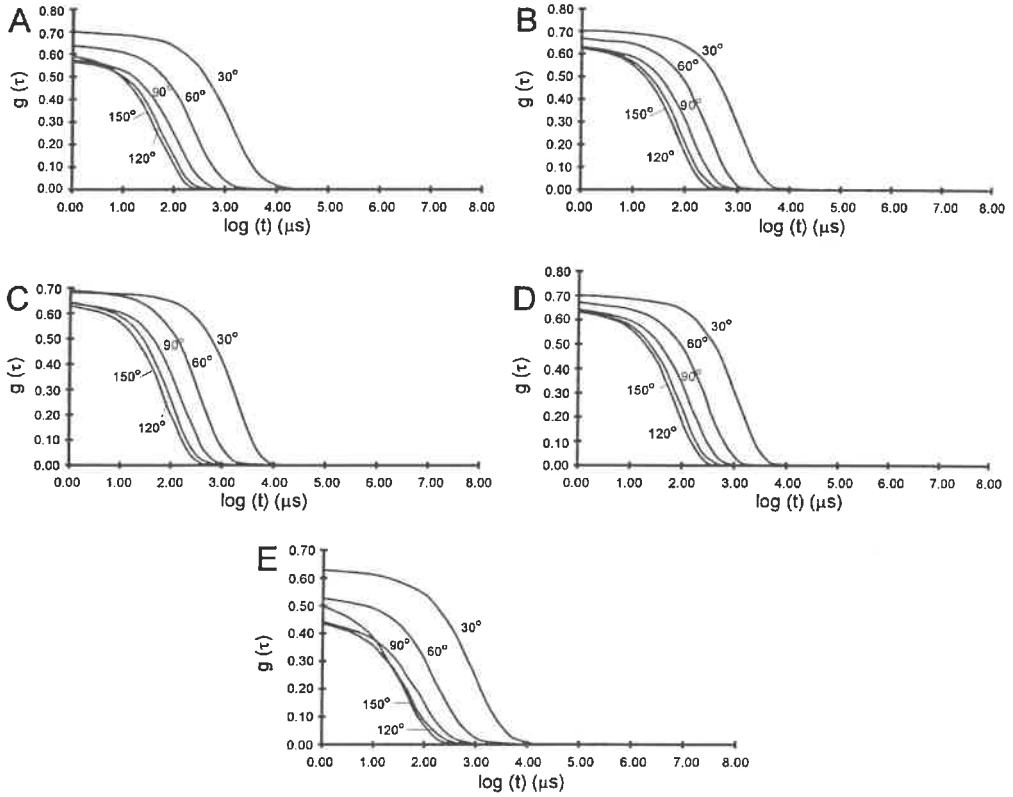

Fig. 5. Angular dependence of time correlation function of β-amylase-hydrolyzed amylopectins from dynamic light scattering. **A,** amaranth; **B,** waxy corn; **C,** normal corn; **D,** commercial corn; **E,** potato.

suggesting that this amylopectin had the most highly branched structure. The values for waxy and commercial corn amylopectins (242 and 273 nm, respectively) were close to those reported for corn amylopectin (214–238 nm) by Aberle et al (1994). Potato amylopectin had the lowest RG (15 nm). Native amaranth and potato amylopectin gave the highest and lowest hydrodynamic radius (RH) values, respectively. The RH value gives information about branching: when the RH value is large, the molecule is more branched (Burchard 1993). Amaranth amylopectins thus, have a more branched structure than do the other amylopectins. This trend is somewhat related to that followed by M_w and RG values. The RG-RH relationship (Table II) gives information about molecular architecture (Burchard 1992). Amaranth, waxy, and commercial corn amylopectins gave values higher than 2.0, which corresponds to a semiflexible chain behavior, whereas normal corn and potato amylopectin gave values lower than 2.0, suggesting a spherical or globular structure. The time correlation function was measured at various scattering angles and analyzed by inversed Laplace transformation. Curves of the time correlation function (Fig. 3) give additional information on amylopectin structure. Amaranth, waxy corn, and commercial corn amylopectins have a pronounced angular dependence, whereas normal corn and potato amylopectins have only slight differences. The smoothness of curves in Figure 3 suggests that there is only one population of molecules in all amylopectins tested.

After debranching with pullulanase, all M_w, RG, and RH values decreased, except those for potato amylopectin, which increased (Table II). The latter trend suggests chain aggregation. RG/RH values for amaranth, waxy, commercial corn, and potato amylopectin (Table II) were lower than 2.0, suggesting as indicated previously, a spherical or globular structure. However, normal corn amylopectin presented a value greater than 2.0, implying a structural change after debranching with pullulanase that may be due to the presence of amylose in this material.

The time correlation function of amylopectins depolymerized with pullulanase was measured at various scattering angles (Fig. 4). All samples showed large clusters at low angles, but at 90–150°C, slight differences existed among the curves. Again, the smoothness of curves (Fig. 4A–E) suggested the presence of a single population. Normal corn at low angles exhibited comparatively a major dependence, most likely due to amylose contaminating this amylopectin as found in the HPSEC study.

After hydrolysis with β-amylase, M_w decreased compared with untreated amylopectins (Table II). All amylopectins showed RG/RH values lower than 2.0. The time correlation function of amylopectins treated with β-amylase and measured at 30–150 scattering angles (Fig. 5) showed, in general, a similar behavior to that of amylopectins treated with pullulanase (Fig. 4).

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