Quantitative Method for the Survey of Starch Phosphate Derivatives and Starch Phospholipids by ^{31}P Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

Phosphorus of different chemical structures (e.g., phospholipids, starch phosphate monoester, and inorganic phosphate) are found in starch. In contrast to the colorimetric chemical method (Smith and Caruso 1964), which determines total phosphorus content in starch without differentiating phosphate monoester from phospholipids, ^{31}P nuclear magnetic resonance (NMR) spectroscopy determines phosphorus chemical structures and their individual contents. The relaxation times (T_1) of starch phosphate monoesters, phospholipids, inorganic phosphate, and phosphate derivatives in nicotinamide adenine dinucleotide (NAD) (internal reference standard) ranged from 1.0 to 2.1 sec. To ensure full relaxation between pulses for quantitative results, a relaxation delay of 11 sec was programmed between data acquisitions. Dimethyl sulfoxide solution (45%, v/v) (DMSO) was used to improve α-limited dextrin solubility. ^{31}P NMR spectroscopy of this solution provided quantitative results. ^{31}P NMR results showed that potato starch contained mainly phosphate monoester (0.086%), wheat starch contained mostly phospholipids (0.058%), mung bean starch contained mainly phosphate monoester (0.0083%) and phospholipids (0.0006%), tapioca starch contained mainly phosphate monoester (0.0065%), high-amylose (50% amylose) maize starch contained mainly phospholipids (0.015%) and phosphate monoester (0.0049%), and waxy maize starch contained only a trace of phosphate monoester. The total phosphorus contents in starches obtained by ^{31}P NMR spectroscopy agreed with those obtained from the colorimetric chemical method. Acid hydrolysis of starch and high-temperature operation were attempted to improve the α-limited dextrin solubility, but the amylose-phospholipid complexes remained insoluble in the aqueous solution, and the structures of phosphate derivatives and phospholipids were altered.

Phosphorus in starch is found in three major forms: starch phosphate monoester, phospholipids, and inorganic phosphate. Phosphorus structures and contents in starches vary with the botanical source, maturity, and growing conditions of the plant (Muhrbeck and Tellier 1991, Bay-Smidt et al 1994, Lim et al 1994, Nielsen et al 1994). Most normal cereal starches contain phosphorus in the form of phospholipids (Schoch 1942, Morrison 1981, Hizukuri et al 1983, Morrison and Gadan 1987, Morrison 1988, Lim et al 1994), whereas phosphorus in root and tuber starches is in the form of starch phosphate monoesters (Schoch 1942, Hizukuri et al 1970, Tabata et al 1975, Lim et al 1994).

Lim et al (1994) characterized phosphorus in a variety of starches by using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. They reported that root and tuber starches (i.e., potato, sweet potato, tapioca, lotus, arrow root, and water chestnut) contain mainly starch phosphate monoesters with some inorganic phosphate; no phospholipids were found in these starches. Normal cereal starches (i.e., maize, wheat, rice, oat, and millet) contain mainly phospholipids. Normal rice starch also contains a small amount of phosphate monoester. Legume starches (i.e., green pea, lima bean, mung bean, and lentils) contain mainly starch phosphate monoester. Waxy starches (i.e., waxy maize, waxy rice, *du*-waxy maize, and amaranth) contain mainly phosphate monoester; *du*-waxy maize and waxy rice starches also contain small amounts of phospholipids. Total contents of phosphorus in the starches were determined by the chemical method of Smith and Caruso (1964).

Phosphorus in starch plays important roles in starch functional properties. For example, phosphate monoester in potato starch accounts for paste clarity, high pasting viscosity, low gelatinization temperature, and slow retrogradation rate. Phospholipids in wheat starch reduce the paste clarity and the pasting viscosity (Schoch 1942, Swinkels 1985, Lim 1990).

Quantification of phosphorus in organic matter has been analyzed by using numerous methods (Telep and Ehrlich 1958, Cincotta 1960, Morrison 1964, Smith and Caruso 1964, Kovacs 1986, Singh and Ari 1987). These methods are based on the destruction of organic matter by incineration or by wet oxidation and converting the phosphorus into its inorganic form. These methods have several disadvantages: requiring large samples, being time consuming, having color instability, and only providing the total phosphorus content.

^{31}P NMR has been used to characterize the phosphorus in starches and also has been used to identify phosphorylation in modified starches (McIntyre et al 1990, Muhrbeck and Tellier 1991, Lim and Seib 1993, Bay-Smidt et al 1994, Kasemsuwan and Jane 1994, Lim et al 1994). Those studies, however, provide only qualitative results. The objective of this study was to develop a quantitative method, by using ^{31}P NMR spectroscopy, to survey starch phosphate derivatives and phospholipids and their contents in starches. This method may help scientists reveal the chemical structures of starch phosphorus, as well as understand the correlation between the structures and functional properties of starch. The results of the total phosphorus content obtained by ^{31}P NMR spectroscopy were compared with those obtained by the colorimetric chemical method.

MATERIALS AND METHODS

Materials

Maize, wheat, potato starches, β-glycerophosphate, a mixture of α- and β-glycerophosphates, and crystalline α-amylase of Bacillus species (Type IIA, 1,270 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO); tapioca and high-amylose maize (Hylon-5) starches were gifts of the National Starch and Chemical Co. (Bridgewater, NJ); waxy maize starch was a gift of the American Maize-Products Co. (Hammond, IN); and mung bean starch was purchased from the Srithinun Co. (Bangkok, Thailand). Dimethyl-d_6 sulfoxide was purchased from Cambridge Isotope Laboratories (Andover, MA). Rice gluten was a gift of Riceland Foods Inc. (Jonesboro, AR).

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α-Limited Dextrin Preparation

Starch (2 g, dsb) was suspended in 6.0 ml of acetate buffer (0.01*M*, pH 6.9). α-Amylase (0.5 mg) was added, and the suspension was heated and stirred in a water bath to boil for ≈10 min, following the method of Lim et al (1994). High-amylose maize starch was autoclaved for 1 hr (with α-amylase added). After the solution was cooled to 70°C, additional enzyme (1 mg) was added, and the digestion was continued by incubation in a waterbath shaker at 70°C for 2 hr. The hydrolysate was heated in a boiling water bath for 10 min to stop the enzyme reaction. The hydrolysate was frozen (–85°C) and dried in a freeze dryer (Virtis, Unitrap II, Gardiner, NY).

^{31}P NMR Spectroscopy

The freeze-dried α-limited dextrin was resuspended in 90% deuterated DMSO (1.5 ml) and heated in a boiling waterbath for 10 min. To develop an internal reference standard, the solution was mixed with 1.0 ml of deuterium oxide and 0.5 ml of nicotinamide adenine dinucleotide (NAD) at a concentration proportional to the phosphorus content in the starch analyzed. The solution was adjusted to pH 8.0 ± 0.1.

In an attempt to improve the solubility of α-limited dextrin in the aqueous solution, the α-limited dextrin (3 ml) was further hydrolyzed by adding hydrochloric acid (HCl, 4*M*) to a final concentration of 0.7*M*. The solution was stirred in a boiling water bath for 4 hr, and the hydrolysate was neutralized with sodium hydroxide (NaOH, 4*M*) (Bay-Smidt et al 1994). The hydrolysate was adjusted to pH 8.0 ± 0.1 before NMR analysis.

^{31}P NMR spectra were acquired by using a Bruker AC-200 NMR spectrometer (USA Bruker Instruments, Billerica, MA) at a frequency of 81 MHz, flip angle 90° (24 μsec), sweep width 31.18 ppm, 8 k data points, and temperature at 298° K. Relaxation times (T_1) of the phosphorus containing compounds were measured by using the inversion recovery method at 180° and 90°. A relaxation delay of 11 sec was inserted to ensure full relaxation between pulses. The Waltz-16 sequence was used for proton decoupling, and 5,000 scans were collected for each spectrum. All chemical shifts were recorded in parts per million (ppm) from 85% phosphoric acid as an external reference (as 0.0 ppm), and the line broadening for all the spectra was 1.0 Hz. A curve fitting software (NMR1 Version 1, 1992, New Methods Research, Inc. East Syracuse, NY) was used to quantify the area of each NMR peak.

Phosphorus Analysis

For colorimetric chemical analysis, total phosphorus content in starch was determined following the Smith and Caruso method (1964). ^{31}P NMR spectroscopy was undertaken based on the phosphorus contents of starch phosphate monoester, phospholipids, and inorganic phosphate. They were calculated on the basis of the ratio of their peak areas compared with the peak area of a known concentration of the internal reference compound (NAD). The total phosphorus content of each starch was calculated as the sum of all the phosphorus contents.

Statistical Methods and Analysis

A two-factor factorial was used to design experiments. The first factor was a method of determination of phosphorus contents (Colorimetric Chemical Method and ^{31}P NMR Spectroscopy Method). The seven starches contributed the second factor. Thus there were 14 treatment combinations. The experiment was conducted by using a randomized complete block design with two blocks. Each block contained a complete set of 14 treatment combinations and was obtained at separate times. For the chemical method, three determinations were obtained and averaged for each of the 14 treatment combinations in each block. A single measurement of each starch sample was obtained with the ^{31}P NMR spectroscopy. Treatment means and standard errors were reported. Least significant difference (LSD) (0.05), was calculated to compare the phosphorus contents of the starches.

RESULTS AND DISCUSSIONS

Phosphorus was found in small quantities in most native starches. Therefore, we needed to increase the concentration of starch solution for the analysis. Starch was hydrolyzed by α-amylase to increase its solubility. Enzymatic hydrolysis decreased the viscosity of the starch paste to prevent peak broadening and concentrated the starch solution to speed up the data acquisition.

^{31}P NMR spectroscopy of starch samples conducted with aqueous solutions gave only qualitative results because of the limited solubility of α-limited dextrin. Fine particles were observed in the hydrolysate, which was attributed to the starch-phospholipid complex (Jane et al 1996). To obtain quantitative results, the α-limited dextrin was solubilized in DMSO solution, and a long pulse relaxation interval (11 sec) was used (five times the relaxation interval of the starch monoester). The relaxation times (T_1) of phosphorus with different chemical structures are shown in Table I.

^{31}P NMR spectrum of potato starch in 45% DMSO solution (Fig. 1) showed signals mainly at chemical shifts 4.1 and 5.2 ppm, which indicated phosphate monoester, and a minor peak at the chemical shift 3.0 ppm, which indicated inorganic phosphate (Kasemsuwan and Jane 1994, Lim et al 1994). Potato starch contains a significant amount of phosphorus, thus the spectrum of potato starch was acquired with only 500 scans. Wheat starch (Fig. 2) showed the signals mainly at chemical shifts between 0.0 and 1.5 ppm, which indicated phospholipids (Kasemsuwan and Jane 1994, Lim et al 1994). The ^{31}P NMR signal of phospholipids showed a broad peak because the phospholipids consisted of a mixture of compounds with similar molecular structures, such as phosphatidylethanolamine and phosphatidylcholine. Those compounds gave similar chemical shifts that overlapped and cannot be resolved by NMR spectroscopy. Mung bean starch (Fig. 3)

TABLE I
Relaxation Times and Chemical Shifts[a]

Types	Chemical Shift, ppm	T_1, sec (SD)[b]
Phosphate monoester	5.39	2.127 (0.006)
	4.08	1.784 (0.004)
Inorganic phosphate	3.00	1.422 (0.009)
Phospholipids	0.77	0.85 (0.02)
	0.54	1.03 (0.03)
NAD[c] (internal reference)	10.37	1.079 (0.004)

[a] Data were obtained in 45% dimethyl sulfoxide solution.
[b] Relaxation time (standard deviation in parentheses).
[c] Nicotinamide adenine dinucleotide.

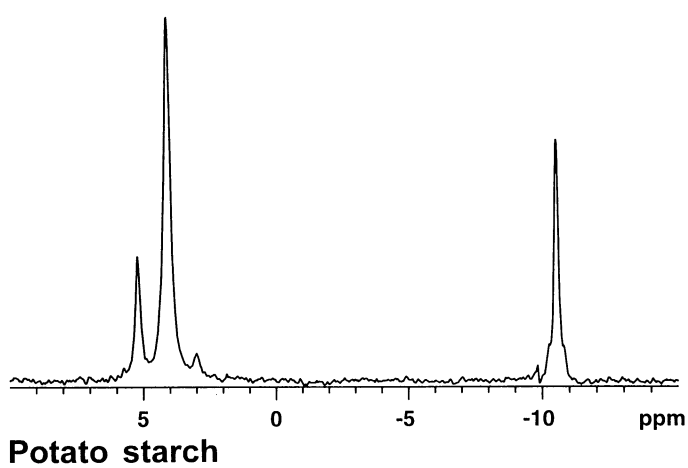

Fig. 1. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from potato starch. Signal at 10.5 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 7.5 mg).

showed signals mainly at 4.0 and 5.5 ppm, which indicated phosphate monoester; the signal at 1.8 ppm indicated phosphoprotein, which coincided with the peaks of rice gluten at the chemical shift range from 1.5 to 3.0 ppm (data not shown), and the signal at 0.7 ppm indicated phospholipids. Tapioca starch (Fig. 4) showed broad signals at the chemical shift between 3.5 and 5.5 ppm which indicated phosphate monoesters. Tapioca starch contained a very small amount of total phosphorus (Tables II and III), thus the ^{31}P NMR spectroscopy required at least 8,000 scans to get a spectrum with an adequate signal-to-noise ratio. Maize starch (Fig. 5) showed a broad signal at the chemical shift between 0.5 and 1.5 ppm, which indicated phospholipids, and a minor peak at 3.0 ppm which indicated a small amount of inorganic phosphate.

High-amylose maize starch (50% amylose) (Fig. 6) showed signals mainly at the chemical shift between 0.5 and 2.0 ppm, which indicated phospholipids. A large peak at 3.0 ppm indicated a large amount of inorganic phosphate, which might have been derived from the hydrolysis of other derivatives as a result of the autoclaving preparation of the starch sample. It also showed a small signal at 4.0 ppm, which indicated phosphate monoester. Waxy maize starch (Fig. 7) showed a very small signal (bump) at chemical shift 5.5 ppm, which indicated phosphate monoester. The spectrum displayed a high noise baseline because of the low concentration of phosphorus in the waxy starch. The high noise level in the ^{31}P NMR spectra limited identification and measurement of the peak area. ^{31}P NMR spectra of the sample in a DMSO solution displayed broader peaks, with a lower resolution, than those prepared in an aqueous solution. For example, the spectrum of potato starch in an aqueous solution displayed split peaks at chemical shifts 4.2 and 4.5 ppm (Fig. 8a), but that obtained in DMSO solution displayed a broad peak (Fig. 1).

TABLE II
Phosphorus Content in Starches[a,b]

Starches	Phosphate Monoester	Phospholipids	Inorganic Phosphate
Potato	0.086 ± 0.007[c]	nd[d]	0.0048 ± 0.0003
Wheat	nd	0.058 ± 0.002	Trace
Mung bean[e]	0.0083 ± 0.0002	0.0006 ± 0.0001	nd
Tapioca	0.0062 ± 0.0004	nd	Trace
Maize	0.003 ± 0.001	0.0097 ± 0.0001	0.0013 ± 0.0007
High amylose maize (50%)	0.005 ± 0.001	0.015 ± 0.003	0.0076 ± 0.0006
Waxy maize	0.0012 ± 0.0006	nd	0.0005 ± 0.0001

[a] Percentage of phosphorus in starch (dsb, w/w).
[b] Analysis was replicated twice.
[c] Standard deviation.
[d] Not detectable.
[e] Mung bean starch also contained phosphoproteins (0.0028 ± 0.0015).

TABLE III
Total Phosphorus Content[a,b]

Starch	Method[c]		
	Chemical Analysis	^{31}P NMR Analysis	Starch Mean[d]
Potato	0.090	0.091	0.090
Wheat	0.057	0.058	0.057
Mung bean	0.012	0.012	0.012
Tapioca	0.007	0.006	0.007
Maize	0.019	0.014	0.016
High amylose maize (50%)	0.030	0.028	0.028
Waxy maize	0.002	0.002	0.002
Method mean	0.031	0.030	
(standard error)	(0.0006)	(0.0006)	

[a] Percentage of phosphorus in starches (dsb, w/w).
[b] Analysis was replicated twice.
[c] Standard error of a starch-method mean is 0.0016.
[d] Least significant difference for starch mean is 0.0035 (LSD 0.05).

The ^{31}P NMR spectra (Figs. 1–7) showed both the structure and the quantity of each form of phosphorus in starch. The quantity of phosphorus in each form is shown in Table II. Tuber starches (e.g., potato and tapioca) contained mainly phosphate monoester derivatives. Cereal starches (e.g., wheat, maize, and high-amylose maize) contained mainly phospholipids. Waxy maize starch contained a trace amount of phosphate monoester. Mung bean starch contained mainly phosphate monoester and a small amount of phospholipids.

To reduce the deviation of the measurement caused by the large difference between peak sizes, the concentration of the reference compound (NAD) was adjusted to a range similar to the phosphorus content of starch. Potato and wheat starch samples each had 7.50 mg of internal reference standard (NAD) added into a 3-ml sample solution; high-amylose maize, normal maize, and mung bean starch each had 1.25 mg of NAD; the tapioca starch sample had 0.625 mg of NAD, and waxy maize starch had 0.312 mg of NAD. The spectra (Figs. 1–7) revealed that each starch contained different phosphorus structures and contents that correlated to its physical properties.

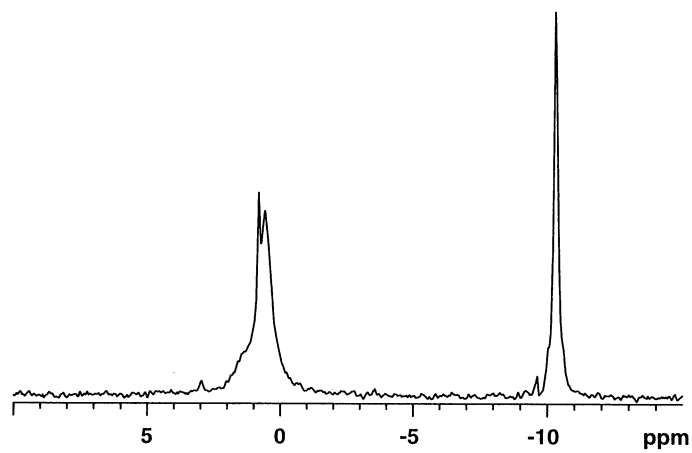

Fig. 2. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from wheat starch. Signal at 10.2 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 7.5 mg).

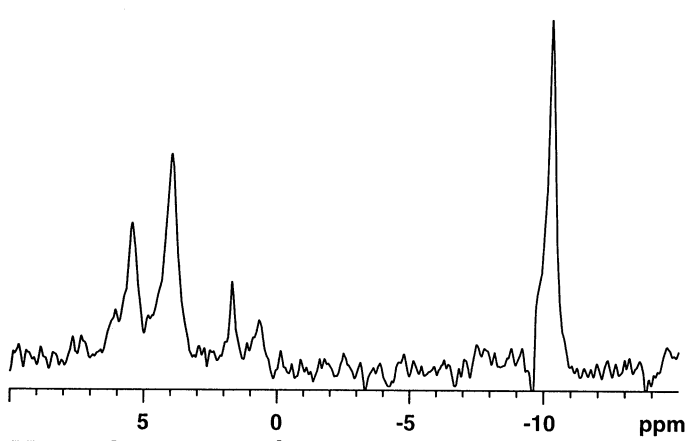

Fig. 3. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from mung bean starch. Signal at 10.2 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 1.25 mg).

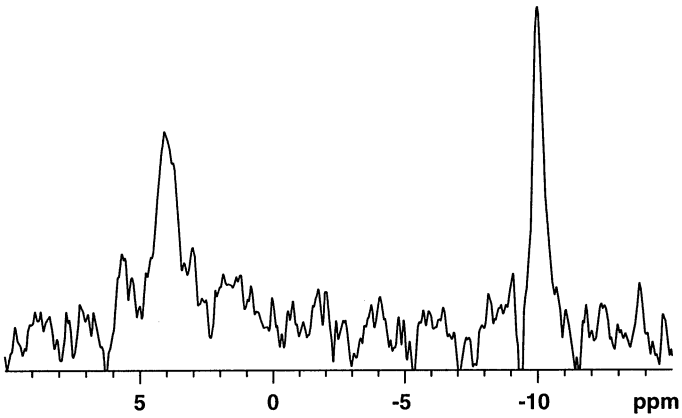

Fig. 4. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from tapioca starch. Signal at 10.1 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 0.625 mg).

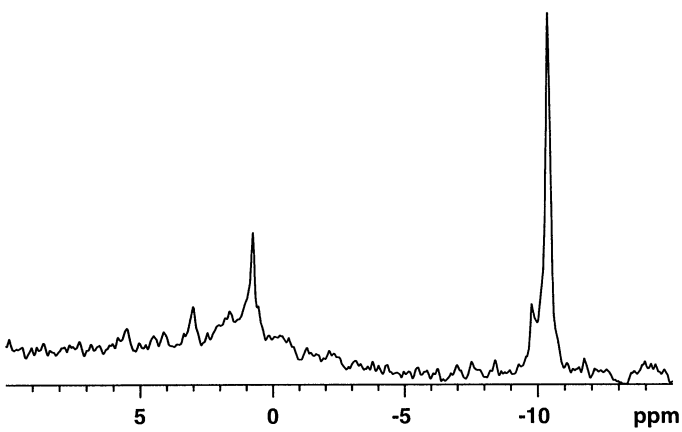

Fig. 5. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from normal maize starch. Signal at 10.4 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 1.25 mg).

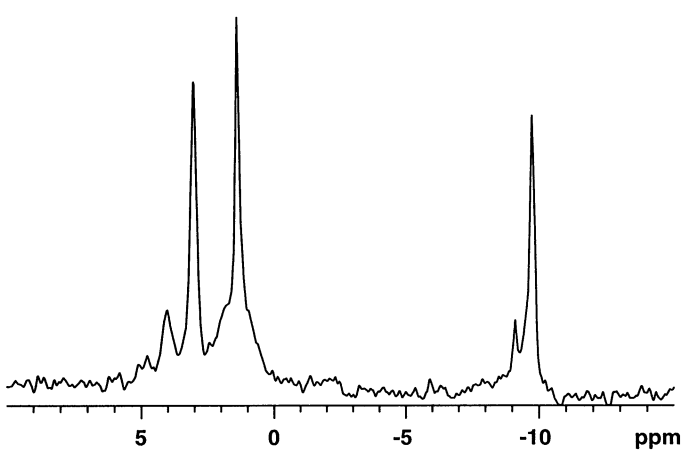

Fig. 6. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from high-amylose maize starch (50% amylose). Signal at 9.8 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 12.5 mg).

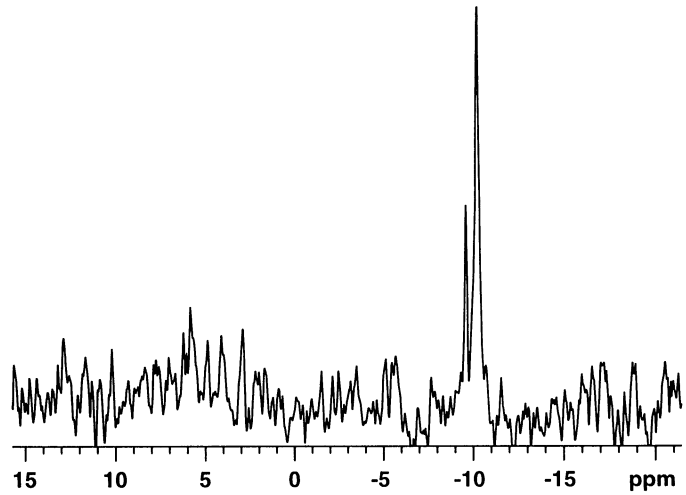

Fig. 7. ^{31}P nuclear magnetic resonance spectrum of α-limited dextrin prepared from waxy maize starch. Signal at 10.2 ppm is an internal reference standard (nicotinamide adenine dinucleotide, 0.3125 mg).

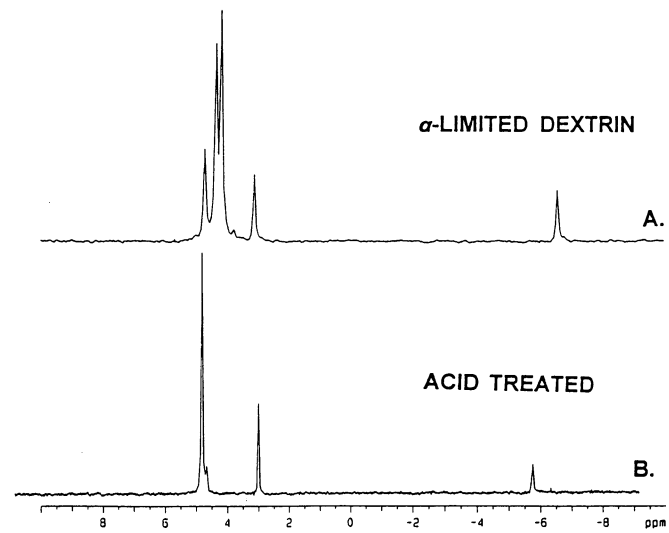

Fig. 8. ^{31}P nuclear magnetic resonance spectra of α-limited dextrin prepared from potato starch: **A,** without acid hydrolysis. Signals between 3.5 and 5.0 ppm are phosphate monoesters. **B,** with acid hydrolysis. Signal at 4.9 ppm is glucose-6-phosphate. Signal at 3.0 ppm is inorganic phosphate.

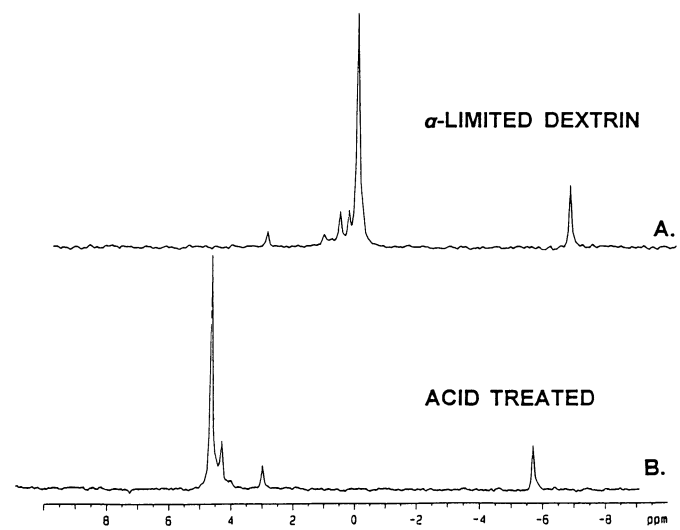

Fig. 9. ^{31}P nuclear magnetic resonance spectra of α-limited dextrin prepared from wheat starch: **A,** without acid hydrolysis. Signals between –1.0 and 1.0 ppm are phospholipids. **B,** with acid hydrolysis. Signal at 4.6 ppm is glycerophosphate. Signal at 3.0 ppm is inorganic phosphate.

The total phosphorus content determined by chemical analysis was compared with that calculated from the total peak area of ^{31}P NMR spectra (Table III). Data obtained for potato, wheat, mung bean, and waxy maize were in good agreement; however, maize, tapioca, and high-amylose maize had fairly large deviations because of the broad peaks, low phosphorus concentrations, and a high noise ratio in the spectra. The statistical analysis indicated that the phosphorus contents varied between the varieties of starches, but the phosphorus contents obtained by the two methods of analysis did not vary. There was no significant difference (at 0.05) in phosphorus contents between these two analyses.

Because α-limited dextrin was not completely soluble in the aqueous solution, a quantitative analysis by liquid NMR spectroscopy was impaired. Acid hydrolysis was attempted to improve the solubility of α-limited dextrin. The α-limited dextrin was hydrolyzed with hydrochloric acid (0.7*M*) in a boiling water bath for 4 hr (Bay-Smidt et al 1994). ^{31}P NMR spectra of the aqueous acid hydrolysates were different from those of aqueous α-limited dextrin. The spectrum of potato starch acid hydrolysate (Fig. 8b) showed a high intensity phosphate monoester signal at 4.9 ppm, a small signal at 4.7 ppm, and an increased intensity of inorganic phosphate signal (3.0 ppm). Compared with the α-limited dextrin spectrum (Fig. 8a), the phosphate monoesters, which occurred at different positions (C-6, C-3, C-2) of α-limited dextrins (Lim and Seib 1993), were hydrolyzed and produced mainly glucose-6-phosphate (4.9 ppm) and a small amount of glucose-3-phosphate (4.7 ppm) (Lim and Seib 1993, Kasemsuwan and Jane 1994). The signal at 3.0 ppm (inorganic phosphate) also increased. The result indicated that the phosphate monoesters of C-2 and C-3 were acid labile. The phosphate monoester of C-2, constituting ≈1% of total phosphate in potato starch (Tabata and Hizukuri 1971), was undetectable or hydrolyzed as indicated by the absence of glucose-2-phosphate signal at 4.3 ppm (Lim and Seib 1993, Kasemsuwan and Jane 1994). Whereas, the phosphate monoester of C-3, constituting 38% of the total phosphate content (Tabata and Hizukuri 1971), was partially hydrolyzed with a small amount of remaining glucose-3-phosphate (4.7 ppm). This result agreed with that reported by Tabata and Hizukuri (1971).

The spectrum of wheat starch acid hydrolysate (Fig. 9b) showed a phosphate monoester signal at 4.6 ppm, which coincided with the chemical shift of β-glycerophosphate and a mixture of α- and β-glycerophosphates (4.6 ppm) (data not shown). The results indicated that phospholipids were hydrolyzed during the acid hydrolysis and produced glycerophosphate (Christie 1982). The phosphate diesters of phospholipid signals in the α-limited dextrin spectrum (–1.0 to 1.0 ppm) (Fig. 9b) disappeared after the acid hydrolysis. The inorganic phosphate signal remained the same. The spectrum of rice starch acid hydrolysate also showed a high intensity of phosphate monoester signal at 4.6 ppm, whereas the phospholipid signals of the α-limited dextrin disappeared, indicating the hydrolysis of phospholipids. The phosphoprotein signals (1–3 ppm), confirmed by comparing with the spectrum of isolated rice gluten, remained at the same chemical shifts, but the signal intensities reduced, which indicated some hydrolysis occurred (Fig. 10a,b). The results indicate that acid hydrolysis cannot be used to prepare starch samples for the quantitative analysis by ^{31}P NMR spectroscopy. Structures of the phosphorus in starches were obviously changed by the acid hydrolysis, but some dextrin-lipid complex still remained insoluble.

^{31}P NMR spectra of the acid hydrolysate showed that the chemical shift of the pyrophosphate (added as an internal reference) was changed because of the high salt concentration (which came from the neutralization). This was confirmed by the chemical shift of the pyrophosphate peak of the potato α-limited dextrin moved from –6.6 ppm to –5.8 ppm with an addition of 1% sodium chloride, whereas the chemical shifts of the inorganic phosphate and the phosphate monoesters remained the same (data not shown).

High-temperature (70°C) ^{31}P NMR spectroscopy also was attempted to improve the solubility of the α-limited dextrin. The spectra showed high noise, and the insoluble precipitant was still present (data not shown).

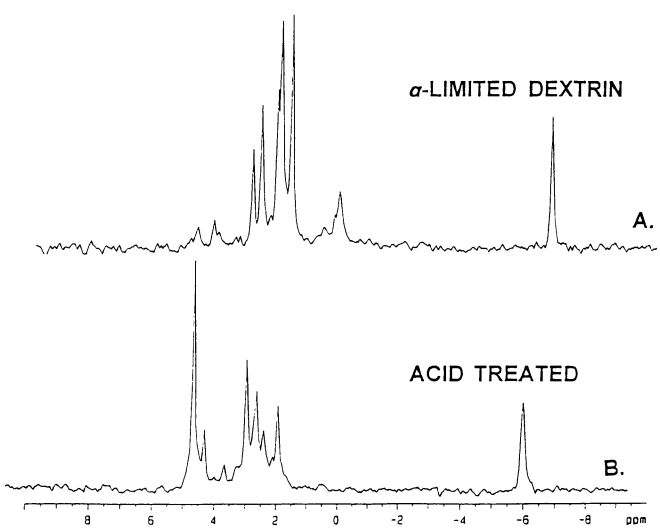

Fig. 10. ^{31}P nuclear magnetic resonance spectra of normal rice starch α-limited dextrin: **A,** without acid hydrolysis. Signals between –1.0 and 1.0 ppm are phospholipids. **B,** with acid hydrolysis. Signal at 4.6 ppm is glycerophosphate. Signals between 1.0 and 3.0 ppm are phosphoproteins.

CONCLUSIONS

^{31}P NMR spectroscopy revealed the chemical identity and contents of each form of phosphorus in starch. ^{31}P NMR spectra of those starch samples solubilized in DMSO solution provided quantitative results, but those prepared in aqueous solution contained amylose-phospholipids complex, which was insoluble and did not provide quantitative results. Results of starch total phosphorus contents in DMSO solution obtained by ^{31}P NMR spectroscopy were in good agreement with those obtained from the chemical method. Acid hydrolysis and the high temperature operation did not improve the quantitative analysis. The acid hydrolysate changed the structures of the phosphorus in the starch.

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