Characterization of Phosphorus in Starch by ^{31}P-Nuclear Magnetic Resonance Spectroscopy[1]

SEUNG-TAIK LIM,[2] TUNYAWAT KASEMSUWAN,[2] and JAY-LIN JANE[2,3]

ABSTRACT

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Phosphorus in starches from various botanical sources (cereals, roots, tubers, and legumes) was examined by ^{31}P-nuclear magnetic resonance spectroscopy. Normal cereal starches contained phosphorus (0.02–0.06% starch dry weight) mainly in the form of phospholipids, whereas waxy starches had much less phosphorus (<0.01%), mainly in the form of starch phosphate monoesters. High-amylose corn starch (70% amylose) contained organic phosphorus (0.02%) as starch phosphate monoesters and phospholipids in a 1:4 ratio. Root and tuber starches were phospholipid-free, and the residual organic phosphorus was exclusively phosphate monoesters. Phosphate monoesters were exceptionally high in potato starch (0.089%). Legume starches contained phosphorus (~0.01%), mainly in the form of starch phosphate monoesters. Rice and lentil pea starches displayed signals absent in the other starches at 1.5–2.5 ppm on ^{31}P-nuclear magnetic resonance spectra. Phosphate monoesters of all the starches were located more on the primary carbon (C-6) than on the secondary carbon (C-3) of the anhydrous glucose unit.

Most cereal starches contain phosphorus that is mainly in the form of phospholipids (Schoch 1942a,b; Tabata et al 1975; Meredith et al 1978), whereas root and tuber starches contain phosphorus in the form of starch phosphate monoesters (Posternak 1935, 1951; Hodge et al 1948; Hizukuri et al 1970). Several cereal starches from corn, waxy corn, rice, and waxy rice were reported to contain minor amounts (6–15 ppm) of starch phosphate monoesters located mostly at C-6 of their anhydrous glucose units (Tabata et al 1975).

As major phosphoryl sources in cereal starches, lysophospholipids from helical complexes with starch. This reduces water-binding capacity (Tester and Morrison 1990) and increases opaqueness of a starch paste (Schoch 1942a, Swinkels 1985). Phosphate monoesters in root or tuber starches, however, promote its hydrophilic nature by "wedging" apart individual starch chains with negatively charged phosphate groups, increasing water-binding capacity, swelling power, and paste clarity (Swinkels 1985, Lim 1990).

^{31}P-nuclear magnetic resonance (NMR) spectroscopy has been used to locate the phosphorylations in modified wheat and corn starches and in native potato and taro starches (Lim 1990, Muhrbeck and Tellier 1991, Jane et al 1992, Lim and Seib 1993) and locations of phosphodiester cross-linkage in corn starch (Kasemsuwan and Jane 1994).

The objective of this study was to use ^{31}P-NMR spectroscopy to characterize naturally existing phosphorus in starches from various botanical sources.

MATERIALS AND METHODS

Corn, rice, and potato starches were purchased from Sigma Chemical Company (St. Louis, MO). Several starches were gifts of respective companies: waxy and *du*-waxy corn starches (American Maize-Products Co., Hammond, IN); oat starch (ConAgra, Omaha, NE); wheat starch (Midwest Grain Products Co., Atchison, KS); high-amylose corn starch (70% amylose) (National Starch and Chemical Co., Bridgewater, NJ); tapioca starch (A. E. Staley Mfg. Co., Decatur, IL). Arrowroot starch was purchased from Frontier Cooperatives (Norway, IA). Other starches were isolated in our laboratory.

α-L-Lysophosphatidyl choline, α-L-lysophosphatidyl ethanolamine, and crystalline α-amylase of *Bacillus* species were purchased from Sigma. The activity of α-amylase was 2,100 U/mg. One unit is defined as release of 1 mg of maltose from starch in 3 min.

Starch Isolation

Starches were isolated from a number of sources. Cattail millet (*Pennisetum americanum*) and waxy amaranth (*Amaranthus hypochondriacus*) starches were isolated by the method of Yanez and Walker (1986). Waxy rice starch was isolated by the method of Tester and Morrison (1990). Sweet potato (*Ipomoea batatas*), lotus (*Nelumbo nucifera*), and water chestnut (*Trapa natans*) starches were isolated by the method of Shiotani et al (1991). Legume starches from mung bean (*Vigna radiata*), green pea (*Pisum sativum*), lentil pea (*Lens culinaris*), and lima bean

[1]Journal paper J-15738 of Iowa Agriculture and Home Economics Experiment Station, Ames. Project 2863.
[2]Department of Food Science and Human Nutrition, Center for Crops Utilization Research, Iowa State University, Ames.
[3]Corresponding author.

(*Phaseolus lunatus*) were isolated by the method of Schoch and Maywald (1968).

α-Limit Dextrin Preparation

Starch was excessively digested by α-amylase for NMR measurement following the method of Lim and Seib (1993) with slight modifications. Starch (5 g, dsb) was heated in the presence of α-amylase (1 mg) in 0.1 m*M* calcium chloride aqueous solution (20 ml) in a boiling water bath for 10 min with occasional stirring. The high-amylose corn starch dispersion was heated with α-amylase at 130°C for 30 min in a pressure reactor (Parr Instruments, Moline, IL). Because α-amylase simultaneously digested starch during the process of heating, gelatinization, and pasting, the solution became thin and translucent. The pH of the solution was adjusted to 6.5 by adding 0.1*N* aqueous HCl or NaOH solution. Additional enzyme (2 mg) was added, and the digestion was continued by incubating the solution (2 hr) in a water bath (70°C). The starch hydrolysate was heated in a boiling water bath for 30 min to inactivate the enzyme, and then centrifuged (3,000 × *g*, 10 min) to remove insoluble materials. α-Limit dextrins from the starches with low phosphorus contents (green pea, waxy corn, *du*-waxy corn, and waxy rice starches) were rotary vacuum-evaporated at 40°C to approximately one third of their original volumes.

Starch Defatting

Rice and potato starches were defatted by following the procedure of Vasanthan and Hoover (1992). Starch (10 g, dsb) was refluxed with an *n*-propanol and water mixture (3:1, 300 ml) for 24 hr. An aliquot (200 ml) of the lipid extract was rotary vacuum-evaporated at 40°C to a syrupy material to remove most of the *n*-propanol. The residue was dissolved in water (10 ml) and reevaporated to ~5 ml for NMR measurement. Rice starch also was defatted with water-saturated butanol under the same conditions for comparison.

To achieve complete removal of phospholipids, rice starch (3 g, dsb) was dissolved in 90% aqueous dimethyl sulfoxide (DMSO) solution (100 ml) by heating and stirring the solution in a boiling water bath for 1 hr to remove complexed lipids. The solution was continuously stirred for an additional 24 hr at room temperature. Starch was precipitated by adding absolute ethanol (500 ml) and centrifuging (3,000 × *g*, 10 min). This process was repeated three times, and the final starch was washed with additional ethanol (300 ml) and dried overnight in an oven at 40°C.

^{31}P-NMR Spectroscopy

α-Limit dextrin aqueous solution (3 ml) or lipid extract aqueous solution (3 ml) was mixed with D_2O (1 ml), ethylenediaminetetraacetic acid (EDTA) dihydrate disodium salt (20 mg), and 0.2% sodium azide solution (40 μl). After the EDTA was completely dissolved, the solution was adjusted to pH 8.0–8.5 by adding 0.5*N* NaOH solution. ^{31}P-NMR spectra were obtained using a Bruker WM200 NMR spectrometer (USA Bruker Instruments, Mountain View, CA) at frequency 81 MHz, flip angle 65° (15 μsec), recycle time 1.4 sec, and sweep width 20,000 Hz. During data acquisition, proton resonance was broad-band decoupled. All chemical shifts were recorded in parts per million from an 85% H_3PO_4 external reference (as 0 ppm).

Phosphorus Contents

Total phosphorus contents in native starches and in α-limit dextrin solutions were determined by the method of Smith and Caruso (1964). α-Limit dextrin solutions were heated to dryness on a hot plate before analysis. Approximate concentrations for phosphorus of different chemical structures (i.e., starch phosphate monoester, phospholipids, and inorganic phosphate) were calculated from the ratio of the integrated NMR peak area and the total phosphorus content of the starch. All the calculated amounts of starch phosphate monoester, phospholipids, or inorganic phosphate were displayed as percent phosphorus based on the dry weight of starch.

RESULTS AND DISCUSSION

Chemical Shift of Phosphorus

Phosphomonoesters usually appear on a ^{31}P-NMR spectrum downfield from phosphodiesters because of their deshielding effects (Gorenstein 1984). Raising the pH causes dianion formation of phosphomonoesters and a further downfield shift (at ~4 ppm) (Glonek and Kopp 1985, James 1985). Phosphodiesters, however, are not significantly affected by the pH change, because their monoanionic structures are not changed.

Organic phosphorus in starch exists in two major forms, starch phosphate monoesters (Starch-P) and phospholipids (Lipid-P). Phospholipids in starch usually exist as phosphodiesters. Dianionic Starch-P were located in a range of 4.0–4.5 ppm from the external phosphoric acid, whereas Lipid-P was located ~0–1 ppm (Lim 1990, Lim and Seib 1993). Commercial lysophosphatidyl choline and lysophosphatidyl ethanolamine appeared at 0.2 and 0.3 ppm, respectively, consistent with the chemical shift assignment for Lipid-P (spectra not shown).

Normal Cereal Starches

Normal cereal starches from corn, wheat, rice, oat, and millet contained 0.016–0.065% organic phosphorus based on the dry starch weight (Table I). Corn starch had the least phosphorus content (0.016%) among normal cereal starches, whereas rice starch showed the greatest phosphorus content (0.065%). Genetic variations within each species also could differ in the phosphorus content. More than 85% of the phosphorus in the starch was solubilized by α-amylolysis, except rice starch (47%). Phosphorus in normal cereal starches existed mainly as Lipid-P appearing between 0 and 1 ppm on NMR spectra (Fig. 1 and Table II). Oat and millet starches showed three major Lipid-P signals at 0.1, 0.7, and 1.2 ppm, whereas corn and wheat starches did not show the 1.2 ppm signal. Corn and wheat starches displayed trace amounts (<0.001% based on the peak area) of Starch-P at 4.4 and 4.1 ppm, respectively (Fig. 1, marked by an arrow). Rice starch showed multiple peaks of Starch-P signals with relatively higher intensity than that of wheat or corn starches.

TABLE I
Phosphorus (P) Contents in Starches and Percent Recoveries of Phosphorus in α-Limited Dextrin (LD)[a]

Starch	Organic P in Starch (% dsb)	Inorganic P in Starch (% dsb)	P Recovery in α-LD (%)
Cereal starches			
Normal starches			
Corn	0.016	0.002	100
Wheat	0.054	ND[b]	85
Rice	0.065	0.001	47
Oat	0.056	ND	85
Millet	0.058	0.001	85
Waxy starches			
Amaranth	0.003	0.005	92
Waxy corn	0.002	0.001	79
du-Waxy corn	0.003	0.001	98
Waxy rice	0.005	ND	82
High-amylose corn starch	0.020	0.013	67
Root and tuber starches			
Potato	0.089	0.001	101
Sweet potato	0.012	ND	99
Tapioca	0.008	0.001	73
Lotus	0.005	ND	99
Arrowroot	0.021	0.001	98
Water chestnut	0.004	0.007	92
Legume starches			
Green pea	0.006	0.001	96
Lima	0.011	ND	100
Mung bean	0.012	0.001	83
Lentils	0.008	0.001	80

[a]Organic and inorganic P contents were calculated from the integrated ^{31}P-signal area and total P content in starch.
[b]Not detected on spectrum.

Rice starch displayed poor phosphorus recovery (47%) in the α-limit dextrin solution compared with other cereal starches (≥85%). Half of the phosphorus in the starch was lost in the starch hydrolysate precipitate. Defatting the rice starch by refluxing with an aqueous propanol (water and alcohol 1:3) for 24 hr did not improve phosphorus recovery. The defatted starch still showed the Lipid-P signals with significantly high intensity (Fig. 2a), indicating that the defatting procedure had not removed all the residual phospholipids from the rice starch. The lipid extracted from rice starch showed signals between 0 and 1 ppm (Fig. 2b), similar to those observed on the spectra of oat and millet starches. Water-saturated butanol (WSB) also was used to defat rice starch, and the WSB-defatted rice starch contained Lipid-P signals like the propanol-treated starch (Fig. 3), indicating that WSB treatment also was insufficient for complete removal of rice starch phospholipids.

Rice starch showed unusual signals (1.7 and 2.1 ppm) on the NMR spectrum, which were absent in the spectra of the other cereal starches (Fig. 1 and Table II). These signals were not found either on the aqueous propanol defatted rice starch (Fig. 2a) or on the lipid extract (Fig. 2b) spectra. Instead, the aqueous propanol defatted rice starch showed an intensified inorganic phosphate signal (2.9 ppm), which could have resulted from hydrolysis of the phosphorus components displaying 1.7 and 2.1 ppm signals. In contrast to the propanol-defatted rice starch, WSB-defatted rice starch retained the signals.

TABLE II
Organic Phosphorus (P) Contents (%, dry basis) in Starch[a]

Starch	Mono-P[b]	Lipid-P[c]	Unknown[d]
Cereal starches			
Normal starches			
Corn	Trace	0.016	ND[e]
Wheat	0.001	0.053	ND
Rice	0.013	0.048	0.004
Oat	ND	0.056	ND
Millet	ND	0.058	ND
Waxy starches			
Amaranth	0.003	Trace	ND
Waxy corn	0.002	Trace	ND
du-Waxy corn	0.002	ND	0.002
Waxy-rice	0.003	ND	0.002
High-amylose corn starch	0.013	0.014	ND
Root and tuber starches			
Potato	0.089	ND	ND
Sweet potato	0.011	ND	0.001
Tapioca	0.008	ND	ND
Lotus	0.005	ND	ND
Arrowroot	0.021	ND	ND
Water chestnut	0.004	ND	ND
Legume starches			
Mung bean	0.011	0.001	ND
Green pea	0.004	0.001	ND
Lentils	0.002	ND	0.006
Lima	0.011	Trace	ND

[a] Calculated based on integrated area of P-signals.
[b] Phosphate monoesters P-signals located at 4.0–4.5 ppm relative to external 80% orthophosphoric acid.
[c] Phospholipids P-signals located at −0.4 to 1.2 ppm.
[d] Three types of unknown signals: 1) 1–4 major signal at 1.5–2.5 ppm on the spectra for rice, waxy rice, and lentils starches; 2) a sharp signal at 8–11 ppm on the spectra of waxy rice and sweet potato starches; and 3) six major signals on green pea starch spectrum divided into groups; three signals each at −5 to −7 and −10 to −12 ppm.
[e] Not detectable.

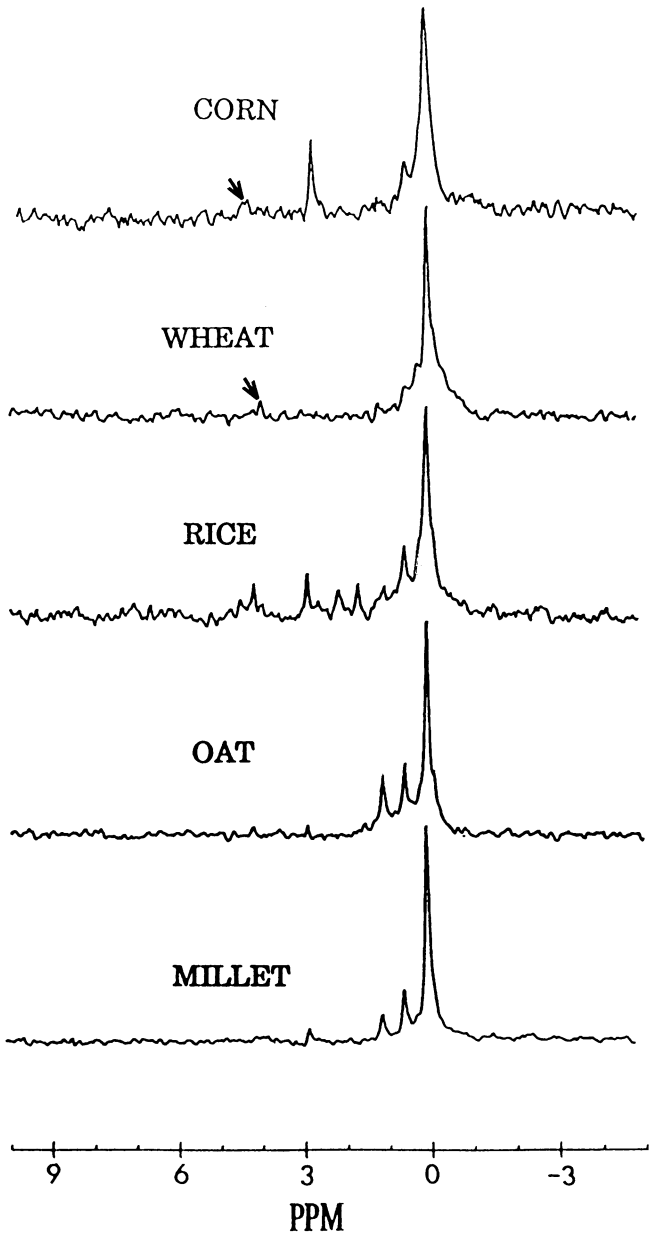

Fig. 1. ^{31}P-nuclear magnetic resonance spectra of normal cereal starches (corn, wheat, rice, oat, and millet). Trace amounts of Starch-P are indicated by arrow.

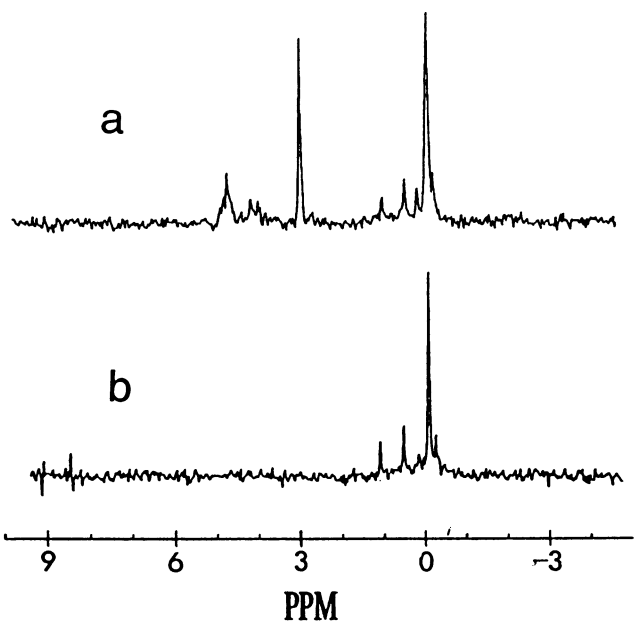

Fig. 2. ^{31}P-nuclear magnetic resonance spectra of rice starch defatted with an aqueous propanol (a), and the lipid extract (b). Starch was refluxed for 24 hr with a mixture of *n*-propanol and water (3:1).

Rice starch, after being treated with an aqueous DMSO solution (90%) and precipitated by ethanol, was completely free of Lipid-P (Fig. 4). The treated starch was readily solubilized by α-amylolysis. This may be attributed to a complete removal of complexed phospholipids from rice starch by the aqueous DMSO solution. The removal of the phospholipids destroyed helical complex crystals and rendered the starch susceptible to α-amylolysis. Four intense signals between 1.5 and 2.5 ppm appeared on the NMR spectrum of the treated-rice starch as the major phosphorus components in the defatted rice starch. Among the possible structures of phosphorus appearing in this region are anomeric sugar phosphates, which have been reported to display signals with a chemical shift range of 1.2–2.4 ppm (Glonek and Kopp 1985). Phosphoprotein was reported to have signals in a chemical shift range of 1.0–4.0 ppm at a neutral pH (James 1985). It would be rare to have a substantial amount of anomeric phosphate sugar in starch. Protein contents in the α-limited dextrin were ~0.4%. It is not known what structures of phosphorus these signals represent. More studies are needed to identify the nature of the phosphorus.

Waxy and High-Amylose Starches

Waxy cereal starches from waxy amaranth, waxy corn, *du*-waxy corn, and waxy rice contained much less phosphorus (0.002–0.005%) than did normal cereal starches (Table I). Waxy amaranth and waxy corn starch displayed an intense inorganic phosphate signal at 2.9 ppm. The inorganic phosphate in these starches was not solubilized in water (starch and water 1:4) by stirring the suspension for 24 hr at room temperature. This may indicate that the phosphate existed in starch granules with strong molecular interactions. The low phosphorus content in waxy starch was reported by Morrison et al (1984). They also reported a positive correlation between amylose and lipid contents in starch. Organic phosphorus in waxy starches existed mainly as Starch-P and appeared on the spectra at 4.0–4.5 ppm (Fig. 5 and Table II). Tabata et al (1975) reported that Japanese waxy rice starch contained Starch-P mainly as phosphate esters on the hydroxyl group of the primary carbon (C-6), whereas Japanese normal rice starch granules contained phosphorus in the form of Lipid-P. Based on the peak area, approximate phosphorus concentrations for Starch-P in the waxy starches were in the 0.002–0.003% range. *du*-Waxy corn starch showed a relatively strong signal at 0.3 ppm, indicating the presence of Lipid-P (Fig. 5). The phospholipids were correlated to the extensively long, long-B chain of the amylopectin (J. Chen and J.-L. Jane, *unpublished data*).

Like normal rice starch, waxy rice starch contained phosphorus components that appeared in the 1.5–2.5 ppm range of on the NMR spectrum (Fig. 5).

High-amylose (70% amylose) corn starch contained 0.020% phosphorus, which consists of Starch-P and Lipid-P in an approximate 1:4 ratio (Table I, Table II, and Fig. 6). The approximate phosphorous content of Starch-P in high-amylose corn starch was 0.004%. This value was greater than those of normal or waxy corn starches (0.001–0.002%).

Root and Tuber Starches

Organic phosphorus contents in root and tuber starches (potato, sweet potato, tapioca, lotus, arrowroot, and water chestnut) varied widely from 0.004 to 0.089% (Table I). Potato starch contained an exceptionally large amount of Starch-P (0.089%) compared with other root or tuber starches (0.004–0.021%) (Table II). All the tested root and tuber starches seemed to be free of lipid-P on ^{31}P-NMR spectra (Fig. 7), supporting early findings (Posternak 1935, 1951; Hodge et al 1948; Hizukuri et al 1970). No phosphorus was extracted from potato starch in aqueous propanol during

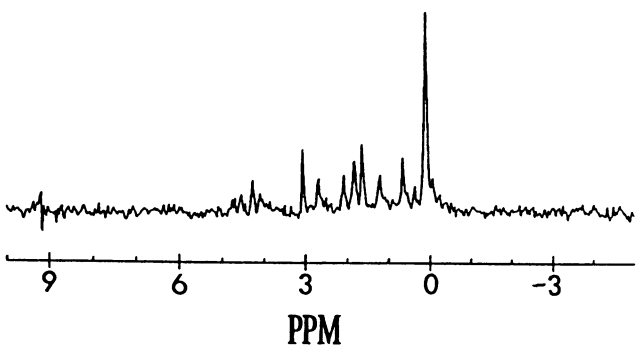

Fig. 3. ^{31}P-nuclear magnetic resonance spectrum of rice starch defatted with water-saturated butanol by refluxing 24 hr.

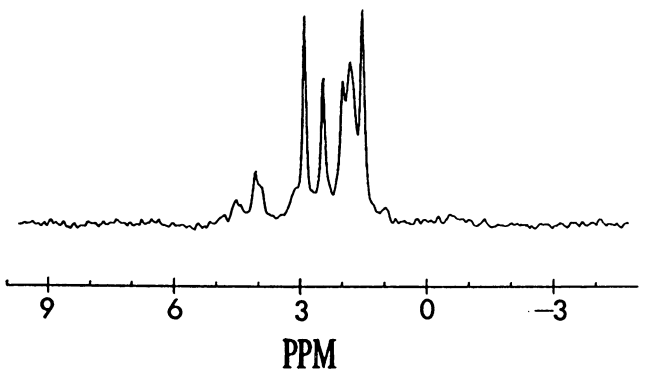

Fig. 4. ^{31}P-nuclear magnetic resonance spectrum of treated rice starch by using dimethyl sulfoxide and ethanol.

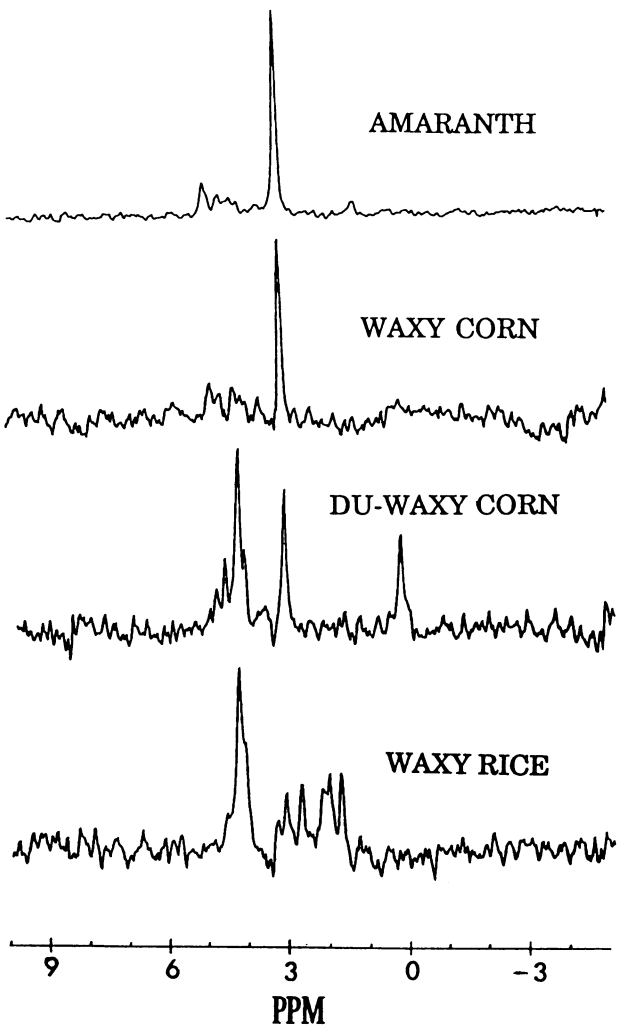

Fig. 5. ^{31}P-nuclear magnetic resonance spectra of waxy cereal starches (amaranth, waxy corn, *du*-waxy corn and waxy rice).

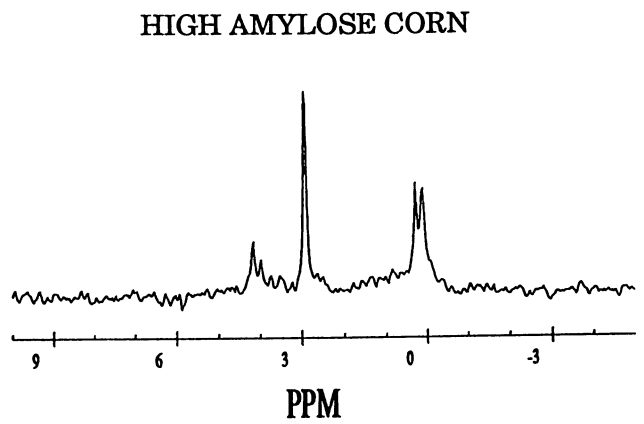

Fig. 6. ^{31}P-nuclear magnetic resonance spectrum of high-amylose corn starch (70% amylose).

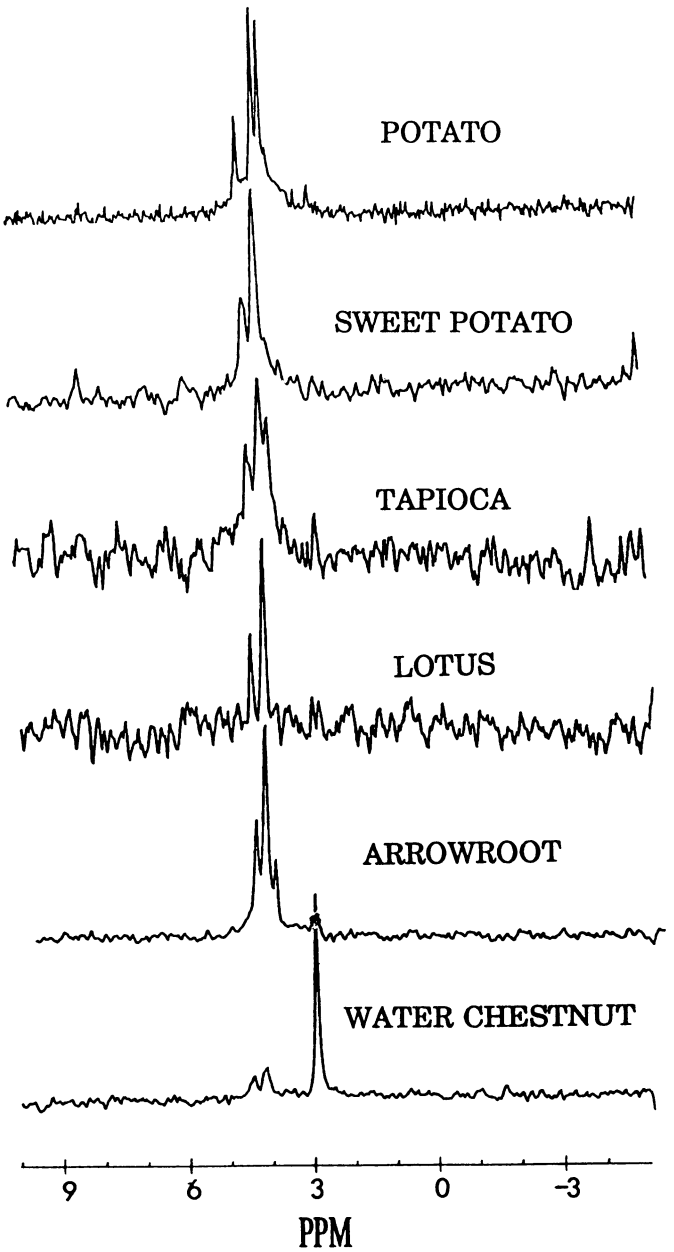

Fig. 7. ^{31}P-nuclear magnetic resonance spectra of root and tuber starches (potato, sweet potato, tapioca, lotus, arrowroot, water chestnut).

24 hr of refluxing, which also confirmed that the starch was free of Lipid-P. Root and tuber starches contained only trace amounts of inorganic P, except water chestnut starch.

Lim and Seib (1993) reported that two adjacent signals (at ~4.0 ppm) among the three major signals on the potato starch spectrum indicated phosphate monoesters on the hydroxyl groups of the primary carbons (C-6) of inner anhydrous glucose units. The third signal, at 4.4 ppm, was assigned as the phosphate on the secondary hydroxyl group on C-3 of inner anhydrous glucose units. Based on the ^{31}P-NMR spectra, the root or tuber starches contained more phosphate derivatives on C-6 than they did on C-3 (Fig. 7). Sweet potato starch and lotus starch gave a single signal for the primary carbon phosphates, whereas other starches produced dual signals. This result indicates that the phosphates on the primary carbon (C-6) in sweet potato and lotus starches were located at uniform positions on the inner anhydrous glucose units.

Legume Starches

Four legume starches from green pea, lima bean, mung bean, and lentil pea were examined on ^{31}P-NMR spectra. The legume starches had organic phosphorus in the 0.006–0.012% range, which appeared mainly as Starch-P on NMR spectra (Table I, Table II, and Fig. 8).

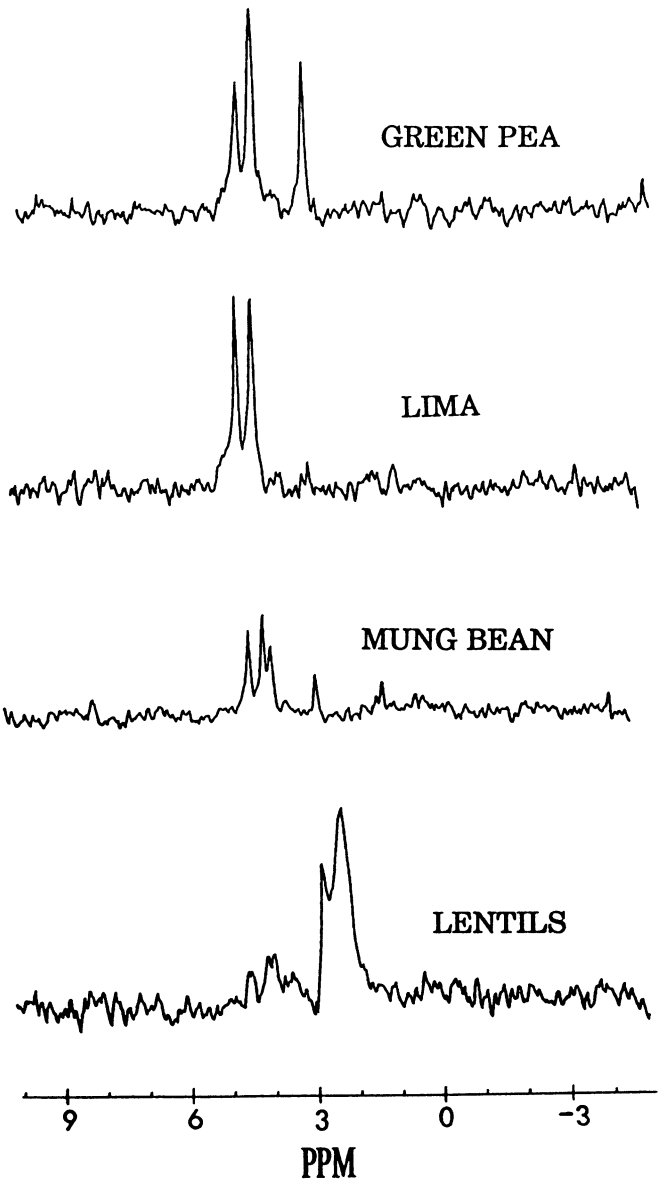

Fig. 8. ^{31}P-nuclear magnetic resonance spectra of legume starches (green pea, lima bean, mung bean, and lentil pea).

Lentil pea starch showed a broad and large signal at ~2.1 ppm, with threefold intensity of the signals at ~4.0 ppm (Fig. 8). The chemical shift of the signal was identical to one of the unknown phosphorus signals from rice starch, possibly indicating similar structures.

Green pea and lima bean starches showed two signals for Starch-P, as phosphomonoesters on the primary (C-6) and secondary (C-3) hydroxyl groups, as did the sweet potato and lotus starches. The single peak for the phosphorylation on the primary carbon may indicate its uniform location on the anhydrous glucose units (Lim and Seib 1993). Lima bean starch showed almost the same signal intensity for both Starch-P, indicating an approximately equivalent degree of phosphorylation on C-6 and C-3, whereas green pea and mung bean starches showed an approximate 2:1 ratio for C-6 and C-3 phosphorylations.

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