Location of Amylose in Normal Starch Granules. II. Locations of Phosphodiester Cross-Linking Revealed by Phosphorus-31 Nuclear Magnetic Resonance[1]

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ABSTRACT

Amylose locations were revealed by phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy and gel-permeation chromatography. Native maize starch was cross-linked with $POCl_3$ at pH 12 at 25°C. The cross-linked starch was solubilized in a dimethyl sulfoxide (DMSO) solution and was separated into soluble and insoluble fractions. Gel-permeation chromatograms showed that the soluble fraction contained amylose molecules of small molecular size only. The amylopectin and large amylose molecules were cross-linked and became insoluble in the DMSO solution. The chromatograms showed no cross-linkage among amylose molecules. Phosphorus-31 NMR was used to determine the phosphate structure in the starch molecules. Chemical shift of phosphate diester (phosphate cross-linking) was between −1.0 and 1.0 ppm, and that of a phosphate monoester (phosphate derivatives) was between 4.3 and 4.9 ppm. The phosphorus-31 NMR spectra also confirmed that the amylopectin and amylose molecules cross-linked in the insoluble fraction, whereas the spectra of soluble fraction (containing amylose) displayed only a small amount of phosphomonoester derivative. The results of gel-permeation chromatography and phosphorus-31 NMR spectroscopy indicated that the amylose molecules were randomly interspersed in the starch granule instead of being in bundles.

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Normal starch consists of two major components: amylose and amylopectin. Normal maize starch contains 28% amylose and 72% amylopectin (Swinkels 1985). Amylopectin, which is a branched molecule, has been extensively studied for its structure (Greenwood 1964, French 1972, Hizukuri et al 1983, Zobel 1984, Manners 1985). Amylose is essentially a linear molecule containing glucose units linked by α-1,4 linkages with few branches (Hizukuri et al 1981). The location of amylose in a starch granule is not well understood. Amylose has been located in bundles between amylopectin clusters (Nikuni 1978, Blanshard 1986, Zobel 1992), randomly interspersed among amylopectin clusters in both the amorphous and crystalline regions (Jane et al 1992). Although chemical structure features of the starch components are now well established, studies on the organization of the starch molecules yield only limited and often conflicting reports.

Jane et al (1992) studied the location of amylose by using epichlorohydrin cross-linking. At low levels of cross-linking, the amylose and amylopectin molecules were cross-linked and eluted together as indicated by the increased blue value in the amylopectin peak in the gel-permeation chromatograms. Gel-permeation chromatography did not show any increase in molecular size of the amylose peak, which indicated that there was no cross-linking between amylose molecules. The susceptibility of the amylose to sequential hydrolysis by isoamylase and β-amylase, however, decreased. This resistance to enzyme hydrolysis was attributed to phosphomonoester derivatives, but no structural data were given.

Phosphodiester cross-linked starch can be prepared by reagents such as phosphorus oxychloride ($POCl_3$) and sodium trimetaphosphate (Wurzburg 1986). Most of the cross-linking reagents also produce phosphomonoester derivatives as by-products. The ratio of the cross-linking and monoester derivatives can be controlled by the pH of the reaction. At pH levels between 8 and 12, the cross-linking is predominant; at pH levels below 6, the

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reaction shifts to produce phosphomonoester derivatives (Felton and Schopmeyer 1943, Wetzstein and Lyon 1956, Patten et al 1969, Lloyd 1970, Rogols and Salter 1979). $POCl_3$ is a very active reagent, producing a very fast reaction rate (Wurzburg 1986, Wu and Seib 1990). Starch modified with $POCl_3$ results in random cross-linking in starch molecules (Rutledge et al 1974). Because the degree of cross-linking in starch is normally quite low, it is difficult to quantify directly with chemical methods. The physical properties of the cross-linked starch, such as viscosity (detected by a viscoamylograph) and swelling power, are generally used to estimate the degree of cross-linking (Hullinger 1967, Jarowenko 1971, Rutenberg and Solarek 1984). The cross-linking also limits amylose leaching from the granule and decreases starch solubility in aqueous and dimethyl sulfoxide (DMSO) solutions at 95°C (Kerr and Cleveland 1957, Leach and Schoch 1962, Wurzburg 1986). The molecular weight distribution of native starch and the soluble fractions of modified starch can be determined by gel-permeation chromatography.

In this study, $POCl_3$ was used to cross-link normal maize starch, and phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy was used to detect the phosphate esters in the starch (Lim 1990, Lim and Seib 1993, McIntyre et al 1990). Phosphorus-31 NMR can detect and differentiate phosphomonoesters from the diesters within a reasonable data-acquisition time (McIntyre et al 1990). A constant temperature (25°C) and pH (8.0 ± 0.1) was used throughout the analysis to avoid temperature and pH effects on chemical shifts (Bock and Sheard 1975, Barany and Glonek 1982, Jame 1985, Tebby and Glonek 1991).

MATERIALS AND METHODS

Normal maize starch was purchased from Sigma Chemical Company (St. Louis, MO). $POCl_3$ (99.999%) was purchased from Aldrich Chemical Company (Milwaukee, WI). Other chemicals were reagent grade and were used without further treatment. α-D-Glucose-2-phosphate and α-D-glucose-3-phosphate used as the phosphorus-31 NMR reference compounds were provided by S. Lim of Korea University (Seoul, Korea).

Starch Cross-Linking with $POCl_3$

Normal corn starch (100 g, dsb) was suspended in distilled water (120 g). The starch slurry was adjusted to each designated pH with 1.0*M* NaOH. $POCl_3$ was diluted with tetrahydrofuran (for the low levels of modification) and then added to the slurry. The mixture was mechanically stirred. The pH was controlled by a pH controller (Chemcadet, Cole Parmer Instrument Co., Chicago, IL) at 25°C. After 30 min, the slurry mixture was adjusted to pH 5.5 to stop the reaction. The slurry was washed three times with distilled water and twice with 100% methyl alcohol. The washed starch was then filtered with a Whatman No. 4 filter paper. The modified starch was dried in a draft-air oven at 40°C.

To determine optimal cross-linking conditions, the starch was treated at a range of pH 7–12 and at different $POCl_3$ concentrations. The degree of cross-linking was determined by using a Brabender Viscoamylograph (model VA-VE, C. W. Brabender Instruments Inc., South Hackensack, NJ). The reactions were repeated at least twice.

Starch Phosphomonoester

A starch phosphomonoester derivative was prepared by the method of Paschall (1964). Starch mixed with orthophosphate (a mixture of mono- and di-hydrogen phosphate) at pH 5–6.5 and at a high temperature (155°C) gives a maximum starch phosphomonoester at ~0.2 degrees of substitution.

Viscosity and Pasting Properties Determination

Viscosities of the native and modified starches were obtained with a Brabender Viscoamylograph following the method of Smith (1964). The viscosity was determined continuously during the heating, holding, and cooling of the starch paste. Starch (32 g, dsb) was suspended in 368 g of distilled water and put in an amylograph cup. The suspension was stirred and heated (75 rpm, 1.5°C/min) to 95°C. It was held for 30 min, then cooled to 50°C (1.5°C/min). The amylograms were done in duplicate.

Starch Fractionation

$POCl_3$ cross-linked starch was fractionated into soluble and insoluble fractions. Modified starch (30 g) was dissolved in 1 L of 90% DMSO. The slurry was stirred and heated in a water bath (95°C) for 2 hr, allowed to cool to room temperature, and then continuously stirred for 24 hr. The slurry was then centrifuged at 6,400 × *g* for 20 min. The supernatant and precipitate were collected separately. The precipitate was washed twice with 100% methyl alcohol and dried in a draft-air oven (70°C). The supernatant was precipitated with ~10 volumes of 100% methyl alcohol, filtered (Whatman No. 4 filter), and dried in a draft-air oven (70°C).

Determination of Molecular Weight Distribution by Gel-Permeation Chromatography

Molecular weight distributions of native normal maize starch and the soluble fraction of the modified normal maize starch was determined by using gel-permeation chromatography. A 1-g sample was suspended in 100 ml of 90% DMSO. The suspension was stirred and heated in a water bath (95°C) for 2 hr and then continuously stirred at room temperature for an additional 24 hr. Each solution (15 ml containing ~150 mg of sample) were precipitated by using ~100 ml of 100% methyl alcohol. The precipitate was separated by centrifugation (5,000 × g for 20 min).

For native maize starch, each precipitate (150 ± 10 mg) was dissolved in distilled water (50 ml) by heating and stirring in a water bath (96°C) for 30 min; 10 mg of glucose was added as a marker. For modified starch, each of the precipitates from soluble fractions (150 ± 10 mg) was dissolved in distilled water (150 ml); 30 mg of glucose was added as a marker. Samples (5 ml) contained normal maize starch and the soluble fraction of the modified starch (15 mg and 5 mg, respectively). They were injected into a 2.6 × 80-cm column (Pharmacia Inc., Piscataway, NJ) packed with Sepharose CL-2B gel. Distilled, deionized, and degassed water containing 0.02% Na_2SO_4 was used to elute the samples in ascending order with a flow rate of 30 ml/hr. Fractions of 4.8 ml were collected and analyzed by using an Autoanalyzer II (Technicon Instruments Corp., Elmsford, NJ). The total carbohydrate (anthrone-sulfuric acid reaction) and amylose (iodine blue value) of the fractions were measured at 630 and 640 nm, respectively. A glucose solution (0.005%, w/v) was used as a reference for total carbohydrate determination. Molecular weight distributions were plotted on the basis of total carbohydrate. The blue value was used to identify locations of amylose and amylopectin in the chromatograms.

Preparation of Starch Samples

One gram of each starch sample was dispersed in 20 ml of 1.0*M* acetate buffer (pH 6.9) by heating and stirring in a water bath (96°C) for 20 min. *Bacillus amyloliquefaciens* α-amylase (250U) was added to the starch solution. The mixture was incubated in a water-bath shaker (Versa-bath S, model 236, Fisher Scientific) at 70°C, 100 strokes per minute for 2 hr. The hydrolysate was then boiled for 20 min to terminate enzyme activity.

Phosphorus-31 NMR Spectroscopy

Phosphorus-31 NMR spectra of the modified starches were analyzed by using a Bruker WM-200 NMR spectrometer (USA Bruker Instruments, Mountain View, CA). Phosphorus-31 NMR spectra were acquired with the following conditions: a spectrometer frequency of 81 MHz with a 65° flip angle (15 μsec), 16K data points, 20,000 Hz sweep width, and a recycle time of 1.4 sec. The spectra in this study were collected with about 30,000 scans. All the spectra were replicated at least twice.

The hydrolysate (5%, 2 ml) was mixed with 2 ml of deuterium oxide containing 0.2*M* ethylenediaminetetraacetic acid and adjusted to pH 8.0 ± 0.1. All chemical shifts were reported in parts

per million with 85% H_3PO_4 as an external reference (0.0 ppm). The chemical shifts were recorded with ^1H decoupled spectra at 25°C.

To identify chemical shifts of phosphorus-31 NMR signals, the chemical shifts of reference compounds were determined. Glucose-2-phosphate and glucose-3-phosphate (Lim 1990), glucose-6-phosphate, and starch phosphomonoester were used as references for phosphomonoester derivatives. Lecithin was used as the phosphodiester reference (cross-linking). Sodium phosphate was used as an inorganic phosphate reference. The chemical shifts of the model compounds were obtained by using the same parameters that were used for the starch samples, with only 16 acquisitions. The starch phosphomonoester, however, required about 1,000 acquisitions. Concentrated phosphoric acid (85% H_3PO_4), sealed in a capillary tube, was inserted into the NMR sample tubes as the external reference at 0.0 ppm for calibration.

RESULTS AND DISCUSSION

At alkaline pH, $POCl_3$ modified starch displayed predominant phosphodiester cross-linking with little phosphomonoester derivative. The optimal pH for cross-linking with $POCl_3$ was pH 12 (Fig. 1). The starch modified at pH 12 with 0.04% $POCl_3$ showed the highest viscosity. Further increase of $POCl_3$ decreased viscosity of starch paste, and, eventually, the starch granules were restricted to swelling. The starch modified with 0.2% $POCl_3$ at pH 12 was so highly cross-linked that the viscosity became undetectable by the Brabender Viscoamylograph (Fig. 2). The reactions were replicated, and the amylograms were reproducible.

Starch treated with $POCl_3$ at levels above 0.2% remained insoluble and showed no soluble starch in 90% DMSO after heating at 96°C for 2 hr. Starch treated with less than 0.1% $POCl_3$ formed a viscous gel and also could not be separated by centrifugation into soluble and insoluble fractions. The starches modified with $POCl_3$ at levels between 0.1% and 0.2% were fractionated into soluble and insoluble fractions. The molecular weight distributions of the soluble fractions were analyzed by gel-permeation chromatography. The chromatogram of the soluble fraction of the 0.2% $POCl_3$ modified starch showed only small amylose molecules (compared to those of the native maize starch chromatogram) (Fig. 3). The soluble fraction also showed that the molecular weight of the amylose did not increase with cross-linking. In contrast, the molecular weight of the soluble fraction decreased with increasing degree of cross-linking (Fig. 3). The decrease in the molecular weight of the soluble fraction could be attributed to the large amylose molecules being more susceptible to cross-linking with amylopectin and thus becoming insoluble. These results were consistent with those previously reported (Jane et al 1992). At a low degree of cross-linking, Jane et al (1992) reported that the blue value of the amylopectin peak increased because of cross-linking between amylopectin and amylose. They also reported that the amylose was eluted with the amylopectin.

Phosphorus-31 NMR chemical shifts of reference compounds are listed in Table I. These values are similar to those reported by Lim and Seib (1993). Lim (1990) reported that the phosphomonoester at carbon-3 and at carbon-6 of the nonreducing end of α,γ phosphodextrin from potato starch showed signals at chemical shifts of 1.62 and 1.95 ppm, respectively, using

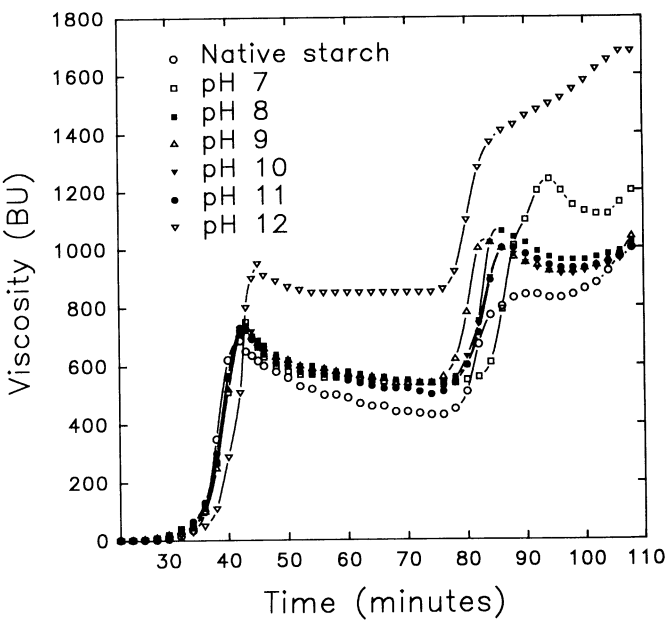

Fig. 1. Amylogram of slurries (8%, dsb) of native and cross-linked normal maize starches in water prepared with 0.05% $POCl_3$ at pH 7–12.

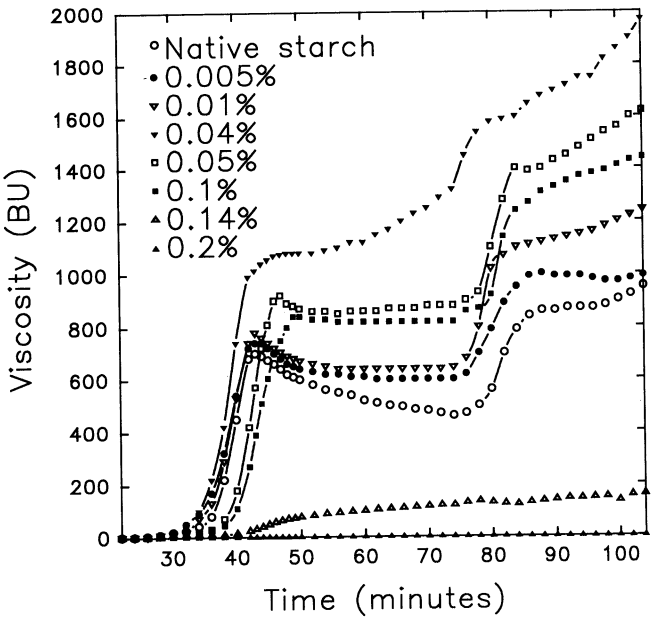

Fig. 2. Amylogram of slurries (8%, dsb) of native and cross-linked normal maize starches in water prepared at pH 12 and modified with 0.005–0.2% $POCl_3$.

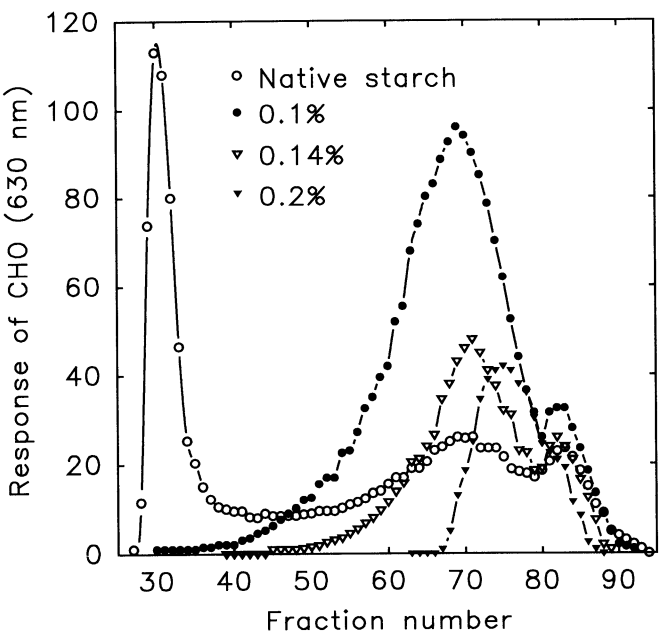

Fig. 3. Chromatography profile of 10 mg of normal maize starch and the soluble fractions fractionated from cross-linked maize starch prepared at pH 12 and modified with 0.1%–0.2% $POCl_3$. Column was eluted with 0.02% sodium sulfate aqueous solution. Flow rate was 0.5 ml/min. Fractions (4.8 ml) were analyzed for total carbohydrate by the anthrone-sulfuric acid procedure. Glucose was used as a marker.

inorganic phosphate as an internal standard. The chemical shift of inorganic phosphate is 2.9 ppm more upfield than that of 95% phosphoric acid; therefore, when using 95% phosphoric acid as an external standard, the chemical shift should be at 4.52 and 4.85 ppm, respectively. Lim and Seib (1993) also reported that phosphomonoester at carbon-3 and carbon-6 on the internal chain of the α,γ phosphodextrin from potato starch showed signals at the chemical shifts of 0.79 and 1.15 ppm, respectively, using inorganic phosphate as an internal standard. When using 95% phosphoric acid as an external standard, the chemical shift should be at 3.69 and 3.95 ppm, respectively. Lecithin carrying phosphodiester linkages, showed a broad peak between −1.0 and 1.0 ppm. The broad peak could be attributed to a mixture of different fatty acids. Tebby and Glonek (1991) reported that myo-inositol 1,2-cyclic phosphate, which has two hydroxy groups from the same sugar ring, forms a cyclic phosphate diester and gives a signal at the chemical shift of 15.5 ppm.

Phosphorus-31 NMR spectrum of the insoluble fraction (solubilized by enzyme hydrolysis) (Fig. 4) showed signals at the chemical shifts of 0.0, 3.6, 4.6, and 15.5 ppm. The signal at δ 0.0 ppm had the highest intensity, indicating a large number of phosphodiester linkages. The signal at δ 3.6 ppm indicated the presence of phosphomonoester derivative in the internal chain of α-limit dextrins. The signal at δ 4.6 ppm indicated the phosphomonoester at the nonreducing end of the α-limit dextrins (Lim 1990). The signal at 15.5 ppm is attributed to the cyclic phosphate glucan. The phosphorus-31 NMR spectrum confirmed that cross-linkages, as well as monophosphate derivatives and other types of phosphate esters, were in the insoluble fraction that contained amylose and amylopectin.

TABLE I
Phosphorus-31 Nuclear Magnetic Resonance Chemical Shift of Reference Compounds

Reference Compounds	Chemical Shift (ppm)
Glucose-2-phosphate	4.3
Glucose-3-phosphate	4.7
Glucose-6-phosphate	4.9
Inorganic phosphate	2.9
Inorganic pyrophosphate	−6.6
Starch phosphomonoester	−6.6, 2.9, 3.7, 4.3, 4.7, and 4.9
Lecithin	−1.0 to 1.0
Myo-inosital 1,2-cyclic phosphate[a]	15.5

[a] Tebby and Glonek 1991.

The phosphorus-31 NMR spectrum of the soluble fractions, however, showed signals at 2.9 ppm, which indicates inorganic free phosphate, and at 4.5 ppm, corresponding to the phosphomonoester derivative at nonreducing terminal of α-limit dextrins (Fig. 5). The spectra indicated that the DMSO-soluble fraction, containing amylose only, had only phosphomonoester derivatives and had no phosphodiester linkage (cross-linking). This result also confirmed that the resistance of the amylose isolated from cross-linked starch to amylases hydrolysis was caused by derivatives instead of cross-linkages (Jane et al 1992).

The results from gel-permeation chromatography and phosphorus-31 NMR spectroscopy indicated that the amylopectin and amylose in starch granules were cross-linked. Amylose molecules had less of a tendency to be cross-linked and were separatable as "soluble fractions." Amylose cross-linked with amylopectin (Jane et al 1992). There was no cross-linkage, however, found between amylose molecules. These results rule out amylose molecules being present in bundles. If the amylose molecules were located in bundles, the amylose molecules would be susceptible to cross-linking. Then the amylose peak in gel-permeation chromatograms should be shifted to a higher molecular weight. All the evidence seems to suggest that the amylose molecules were

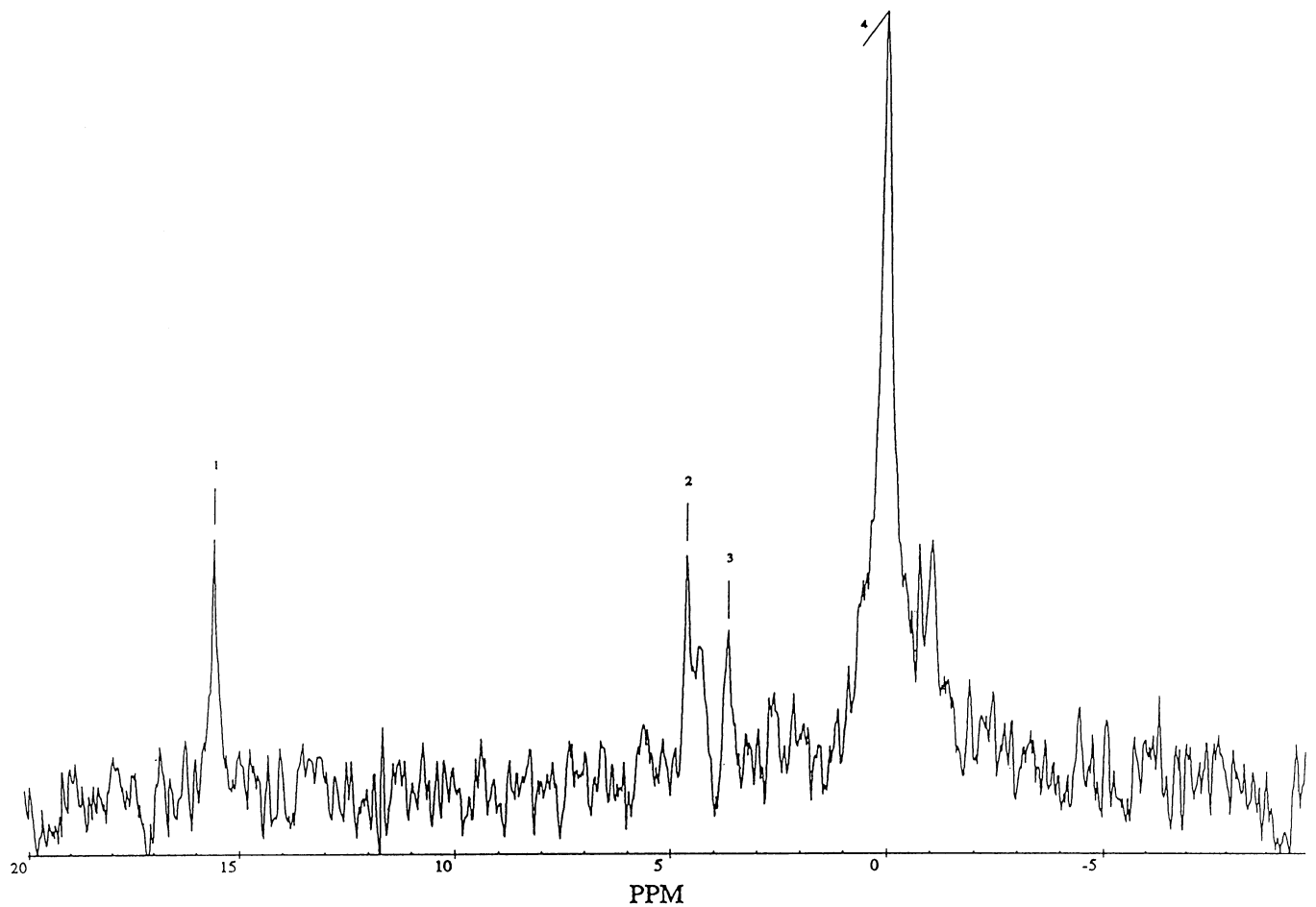

Fig. 4. Phosphorus-31 nuclear magnetic resonance (NMR) spectrum of α-limit dextrins from the insoluble fractions separated from 0.2% $POCl_3$ modified normal maize starch at pH 8.0 ± 0.1 and 81 MHz. Signals 1–4: 15.5, 4.6, 3.6, and 0.0 ppm, respectively.

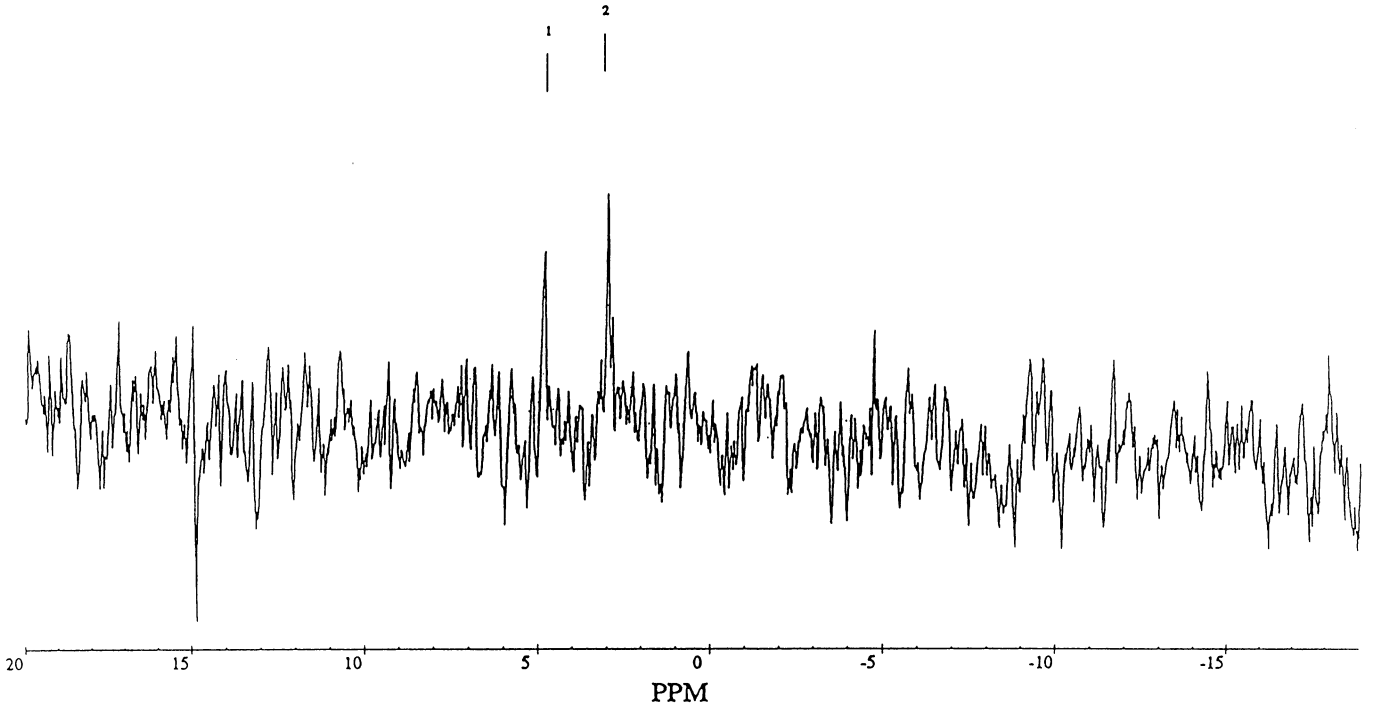

Fig. 5. Phosphorus-31 nuclear magnetic resonance (NMR) spectrum of α-limit dextrins from the soluble fraction separated from 0.2% $POCl_3$ modified normal maize starch at pH 8.0± 0.1 and 81 MHz. Signal 1: 4.5 ppm. Signal 2: 2.9 ppm.

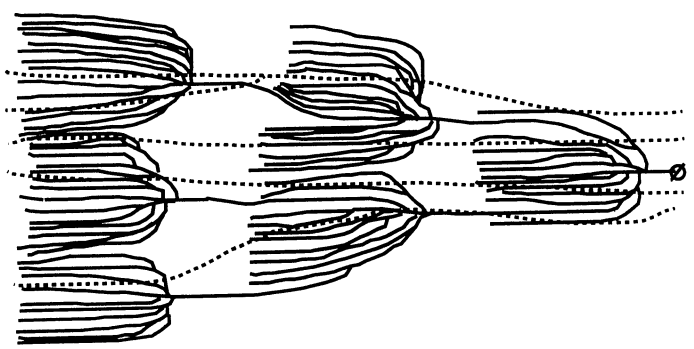

Fig. 6. Schematic model of starch granule organization. Dotted line = amylose molecule, solid line = amylopectin molecule.

randomly interspersed among amylopectin as shown in Figure 6. Amylose must be located in close proximity with amylopectin for them to be cross-linked. It is plausible that some large amylose molecules participate in double helices with amylopectin on the basis that amylose can only be completely extracted at temperatures above 90° C (Banks and Greenwood 1975). Small amylose, located at the periphery of starch granules (Jane and Shen 1993), tends to leach out much more easily.

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