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Characterization of phosphorylated cross-linked resistant starch by ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy

Yijun Sang [a], Om Prakash [b], Paul A. Seib [a,*]

[a] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA
[b] Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA

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Abstract

The structure of phosphate esters in resistant starch (RS_4) is important to understand its digestibility by pancreatic α-amylase. Wheat starch was reacted at pH 11.5, 45 °C, and 3 h with 12% (starch basis, sb) of a 99/1 (w/w) mixture of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP) to give modified starches with ~0.4% phosphorus (P). Exhaustive digestion of the phosphorylated starch with α-amylase followed by amyloglucosidase gave the α,γ-dextrin whose ^{31}P NMR spectrum indicated the presence of a ~1/1 molar mixture of distarch monophosphate (DSMP) and monostarch monophosphate (MSMP). Approximately one-half of STMP/STPP was recovered unreacted after the typical reaction, and it was reacted with a second batch of starch after replenishing with 7% (sb) STMP/STPP. RS_4 wheat starches prepared at pH 9.5–10.5 showed a decreasing ratio of DSMP/MSMP plus an increasing level of monostarch diphosphate (MSDP) and cyclic monostarch monophosphate as reaction pH decreased. Starch triphosphate ester was not detected in the RS_4 products, all of which had been prepared at reaction times greater than three hours. A series of RS_4 wheat starches were prepared where all contained ~0.4% P but with varying levels of DSMP from 41% to 80% of organophosphates. The level of DSMP was positively correlated with the level of RS_4 ($r = 0.96$) and with the level of total dietary fiber ($r = 0.90$).

Keywords: Phosphorylated resistant starch; ^{31}P NMR spectroscopy; Distarch monophosphate; Monostarch monophosphate

1. Introduction

Resistant starch (RS) may be desirable in the human diet principally because of its prebiotic effects and associated health benefits for the colon. RS is fermentable in the large intestine causing little, if any, discomfort, and producing mild laxation due to increased microbial mass. The principal products of fermentation are short-chain fatty acids, which lower colon pH by 1–2 U, plus some carbon dioxide and hydrogen. The acidic pH in the colon prevents the proliferation of pH-sensitive toxic bacteria and lowers the level of secondary bile acids that are cytotoxic at alkaline pH. Among the short-chain fatty acids, butyrate is the preferred energy source of the colonocytes lining the large intestine, and it induces cell death (apoptosis) in tumor cell lines. The high levels of butyric acid in the proximate colon where most fermentation of RS occurs may explain the prevalence of cancer and ulcerative colitis in the distal colon (Topping & Clifton, 2001). Starches rich in RS also contain elevated levels of slowly digestible starch and reduced levels of rapidly digestible starch. Thus, the glycemic index of RS_3 is ~44% compared to starch in bread (Vonk et al., 2000). Foods with a low glycemic index improve satiety (Roberts, 2000; Warren, Henry, & Simonite, 2003) and may help prevent obesity and Type-II diabetes. The reported satiety caused by RS may be explained by its low glycemic index.

RS is divided into physically inaccessible starch (Type I), granular starch with the B-type crystal pattern (Type II), retrograded starch (Type III), and chemically modified starch (Type IV). A general method to convert a granular

* Corresponding author. Tel.: +785 5324088; fax: +785 532 7010.
E-mail address: paseib@ksu.edu (P.A. Seib).

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starch to a food-grade RS was developed by Woo and Seib (2002). In that method starch is phosphorylated at alkaline pH with a 99/1 (w/w) mixture of STMP/STPP to give highly cross-linked starch. In one example, wheat starch was phosphorylated to ~0.4% phosphorus (P) and the cross-linked starch was found to have ~90% dietary fiber by the Prosky method and ~66% RS by the Englyst method (Woo & Seib, 2002). When assaying RS_4 by the Englyst method the level of RS_4 was determined by subtracting the rapidly and slowly digestible starch from 100%.

Sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), and phosphoryl chloride are used to prepare food-grade phosphorylated starch (CFR, 1995; EEC, 2000). Kerr and Cleveland (1957) pioneered the use of STMP to cross-link starch in an alkaline slurry. Those workers produced cross-linked starches with a phosphorus add-on of less than 0.04%. Such cross-linked starches gave improved thickening in foods because of their stability to heat, acid, and shearing. The reaction of starch with STMP/STPP at pH > 9.5 was shown to effect cross-linking, which was indicated by changes in the physical properties of starch (Woo & Seib, 1997). It is generally believed that the phosphorylation reaction also generated monostarch phosphate esters which theoretically could have been monostarch monophosphate, diphosphate or triphosphate. However, the chemical structures of the phosphate cross-links (distarch phosphates) and the monostarch phosphates in STMP-phosphorylated starches have not been determined. In addition, little is known about the mechanism of the reaction of STMP with starch.

The ^{31}P chemical shifts of model compounds are well known (Table 1). Therefore, the chemical structures of phosphorylated starches prepared with STMP can be determined by ^{31}P NMR. The objectives of this study were: (1) to investigate the phosphorylation reaction and the chemical forms of phosphate esters in the STMP phosphorylated starches by ^{31}P NMR; (2) to investigate the effect of reaction pH between 9.5 and 12.5 on the types of phosphate esters in the phosphorylated wheat starches; and (3) to determine the effect of the level of DSMP ester (cross-links) in phosphorylated wheat starches on their levels of resistant starch (RS) and total dietary fiber.

2. Materials and methods

2.1. Materials

Wheat starch (Midsol 50) was provided by MGP Ingredients Inc. (Atchison, KS). Three commercial phosphorylated/cross-linked wheat starches were examined; Products A, B and C.

Sodium trimetaphosphate (STMP), phosphoric acid (85%), nicotinamide adenine dinucleotide (NAD), ethylenediaminetetraacetic acid (EDTA) disodium salt and deuterium oxide were purchased from Sigma Chemical Company (St. Louis, MO). Thermally stable α-amylase (E-BLAAM) (3000 Ceralpha U/mL) and glucoamylase (E-AMGDF) (200 *p*-NP β-Maltoside U/mL) were purchased from Megazyme (Bray, Ireland). All chemicals were reagent-grade.

2.2. General methods

All assays were replicated twice unless otherwise stated. Moisture content was determined by drying at 130 °C for 1 h (AACC, 2000) and phosphorus content by the

Table 1
Phosphorus-31 chemical shifts of inorganic and organic phosphates at pH 7–8 with reference to external 85% phosphoric acid

Compounds	Chemical shift (ppm)	References
Inorganic phosphates		
Na_2HPO_4 (sodium phosphate dibasic)	3.3	Sojka and Wolfe (1978)
$Na_4P_2O_7$ (sodium pyrophosphate)	−5.3	Sojka and Wolfe (1978)
$Na_5P_3O_{10}$ (sodium triphosphate)		
α-P	−4.7	
β-P	−19.1	Sojka and Wolfe (1978)
$Na_5P_3O_9$ (sodium trimetaphosphate)	−21.0	Sojka and Wolfe (1978)
Mono-alcohol monophosphates		
D-Glucose 6-phosphate	4.2–4.7	Barany and Glonek (1982)
D-Glucose 1-phosphate	2.6	Barany and Glonek (1982)
Fructose 6-phosphate	2.6–4.1	Barany and Glonek (1982)
Di-alcohol monophosphates		
DNA, RNA	0–1	Barany and Glonek (1982)
Phospholipids	0–(−1)	Barany and Glonek (1982)
Mono-alcohol diphosphate		
ADP α-P	−10.3	Barany and Glonek (1982)
ADP β-P	−6.1	Barany and Glonek (1982)
Di-alcohol diphosphate		
Nicotinamide adenine dinucleotide (NAD)	−10.5–11.3	Barany and Glonek (1982)
Mono-alcohol triphosphate		
ATP α-P	−10.4	Barany and Glonek (1982)
ATP β-P	−21.2	Barany and Glonek (1982)
ATP γ-P	−5.7	Barany and Glonek (1982)

procedure of Smith and Caruso (1964). Sodium and sulfate ions were determined at Desert Analytics in Tucson, Arizona, and hydroxide ion by titration with standard 0.100 M hydrochloric acid. The digestion profile of a modified starch was determined by the Englyst procedure (Englyst, Kingman, & Cummings, 1992), except % resistant starch (RS) was calculated by 100 – % rapidly digestible starch (RDS) – % slowly digestible starch (SDS). To determine the digestion profile on cooked starch, a raw sample (~0.6 g starch) was digested 30 min at 37 °C with pepsin (50 mg) in 0.05 M hydrochloric acid (10 mL). After adding 0.25 M sodium acetate solution (10.0 mL), the mixture was heated in a boiling water bath for 30 min, cooled to room temperature, and then digested with pancreatin/amyloglucosidase (Englyst et al., 1992).

A phosphorylated wheat starch, which was predominantly monostarch monophosphate (MSMP), was prepared by heating a mixture of wheat starch, 12% (starch basis, sb) sodium tripolyphosphate (STPP) and 5% (sb) sodium sulfate, initially at pH 6.0 and 15% moisture content, for 2 h at 130 °C (Lim & Seib, 1993). The granular product MSMP was washed thoroughly with water, and dried at 40 °C. It was recovered quantitatively and contained 0.22% P (db).

Cross-linking of wheat starch with phosphoryl chloride ($POCl_3$) was done by the method of Felton and Schopmeyer (1943). Wheat starch (50.0 g, db) was slurried in water (70.0 mL) containing sodium sulfate (7.5 g), and the pH of the starch slurry was adjusted to 11.5 by adding 1.0 M sodium hydroxide. Then, $POCl_3$ (4% sb) was added dropwise over a period 20 min to the starch slurry and the pH of the reaction mixture was kept constant at pH 11.5 at 25 °C by periodically and manually adding 1.0 M sodium hydroxide. After stirring an additional hour, the mixture was neutralized to pH 6.5 with 1.0 M hydrochloric acid and the starch was recovered by centrifuging (2000*g*, 10 min). The starch was washed with water (100 mL 4×), dried at 40 °C, and found to contain 0.33% P (db).

2.3. Phosphorylated cross-linked resistant starch; standard reaction conditions and two other conditions

The standard reaction to produce phosphorylated/cross-linked wheat starch with 0.4% P was done as follows: wheat starch (50.0 g, db) was stirred in water (70.0 g) containing a 99/1 (w/w) mixture of STMP/STPP (6.0 g, 12%, sb) and sodium sulfate (5.0 g, 10%, sb). The slurry was adjusted to pH 11.5 by adding 1.0 M sodium hydroxide (~18.5 mL, 1.5%, sb). The reaction mixture was warmed in a water bath of 45 °C and stirred for 3 h. After cooling the mixture was adjusted to pH 6.5 with 1 M hydrochloric acid. The product was washed extensively with water and dried at 40 °C. The yield of the standard phosphorylated/cross-linked wheat starch (Wheat11.5) was quantitative.

Wheat starch also was phosphorylated at pH 10.5 and 12.5 and 45 °C in the presence of 10% sodium sulfate. A series of preliminary experiments were undertaken to determine the level of STMP and the reaction times to produce phosphorylated wheat starches having ~0.4% phosphorus. Wheat starch (50.0 g, db) was stirred in water (70 mL) at 45 °C containing sodium sulfate (5.0 g) and STMP (16.5 g or 6.0 g) at pH 10.5 or 12.5 for 24 or 3 h. After the reaction, the two products, Wheat10.5 and Wheat12.5, which were again obtained in quantitative yield, were purified and dried as described before.

2.4. Phosphorylated cross-linking of starch with STMP/STPP recovered from a standard cross-linking reaction

Starches (wheat and corn) were cross-linked using the standard reaction conditions described above, and the reaction mixture was centrifuged (3000*g*, 10 min) to recover the supernatant (~60 mL). The sedimented starch was washed with another 30.0 mL distilled water, centrifuged, and the supernatant was pooled with the first one. A 99/1 (w/w) mixture of STMP/STPP (3.5 g, 7%, sb), sodium sulfate (2.8 g, 5.6%, sb), and starch (50.0 g, db) were added to the pooled supernatants, and the pH of the slurry adjusted to 11.5 by adding 1.0 M sodium hydroxide. The reaction mixture was stirred at 45 °C for 3 h. The second batch of cross-linked starch was purified and dried as described before.

2.5. Samples preparation for ^{31}P NMR analysis

Purified phosphorylated starch was digested by a modification of the starch-hydrolyzing conditions described in AOAC International Method 991.43, which is used to determine total dietary fiber (AOAC, 2000). Phosphorylated starch (1.0 g, dry basis) was added to water (50 mL) containing 2.0 mM calcium chloride in a tall-form beaker, and the pH adjusted to 8.2 by adding 1 M sodium hydroxide. Heat-stable α-amylase solution (100 μL, 3000 Ceralpha U/mL) was added, and the beaker was covered with aluminum foil and heated in a boiling water bath to 95–100 °C for 30 min with vigorous stirring. The digest was cooled and its pH readjusted from ~7.5 to 8.2. Then α-amylase solution (100 μL) was added and the digestion step was repeated. After cooling, the digest was adjusted to pH 4.5 by adding 1 M hydrochloric acid. Glucoamylase (200 μL) was added, and the digest warmed to 60 °C and allowed to digest for 1 h. The digest was cooled, centrifuged, and the supernatant freeze-dried. The recovered α,γ-dextrins amounted to >950 mg, which represented at least 95% of the weight of the phosphorylated starch.

An enzymatic digest of the total reaction mixture from a standard cross-linking reaction also was prepared by three α-amylase digestions followed by glucoamylase. An aliquot (2.6 mL) of the homogenized reaction mixture (total volume ~130 mL) was digested using the levels of α-amylase and glucoamylase under the conditions described above. The final digest (~50 mL) was concentrated to less than 10 mL using a rotary evaporator at 50 °C instead of freeze-drying. The concentrated solution was centrifuged

(4000*g*, 10 min) and ~88% of the weight of the phosphorylated starch was recovered in the supernatant. The supernatant was adjusted to pH 8.0 and a final volume of 10 mL prior to NMR measurement by adding 1.0 M sodium hydroxide and water, respectively.

2.6. ^{31}P NMR spectra of the digests of phosphorylated starches and of the total standard cross-linking reaction mixture

The freeze-dried digest of a phosphorylated starch (α,γ-dextrin, ~950 mg) was dissolved in deuterium oxide (1.0 mL) containing 0.02% sodium azide as preservative, and the resulting solution was adjusted to pH 8.0 using a pH meter. The proton-decoupled ^{31}P NMR data were acquired on a 11.75 Tesla Varian UNITY plus spectrometer (Varian, Palo Alto, CA), operating at 500 MHz for ^{1}H and 202.34 MHz for ^{31}P, respectively, with a 3 mm NMR probe. The ^{31}P NMR experiments were performed at 25 °C using a delay of 6 s between pulses (pulse width 15.0 μs), sweep width of 12730 Hz and 400 transients for each spectrum. The spectra were processed and analyzed using Varian software VNMR Version 6.1 C. Chemical shifts were reported in δ (ppm) from the reference signal of external 85% phosphoric acid. Relaxation times for monostarch monophosphate esters and phospholipid diester were reported to be, respectively, 1.8–2.1 and 0.9–1.0 s (Kasemsuwan & Jane, 1996).

An aliquot (850 μL) of the concentrated digest (10 mL) prepared from the total standard reaction mixture was added to 100 μL of a deuterium oxide solution of NAD (30 mM, pH 8.0) in an NMR tube. To the tube was added 40 μL of EDTA (500 mM, pH 8.0, in deuterium oxide) and 10 μL of sodium azide (0.2%, pH 8.0, in deuterium oxide).

2.7. Reaction of MSDP and cyclic-MSMP groups on Wheat10.5 at pH 11.5

Wheat starch phosphorylated at pH 10.5 (Wheat10.5, 50.0 g, db) was slurried in water (70.0 mL) containing sodium sulfate (10%, sb). The slurry was adjusted to pH 11.5 by adding 1 M NaOH, and was stirred at 45 °C for 3 h. The starch was collected and washed with water (200 mL × 7). The starch was hydrolyzed by enzymes and the ^{31}P NMR spectrum was recorded as described before.

2.8. Alkaline titration of the acid-form of phosphorylated starches

The acid-forms of phosphorylated starches were titrated according to the procedure developed by Mitchell (1972) with modifications. Phosphorylated starch (15.0 g, db) was suspended in cold 1.0 M hydrochloric acid (75 mL) for 5 h at 4 °C with occasional shaking. The starch was collected by centrifugation and washed with water (125 mL × 5) until no chloride was detected in the washing water with aqueous silver nitrate. The acid-form of the phosphorylated starch then was suspended in 15 mL water overnight and was titrated with 0.100 N sodium hydroxide to pH 11.5 under gentle stirring. Titration curves were obtained by plotting the slurry pH against the volume of 0.100 N sodium hydroxide, or the derivative of that curve against volume of alkali. The peaks in the derivative curve signal the termination of alkali consumed by an ionized acid group. Consumption of alkali by a phosphorylated starch at an ionization constant $pK_1 \sim 3$ was corrected for consumption of 0.07 meq by unmodified wheat starch, and at $pK_2 \sim 7$ of 0.40 meq.

3. Results and discussion

3.1. ^{31}P NMR spectrum of STMP in aqueous sodium hydroxide

The reaction conditions commonly used in our laboratory to prepare cross-linked RS_4 wheat starch with ~0.4% P are: water (70.0 g), starch (50.0 g, db), 12% STMP (sb), 10% sodium sulfate (sb), and 1.5% sodium hydroxide (sb) (pH 11.5), and 45 °C for 3 h. To imitate the continuous aqueous phase in the phosphorylation reaction, we omitted the starch from the reaction. After 3 h of stirring, that reaction mixture was adjusted to pH 8, and EDTA and a known amount of NAD were added. ^{31}P NMR spectroscopy (Fig. 1A) showed the reaction mixture contained a high concentration of unreacted STMP (singlet at δ −21 ppm) together with low concentrations of triphosphate (doublet at δ −4.9 ppm and triplet at δ −19 ppm), pyrophosphate (singlet at δ −5.6 ppm), and orthophosphate (singlet atδ 3.1 ppm).

According to Eq. (1), ring-opening of STMP first gives tripolyphosphate, which then is further hydrolyzed to pyro- and orthophosphate.

$$\text{STMP} \xrightarrow{OH^{\ominus}} \text{P-P-P} \xrightarrow{OH^{\ominus}} \text{P} + \text{PP} \qquad (1)$$

Quantitation of the linear phosphates in Fig. 1A indicated only ~5% of STMP, initially present at a concentration of 0.15 M, reacted in 3 h with hydroxide ion at pH 11.5 and 45 °C. The quantitation of the reaction product was done with reference to the known concentration of standard NAD.

3.2. ^{31}P NMR spectrum of phosphate compounds in the digest of the total standard cross-linking reaction mixture containing starch

In order to record the ^{31}P NMR spectrum on the standard reaction performed on wheat starch for 3 h, the entire reaction mixture was subjected to hot α-amylase digestion followed by glucoamylase digestion at 60 °C. Enzymatic digestion converted the starch phosphate to an α,γ-dextrin that could be detected at a concentration of 5–10% using NMR spectroscopy. Three digestion periods at 95–100 °C with heat-stable α-amylase followed by glucoamylase digestion converted ~88% of the starch phosphates to

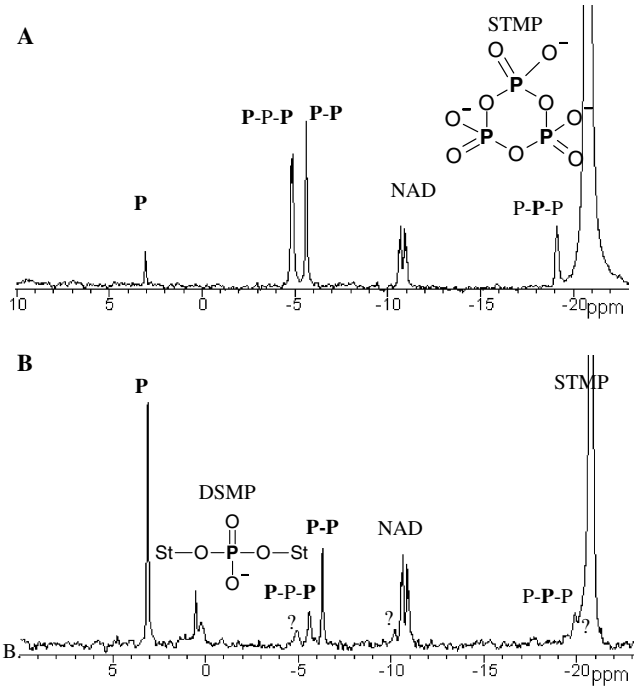

Fig. 1. (A) ^{31}P NMR spectrum of the standard cross-linking reaction mixture without starch. (B) ^{31}P NMR spectrum of α-amylase/glucoamylase digest of the total standard reaction mixture, which contained 35% wheat starch (in the slurry), 12% STMP (sb), 10% sodium sulfate (sb), 1.5% sodium hydroxide (sb), and which was reacted at pH 11.5 for 3 h at 45 °C.

water-soluble α,γ-dextrins. Such severe hydrolysis conditions were necessary probably because the high concentration of STMP in the digest chelated calcium ions. Calcium ions are known to be needed for stability of α-amylase (Whitaker, 1972).

Fig. 1B shows the presence of a high level of unreacted STMP (~80% of initial STMP) in the digest of the standard reaction mixture, along with low levels of linear polyphosphates and orthophosphate. In addition, distarch monophosphate (DSMP, St-P-St) linkages were present in the digest as indicated by the signals between δ 0–1 ppm. Surprisingly, monostarch monophosphate (MSMP, St-P) linkages were not observed (δ 3.5–5.0 ppm) in the digest of the total reaction mixture as they were in the digests of the purified products (see Fig. 4B and discussion below). Apparently, those MSMP structures were lost in the non-soluble (~12%) portion of the digest, perhaps because the monophosphate groups plus STMP gave strong localized chelation of calcium ion and denatured the α-amylase. Comparison of the ^{31}P NMR chemical shifts for the various forms of phosphorus in Figs. 1A and B show some variation. The ^{31}P NMR spectra were recorded at a pH 8.0. Any slight variation in solution pH produces a shift in some phosphate signals because the pK_2 of phosphates occurs between pH 6–9, and the ^{31}P NMR signal is sensitive to pH change near a pK value.

Prior work has shown that wheat starch granules in a 35% aqueous slurry imbibe 88% of sulfate ions, 86% of hydroxide ions, and 38% of sodium ions when the starch is exposed to sodium hydroxide together with 4% sodium sulfate (sb) at pH 12.0 and 25 °C (Matsunaga & Seib, 1997). In the standard reaction mixture described in the present work to produce cross-linked phosphorylated wheat starch having 0.4% P, the continuous phase of the reaction mixture after 3 h reaction was assayed for sulfate, phosphorus, sodium ion, and hydroxide ion. It was determined that 60% of sulfate ion, 42% of phosphorus, 47% of sodium ion, and 76% of hydroxide ion were associated with the starch granules in the discontinuous phase. Those results indicate that it is possible to isolate the continuous phase by centrifugation of the reaction mixture and thereby recover ~50% of STMP, ~50% sodium ions and ~40% sulfate ions. Wheat starch and corn starch cross-linked by the recovered liquid phase which was replenished with STMP/STPP (7%, sb) and sodium sulfate (5.6%, sb), showed similar P content, total dietary fiber content, and enzyme digestion profile after cooking as those cross-linked by the standard reaction (Table 2). The preparation of the second batch of modified starch using the recovered reagents suggests it is possible to reduce the cost of producing RS_4 starch.

3.3. ^{31}P NMR spectra of α,γ-dextrins of purified phosphorylated wheat starches

A phosphorylated wheat starch (WheatSTPP) with 0.22% P was prepared by roasting the starch with STPP at an initial pH 6.0. Those conditions had been previously shown to favor the formation of MSMP ester (Lim & Seib,

Table 2
Phosphorus (P) contents, digestion profiles after heating in boiling water, and total dietary fiber (TDF) contents of starches cross-linked by the standard reaction and by reaction using recovered liquid phase of the standard reaction[A]

Sample	P (%, db)	Starch in boiling water (%, db)[B]			TDF (%, db)
		RDS	SDS	RS	
WheatBatch1	0.38	63^{ab}	29^{a}	8^{a}	91^{a}
WheatBatch2	0.40	65^{a}	27^{a}	8^{a}	90^{a}
CornBatch1	0.31	63^{ab}	30^{a}	7^{a}	57^{b}
CornBatch2	0.35	60^{b}	30^{a}	10^{b}	62^{c}

[A] Values followed by the same letters in the same column are not significantly different ($p < 0.05$).
[B] Rapid digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS); RS is 100% – RDS – SDS.

1993). The ^{31}P NMR spectrum (Fig. 2A) of the α,γ-dextrins of WheatSTPP showed a strong set of three signals for MSMP at δ 3.8–5.2 ppm and a weak set for DSMP at δ 0–1 ppm. The three signals at MSMP range indicated the phosphorylation of hydroxyl groups at C-2, C-3, and C-6. Those results agree with the roasting of both α- and β-cyclodextrins with sodium metaphosphate at pH 4, which also gave a mixture of positional monoesters (Tarelli, Lemercinier, & Wheeler, 1997). On the other hand, phosphorylation of wheat starch with phosphoryl chloride at pH 11.5 (WheatPOCl$_3$) produced predominantly DSMP ester in the modified starch and much less MSMP ester (Fig. 2B). The proportion of the two peak areas in Figs. 2A and 2B showed that WheatSTPP and WheatPOCl$_3$ contained, respectively, 80% MSMP/20% DSMP and 20% MSMP/80% DSMP on a molar basis (Table 3).

Figs. 3A, B, and C show the ^{31}P NMR spectra of α,γ-dextrins from three commercial phosphorylated/cross-linked wheat starches. All three phosphorylated starches showed a set of ^{31}P NMR resonances for both DSMP (δ 0–1 ppm) and MSMP (δ 3.5–5.0 ppm). Product C was unique among the three samples because its spectrum showed three extra ^{31}P NMR resonances, two weak signals at δ −5.0 and −10.3 ppm and a relative strong signal at δ 3.1 ppm. The signal at δ 3.1 ppm was identified to be orthophosphate by spiking the digest with sodium dibasic phosphate. The signals at δ −5.0 and −10.3 ppm were assigned to monostarch diphosphate (MSDP, St-P-P), because they occur in the same region as those of ADP (Table 1) and because the two signals are of equal intensity. The molar proportions (calculated in %) of the organophosphate structures in the three starches are given in Table 3.

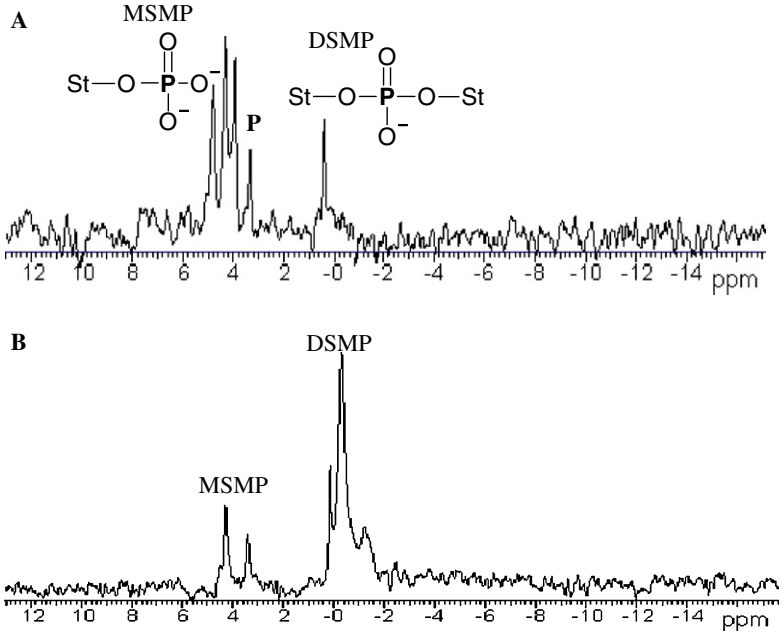

Fig. 2. ^{31}P NMR spectra of α, γ-dextrins prepared from two model phosphorylated wheat starches: (A) Model MSMP starch (WheatSTPP); (B) Model DSMP starch (WheatPOCl$_3$). No signal was observed in the regions of 15 ppm and −20 ppm.

Table 3
Molar proportions of monostarch monophosphate (MSMP), distarch monophosphate (DSMP) and monostarch diphosphate (MSDP) in phosphorylated wheat starches[A,B]

Sample	P % (db)	^{31}P NMR method (% of organophosphates)			Titration method[C] % of organophosphates	
		MSMP	DSMP	MSDP	MSMP	DSMP
WheatSTPP	0.22	80[d]	20[a]	ND[D]	72[e]	18[a]
WheatPOCl$_3$	0.35	20[a]	80[c]	ND	11[a]	89[e]
Product A	0.36	37[c]	63[b]	ND	34[c]	66[c]
Product B	0.45	38[c]	62[b]	ND	39[d]	61[b]
Product C	0.28	31[b]	65[b]	4	22[b]	78[d]

[A] Percentage of phosphate ester based on total moles of organophosphates.
[B] Values followed by the same letters in the same column are not significantly different ($p < 0.05$).
[C] Equivalents of sodium hydroxide to titrate an acidic phosphate group was corrected for the equivalents absorbed by the blank wheat starch.
[D] ND = not detected.

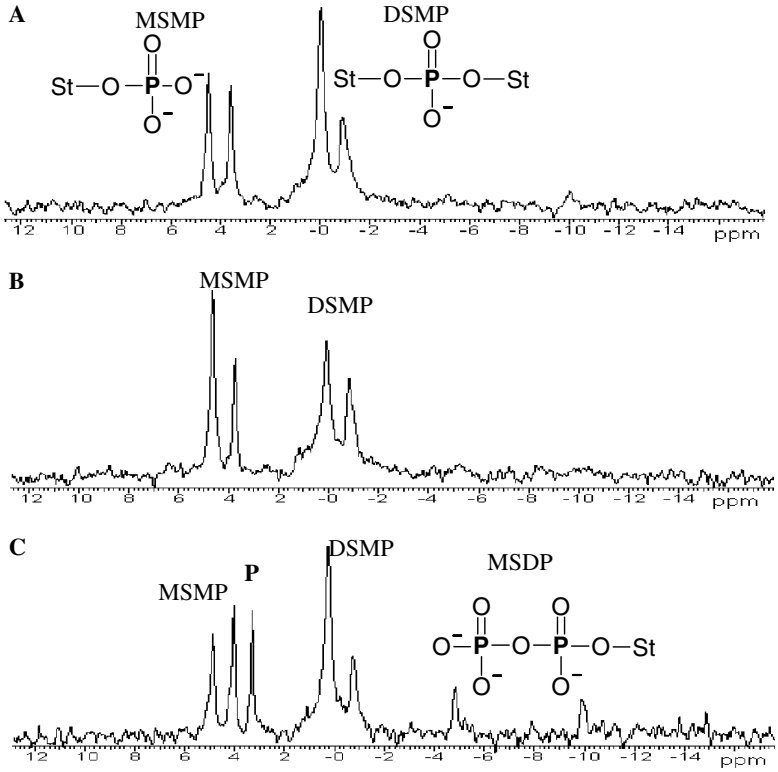

Fig. 3. ^{31}P NMR spectra of α, γ-dextrins prepared from commercial phosphorylated/cross-linked wheat starches: (A) Product A; (B) Product B; and (C). Product C. No signal was observed in the regions of 15 ppm and −20 ppm.

The multiplicity of the two sets of peaks observed in both the δ 3.5–5.0 ppm region and δ 0–1 ppm region of the ^{31}P NMR spectra (Fig. 3) indicate that the phosphate groups on those modified starches occur on at least two positions on the anhydroglucose units along the starch chains. Most likely the 2-OH and 6-OH are the positions of substitution in the DSMP and MSMP structures as has been found for other modified starches prepared by reaction of granular starch with various electrophilic reagents in alkali (Van der Burgt et al., 1998). It appears the reactivity of the 2-OH is caused by its acidity whereas that of the 6-OH is caused by its steric accessibility. Unlike starch, both α- and β-cyclodextrins were selectively phosphorylated at only one (a C-2 OH) of the possible 18 hydroxyls when reacted with trimetaphosphate at pH 12 and 10 °C (Inoue, Tone, Nakayama, & Tsuhako, 2003).

3.4. Effect of reaction pH on the structure of phosphorylated wheat starches

Wheat starch was phosphorylated at 45 °C with STMP at pH 10.5, 11.5 and 12.5. At the different reaction pH's, it was necessary to change the concentration of STMP and the reaction time to achieve 0.4% P incorporation into the three starches (Table 4). Phosphorylation of starch at pH 10.5 (Wheat10.5) was much slower than phosphorylation at pH 11.5 (Wheat11.5) and 12.5 (Wheat12.5). To prepare Wheat10.5 with ∼0.4% P in a time period of one day or less, the concentration of STMP was increased about three times compared to the reaction done at pH 11.5. The hydroxyl groups of starch have ionization constants between pKa 12.5–13.0 at 45 °C (Lammers, Stamhuis, & Beenackers, 1993). At pH 10.5, less than 1% of hydroxyl

Table 4
Molar proportions of monostarch monophosphate (MSMP), distarch monophosphate (DSMP) and monostarch diphosphate (MSDP) in wheat starches phosphorylated at different pH's to a phosphorus level of 0.38%[A,B]

Sample	Reaction conditions	P (%, db)	Chemical forms of P by ^{31}P NMR (% of organophosphates)			
			Cyclic-MSMP	MSMP	DSMP	MSDP
Wheat10.5	STMP (33%, sb), NaOH (0.5%, sb), pH 10.5, 24 h	0.38	19	22[a]	41[a]	18
Wheat11.5	STMP (12%, sb), NaOH (1.5%, sb), pH 11.5, 3 h	0.38	ND[C]	37[b]	63[b]	ND[C]
Wheat12.5	STMP (12%, sb), NaOH (3.2%, sb), pH 12.5, 3 h	0.39	ND[C]	33[c]	67[c]	ND[C]

[A] Percentage of a phosphate ester based on total moles of organophosphates.
[B] Values followed by the same letters in the same column are not significantly different ($p < 0.05$).
[C] Not detected.

groups on starch would be ionized compared to 5–10% ionized at pH 11.5. The different reaction times in Table 4 indicate that ionized hydroxyl groups on starch rather than the protonated hydroxyl groups react more rapidly with STMP.

Figs. 4A, B, and C show the ^{31}P NMR spectra of the α,γ-dextrins produced from the starches phosphorylated at pH 10.5, 11.5 and 12.5, respectively. Wheat12.5 contained 67% DSMP and 33% MSMP, and Wheat11.5 contained 63% DSMP and 37% MSMP on a molar basis of the organophosphates (Table 4). On the other hand, Wheat10.5 contained only 41% DSMP, 22% MSMP, but 18% MSDP, and 19% cyclic-MSMP ($P >$ St). Phosphorylation of corn starch at pH 10.5 and 11.5, respectively, confirmed the pH effect on the structures of the organophosphate esters (data not shown).

3.5. Proposed mechanism for phosphorylation of starch with STMP in alkaline slurry

Based on the various forms of phosphates in the standard cross-linking reaction, as well as on the structures of α,γ-dextrins from the various purified phosphorylated starches, a mechanism of reaction between starch and STMP in alkali is proposed (Fig. 5). The first step involves the ring-opening attack of a starch alcoholate ion on STMP to form the tripolyphosphate intermediate (Reaction 1, Fig. 5). The monostarch triphosphate was not observed in the α,γ-dextrins produced from purified starch phosphates probably because the esterification reactions were carried out for 3 and 24 h. However, a trace of monostarch triphosphate was evident in the α,γ-dextrins produced directly from the standard reaction mixture (3 h) as evidenced by low intensity signals at approximately δ −5.0 ppm (γ-P) and δ −10.2 ppm (α-P) in Fig. 1B. The β-P signal at δ −21 ppm for a possible α,γ-dextrin triphosphate would be under the strong peak for STMP. The monostarch triphosphate is likely to be unstable during the synthesis reaction at an alkaline pH. Tsuhako et al. (1985) reported that an alanine or valine triphosphate intermediate was undetectable during the reaction of those amino acids with STMP.

At an alkaline pH all four ionizable hydrogens on the starch triphosphate intermediate would be negatively charged. All three phosphoryl groups along the triphosphoryl chain have a fairly strong ionizable acidic OH with pK ~3, while the γ-phosphoryl group has a second weak ionization at pK ~8–9. Apparently, pyrophosphate in the fully ionized intermediate is a better leaving group than orthophosphate when hydroxide or starch alcoholate ion attacks the starch triphosphate intermediate at pH 11.5–12.5. Attack of RO^- or OH^- ion at the α-phosphorus of the triphosphoryl group is effective rather than the attack at either the β- or γ-phosphorus atom (Reaction 2, Fig. 5), and forms only DSMP and MSMP. This mechanism explains why MSDP is not formed when starch is

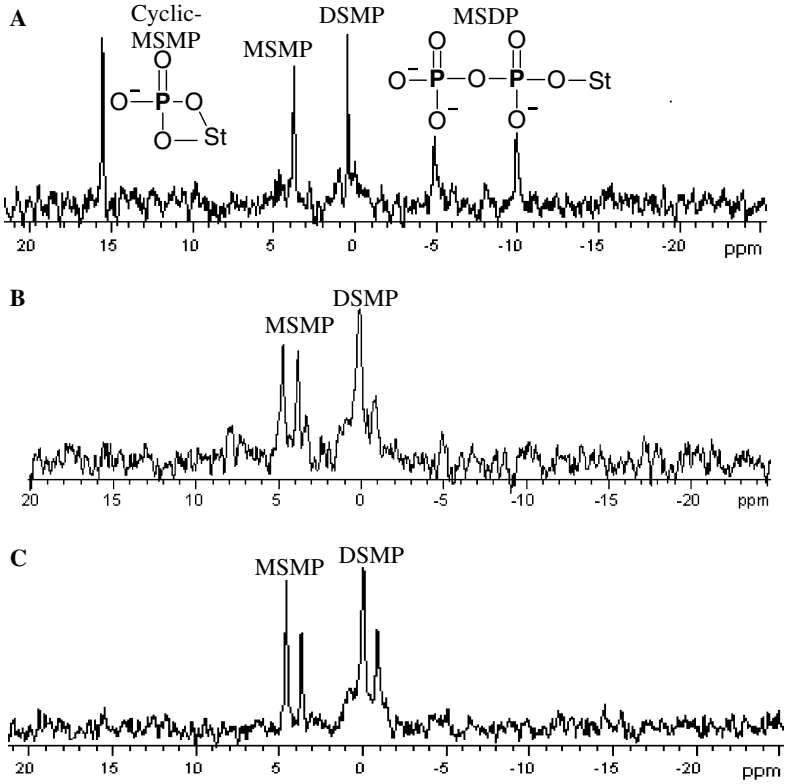

Fig. 4. ^{31}P NMR spectra of α, γ-dextrins prepared from wheat starches phosphorylated to ~0.4% P at 45 °C with STMP at pH: A, 10.5 (Wheat10.5); B, 11.5 (Wheat11.5); and C. 12.5 (Wheat12.5), respectively, for 24, 3, and 3 h.

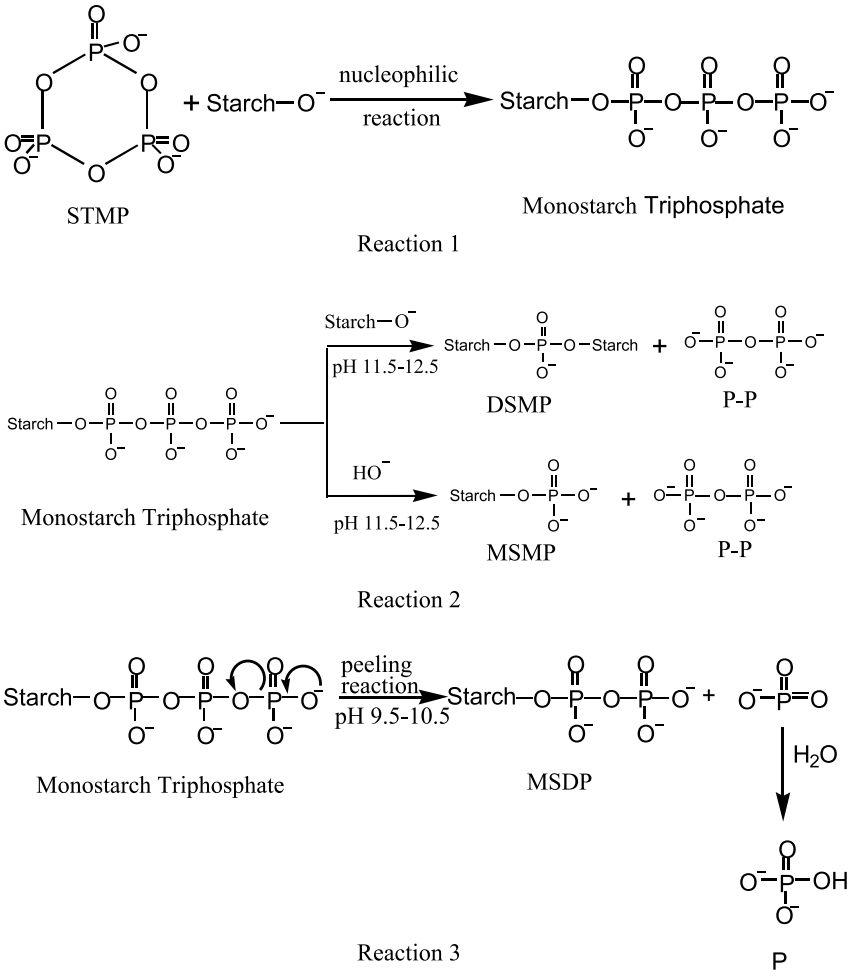

Fig. 5. Proposed pathway of reaction between STMP and starch in aqueous sodium hydroxide at pH 9.5–12.5.

phosphorylated at pH 12.5 as shown in Fig. 4C. However, at pH 10.5 the concentration of starch alcoholate (RO^-) is reduced ~90%, so the reaction of RO^- with the starch triphosphate intermediate is slow. Thus the starch triphosphate intermediate slowly reacts by a competing mechanism. It appears the γ-phosphoryl group on the starch triphosphate intermediate is lost by the so-called peeling mechanism, which is a unimolecular reaction (Reaction 3, Fig. 5). When the peeling reaction on the starch triphosphate intermediate dominates, the product formed is MSDP (Reaction 3, Fig. 5).

The structure of the starch triphosphate intermediate is similar to that of adenosine 5′-triphosphate (ATP). ATP is known to hydrolyze at its α-phosphoryl group and lose the pyrophosphate at alkaline pH (Cook, Lipkin, & Markham, 1957; Lipkin, Markham, & Cook, 1959), but at acidic pH ATP loses the orthophosphate group (Friess, 1953; Miller & Westheimer, 1966).

3.6. Reaction of MSDP and cyclic-MSMP groups on Wheat10.5 at pH 11.5

After stirring Wheat10.5 in a slurry of pH 11.5 for 3 h at 45 °C, cyclic-MSMP disappeared completely and the proportion of MSDP decreased from 17% to 11% (Fig. 6), which indicated the reactivity of the cyclic-MSMP and the MSDP groups at alkaline pH.

3.7. Alkaline titration of phosphorylated starches

Fig. 7 shows a typical titration curve of a phosphorylated wheat starch, Commercial Product A, which was shown to contain only DSMP and MSMP structures by ^{31}P NMR spectroscopy (Fig. 3A). The first-order derivative curve [d(pH)/dV] in Fig. 7 was used to locate the inflection points in the titration curve. The inflection points signal the end-points for the titrations of various acidic phosphate groups (Gramera, Heerema, & Parrish, 1966; Koch, Bommer, & Kleve, 1982). Product A showed an end-point at pH 5.5 for the stronger acidic group and pH 9.6 for the weaker one (Fig. 7). Gramera et al. (1966) titrated a phosphorylated (STPP) corn starch and reported an end-point of pH 5.7 for the more acidic phosphoryl group(s) and an end-point of pH 9.75 for the less acidic group(s).

The alkali consumed in titrating the acidic group with pK 7.5 (Fig. 7) counts the MSMP structure, whereas that consumed by the acidic group at the low pK ~3.0 counts both MSMP and DSMP. The levels of MSMP and DSMP

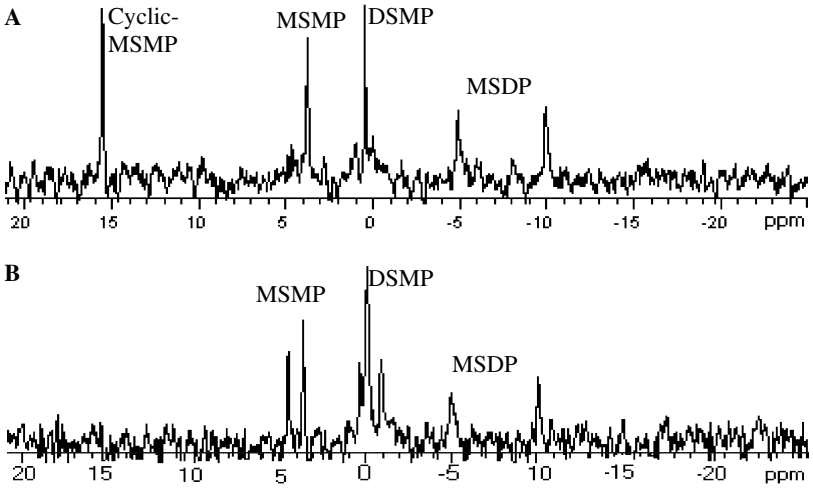

Fig. 6. ^{31}P NMR spectra of α, γ-dextrins prepared from: A,Wheat10.5; and B, Wheat10.5 incubated at pH 11.5, 45 °C for 3 h. The structures of cyclic-MSMP, MSMP, and DSMP were showed in previous figures.

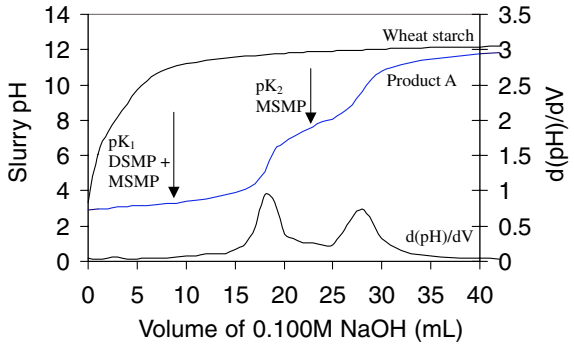

Fig. 7. Titration curves of the acid forms of Product A (15 g) and the wheat starch blank (15 g) with 0.100 M NaOH. The first order derivative of the titration curve for Product A indicated pK_1 3.0 and pK_2 7.5.

determined by titration, which were calculated based on all starch phosphates in Product A, were 34% and 66%, respectively, which was in good agreement with the 37% and 63% determined by ^{31}P NMR (Table 3).

Briggs and Hanig (1946) found that alkali titration of the second ionizing hydroxyl on the phosphoric acid group in MSMP was higher than predicted by the modified starch's phosphorus content. In the present study the titration of the wheat starch blank in Fig. 7 absorbed 0.47 meq of sodium hydroxide during titration from pH ~3 to pH 9.7, and 0.07 meq between pH 3 and 5.5. When titrating phosphorylated wheat starches, the alkali absorbed by the unmodified starch was subtracted from the amount of alkali consumed during the titration of the phosphoric acid group on the modified starches.

The molar proportions (%'s) of MSMP and DSMP in six phosphorylated wheat starches are given in Table 3. The contents of MSMP and DSMP determined by the titration method agreed with those determined by their ^{31}P NMR spectra except for Product C, which contained MSDP in addition to MSMP and DSMP. The alkali titration method used to determine the proportion of phosphate cross-links (DSMP) in starch is limited to starches containing only DSMP and MSMP.

3.8. Effect of DSMP on starch digestion profiles by porcine pancreatic α-amylase and total dietary fiber content

In vitro digestion profiles of four phosphorylated starches all with 0.35–0.39% P but with different DSMP contents are given in Table 5. Wheat-$POCl_3$ had the highest DSMP content (80%), the highest RS content when uncooked (86%) and cooked (12%), and highest TDF content (99%), whereas Wheat10.5 had the lowest DSMP content (41%), the lowest RS when uncooked (33%) and cooked (3%), and the lowest TDF (2.5%). The data in Table 5 indicate a positive correlation between % DSMP and % RS of

Table 5
Molar proportions of distarch monophosphate in STMP-phosphorylated wheat starches and their starch digestion profiles[A,B]

Sample	P (%, db)	DSMP (% of organophosphates)	Starch fraction (%, db)						TDF (%, db)
			Raw starch			Starch in boiling water			
			RDS	SDS	RS	RDS	SDS	RS	
Wheat10.5	0.38	41[a]	20[d]	48[d]	33[a]	68[b]	29[a]	3[a]	2.5[a]
Wheat11.5	0.36	63[b]	8.3[c]	34[c]	57[b]	63[a]	29[a]	8[b]	91[b]
Wheat12.5	0.39	67[c]	5.6[b]	25[b]	70[c]	60[a]	28[a]	12[c]	99[c]
Wheat$POCl_3$	0.35	80[d]	1.7[a]	12[a]	86[d]	61[a]	27[a]	12[c]	99[c]

[A] Starch digestion profiles were done according to Englyst et al. (1992). See Footnote to Table 2.
[B] Values followed by the same letters in the same column are not significantly different ($p < 0.05$).

uncooked ($r = 0.96$, $p = 0.02$) as well as between % DSMP and % TDF ($r = 0.90$, $p = 0.05$). Therefore, wheat starch phosphorylated with STMP under different conditions to ~0.4% P gave a wide variation in the extent of *in vitro* digestion by pancreatic α-amylase.

The phosphorus in modified starch for food use is regulated by the Code of Federal Regulation (CFR, 1995) of the U.S. Food and Drug Administration or by the Directive of the EEC (2000). If STMP/STPP is used to phosphorylate starch for food use, the modified starch can not contained more than 0.4–0.5% P. Even at a limited level of % P in a phosphorylated starch, increasing DSMP contents in the phosphorylated starch has the potential to increase slowly digestible starch (SDS) and resistant starch (RS).

4. Conclusions

^{31}P NMR spectroscopy provided an improved understanding of how starch reacts with STMP. Most STMP remains unreacted in a standard cross-linking reaction that produces RS_4 starch. It appears that unreacted STMP and other reagents can be recovered by separating the continuous aqueous phase from the granular starch product. After replenishing approximately 7% (sb) of STMP/STPP and adding starch, a new batch of phosphorylated starch can be made. Choosing a pH of 9.5–12.5 for reaction of starch with STMP controls the level of cross-links in the modified starch, which in turn controls the rate and extent of its *in vitro* digestion by pancreatic α-amylase.

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