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Effects of the order of addition of reagents and catalyst on modification of maize starches

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ABSTRACT

The objective of this research was to determine if adding reactive reagents to starch granules before addition of alkali (TRF method) would produce products that are different than those obtained by adding alkali before addition of reagent. Normal (NMS) and waxy (WMS) maize starches were each reacted with acetic-adipic mixed anhydride (AAMA), phosphoryl chloride ($POCl_3$), sodium trimetaphosphate (STMP), acetic anhydride (AA), succinic anhydride (SA), and octenylsuccinic anhydride (OSA). Almost no or no starch polymer molecule modification occurred when the TRF method and AAMA, AA, or $POCl_3$ were used; less than half as much reaction when SA was the reagent used, and about the same amount of reaction when STMP or OSA were the reagents used (for different reasons). It was concluded that most AAMA, AA, SA, and $POCl_3$ reacted with surface protein molecules when the TRF method was used and that OSA molecules were driven into the structured internal water of granules.

1. Introduction

The universal procedure for modifying a starch using crosslinking or stabilization reactions is to add a base to a starch-in-water slurry to raise its pH to the desired level, followed by addition of the derivatizing reagent. Phosphoryl chloride, a very reactive and rapidly reacting reagent, is known to react with the first starch molecules it encounters, i.e., the molecules at granule surfaces (Huber & BeMiller, 2001; Gray & BeMiller, 2004). It can be assumed that other rather reactive reagents would react in a similar manner. We therefore wondered whether allowing reagents, especially quite reactive reagents that are likely to react with the first ionized starch hydroxyl groups they encounter, to penetrate more evenly throughout granules before adjusting the pH to an alkaline value might result in more efficient reactions or modified starches with the substituent groups more evenly distributed throughout granules and, therefore, with different properties.

2. Materials and methods

2.1. Materials

Commercial normal and waxy maize starch and normal and waxy maize kernels were donated by Tate & Lyle, NA (Decatur, IL USA). Acetic anhydride, succinic anhydride, 2-octen-1-ylsuccinic anhydride, phosphoryl chloride, adipic acid, sodium trimetaphosphate, and thermolysin (from *Baccillus thermoproteolyticus*, p1512) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Laboratory-isolated starch

Starch was isolated from whole maize kernels using the method described by Wongsagonsup, Varavinit, and BeMiller (2008) and Sui, Shah, and BeMiller (2011), which is a standard laboratory procedure for isolation of maize starch involving steeping whole maize kernels in a solution of sodium bisulfite/sulfurous acid, grinding the softened kernels, sieving the resulting mixture, mixing with 70% ethanol, and centrifugation to remove remaining protein.

2.2.2. Standard/conventional reaction (adding alkali before addition of reagent)

Starch was dispersed in reverse-osmosis and distilled (ROD) water with stirring at room temperature. The starch slurry was adjusted to the specified pH value with 1 M NaOH. Reagent was added with vigorous stirring. During reaction, the pH was maintained using an auto-titrator (TIM 854, Radiometer, Brønshøi, Denmark). After reaction, the reaction mixture was neutralized with dilute HCl. The modified starch was collected by centrifugation, washed three times with ROD water and once with 100% ethanol, and air dried.

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2.2.3. Alternative reaction (adding alkali after impregnating granules with reagent)

Starch was dispersed in ROD water with stirring at room temperature. Reagent was added to the starch slurry with vigorous stirring, which was maintained for 3 h. Then, the starch slurry was adjusted to the specific pH value with 1 M NaOH. The pH was maintained at the same value as for the standard reaction via use of an auto-titrator. The time of reaction (after adjustment of the system pH) was also the same. After reaction, the reaction mixture was neutralized with dilute HCl. The modified starch was collected by centrifugation, washed three times with ROD water and once with 100% ethanol, and air dried.

2.2.4. Incubation of starch with thermolysin

The two commercial starches were treated with thermolysin (Sigma–Aldrich Corp., St. Louis, MO, USA) for 30 min at 23 °C as described by Mu-Forster and Wasserman (1998) to remove surface protein.

2.2.5. Crosslinking with phosphoryl chloride ($POCl_3$)

Starch (20.0 g, db) was dispersed in ROD water (40 mL) with stirring at rt. The pH of the slurry was adjusted to 11.3 with 1 M NaOH either before or after adding phosphoryl chloride (0.01% of the weight of starch added slowly and dropwise as a 3% solution in 1,4-dioxane). The reaction was maintained at pH 11.3 for 1 h using an auto-titrator. After reaction, the reaction mixture was neutralized to pH 6.5 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water and once with 100% ethanol, and air-dried.

2.2.6. Crosslinking with sodium trimetaphosphate (STMP)

STMP (0.040 g) and sodium sulfate (1.00 g) were dissolved in 5 mL of ROD water. Starch (20.0 g, db) was dispersed in ROD water (35 mL) with stirring at rt. The pH of the slurry was adjusted to 10.0 with 1 M NaOH either before or after adding the sodium trimetaphosphate-sodium sulfate solution dropwise. The reaction mixture was maintained at pH 10.0 for 1 h at room temperature using an auto-titrator. The dispersion was put in a Petri dish and dried at 40 °C until the moisture content was ~10%. The modified starch was then heated at 130 °C for 2 h. After cooling to room temperature, the starch was dispersed in 40 mL of ROD water. The reaction mixture was neutralized to pH 6.5 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water, and dried at 40 °C overnight.

2.2.7. Modification with acetic-adipic mixed anhydride (AAMA)

After adipic acid (0.20 g) was clearly dissolved in acetic anhydride (5.6 mL), the acetic-adipic mixed anhydride (AAMA) reagent was diluted with 28 mL of 1,4-dioxane. Starch (25.0 g, db) was dispersed in ROD water (46 mL) with stirring at rt. The pH of the slurry was adjusted to 9.0 with 1 M NaOH either before or after adding the diluted acetic–adipic mixed anhydride solution (6.1 mL) dropwise. The reaction was maintained at pH 9.0 for 2 h using an auto-titrator. After reaction, the reaction mixture was neutralized to pH 6.0 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water and once with 100% ethanol, and air dried. The dried preparations were stored in a refrigerator.

2.2.8. Esterification with acetic anhydride (AA)

Starch (20.0 g, db) was dispersed in ROD water (50 mL) with stirring at room temperarure. The pH of the slurry was adjusted to 8.2 with 1 M NaOH either before or after adding acetic anhydride (AA) (1.7 mL) dropwise. The reaction was maintained at pH 8.2 for 6 h using an auto-titrator. After reaction, the reaction mixture was neutralized to pH 6.5 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water and once with 100% ethanol, and air-dried.

2.2.9. Esterification with succinic anhydride (SA)

Starch (20.0 g, db) was dispersed in distilled water (45 mL) with stirring at rt. The pH of the slurry was adjusted to 8.5 with 1 M NaOH either before or after adding succinic anhydride (SA) (0.80 g) dropwise. The reaction was maintained at pH 8.5 for 6 h using an auto-titrator. After reaction, the reaction mixture was neutralized to pH 6.5 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water and once with 100% ethanol, and air-dried.

2.2.10. Esterification with octenylsuccinic anhydride (OSA)

Starch (20.0 g, db) was dispersed in ROD water (45 mL) with stirring at rt. The pH of the slurry was adjusted to 8.5 with 1 M NaOH either before or after adding 2-octen-1-ylsuccinic anhydride (OSA) (0.6 mL) dropwise. The reaction was maintained at pH 8.5 for 6 h using an auto-titrator. After reaction, the reaction mixture was neutralized to pH 6.5 with dilute HCl. The modified starch was collected by centrifugation, washed three times with 40 mL of ROD water and once with 100% ethanol, and air-dried.

2.2.11. Pasting characteristics

Pasting characteristics of the modified starches were determined with a Rapid Visco-Analyzer (Model 4, Newport Scientific, Warriewood, Australia) using standard profile 1. Modified starch (1.96 g, db) and distilled water (26.04 g) (sample + water = 28.00 g) (7.0% starch; OSA products were measured at 5.0% starch) were mixed and stirred within the analyzer's aluminum sample canister. A 13-min analysis was used with a programmed heating and cooling cycle. The sample was heated to and held at 50 °C for 1 min; the temperature was raised to 95 °C within 3.7 min and held there for 2.5 min, then cooled to 50 °C within 3.8 min, and finally held there a further 2 min. Triplicate analyses were performed on each sample, and the results were averaged.

2.2.12. Differential scanning calorimetry (DSC)

Thermal properties of starch samples were investigated with a differential scanning calorimeter (DSC; TA 2910, TA Instruments, Wilmington, DE, USA). The starch sample (5.0 mg, db) was placed in a pan; ROD water was added to a total of 20.0 mg (starch to water ratio = 1:3, w/w). The pan was sealed and equilibrated at room temperature for at least 2 h before the scan. Samples were heated from 20 °C to 120 °C at a heating rate of 10 °C/min. A sealed empty pan was used as a reference, and indium was used as a calibration standard. Triplicate analyses were performed on each sample, and the results were averaged.

After being stored 7 days at 4 °C, gelatinized samples were rescanned from 20 °C to 140 °C at a heating rate of 10 °C/min. Thermal transitions for gelatinization and retrogradation were characterized by T_o (onset temperature), T_p (peak temperature), T_c (conclusion temperature) and ΔH (enthalpy). Triplicate analyses were performed on each sample, and the results were averaged.

2.2.13. Reflectance confocal laser scanning microscopy (R-CLSM)

Starch samples (0.6 g) were shaken in 0.25 M $AgNO_3$ solution in the dark for 24 h at room temperature, then recovered by centrifugation. The exchange procedure was repeated twice more. Silver ion-exchanged starch pellets were washed with 85% (v/v) aqueous ethanol for 30 min to remove excess $AgNO_3$. The washing procedure was repeated twice more. The starch pellet was recovered by vacuum filtration. Starch granules were prepared for R-CLSM analysis by dusting a small amount of Ag^+-exchanged starch granules on a microscope slide. Slides were exposed to a UV light source for >24 h to fully reduce silver ions to silver atoms. Otherwise, samples were

protected from light during experiments and throughout storage with aluminum foil.

Two or three drops of aqueous sucrose solution (20%, w/v) were added to the starch specimen, and the microscope slide was covered with a glass cover slip and loaded onto a Zeiss LSM 510 confocal laser scanning microscope (CLSM) coupled to an inverted microscope with a 63 × DIC objective lens (Thornwood, NY, USA). An argon laser (488 nm) operating at 30% capacity was used. Light reflected from specimens was detected through a LP 475 emission filter. The images were processed using Zeiss LSM Image Examiner software.

2.2.14. Degree of substitution of OSA- and SA-derivatized starch

Modified starch samples (1 g, db) were weighed accurately, wetted with a few mL of reagent-grade 2-propanol, and dispersed in 25.0 mL of 2.5 M HCl in 2-propanol. After stirring the slurry 30 min, aqueous 2-propanol (90% v/v, 100 mL) was added, and the slurry was stirred an additional 10 min. The slurry was filtered through a glass filter, and the residue was washed with 90% 2-propanol until no Cl^- could be detected by a 0.1 M $AgNO_3$ solution. The starch was re-dispersed in 300 mL of distilled water, and the slurry was heated 15 min in a boiling water bath. The starch solution was titrated while hot with standardized 0.1 M NaOH to pH 8.6 using an auto-titrator. The degree of substitution (DS) of OSA- or SA-derivatized starch was expressed as

$$DS_{OSA} = \frac{0.162 \times (A \times M)/W}{1 - [0.210 \times (A \times M)/W]}$$

or

$$DS_{SA} = \frac{0.162 \times (A \times M)/W}{1 - [0.100 \times (A \times M)/W]}$$

where 162 = molecular weight of a glucosyl unit; 210 = molecular weight of an octenylsuccinyl group; 100 = molecular weight of a succinyl group; A = tritration volume of NaOH solution; M = molarity of NaOH solution; and W = dry weight of starch.

2.2.15. Degree of substitution of AA- and AAMA-derivatized starch

Modified starch samples (1 g, db) were weighed accurately and suspended in 50 mL of ROD water. The resulting slurry was adjusted to pH 8.6 with standardized 0.1 M NaOH to neutralize excess acid. After adding 25.0 mL of standardized 0.45 M NaOH, the flask was stoppered with a septum and kept in a 40 °C water bath with vigorous stirring for 3.5 h. Excess alkali was titrated with standardized 0.2 M HCl to pH 8.6 using a auto-titrator. A blank was titrated at the same time. The degree of substitution (DS) of AA- or AAMA-derivatized starch was expressed as

$$DS_{AA} = \frac{0.162 \times (B - A) \times M/W}{1 - [0.043 \times (B - A) \times M/W]}$$

or

$$DS_{AAMA} = \frac{0.162 \times (B - A) \times 0.5M/W}{1 - [0.112 \times (B - A) \times 0.5M/W]}$$

where 162 = molecular weight of a glucosyl unit; 43 = molecular weight of an acetyl group; 112 = molecular weight of an adipyl group; B = volume of standardized 0.2 M HCl used to titrate the blank; A = volume of standardized 0.2 M HCl used to titrate the sample; M = molarity of HCl solution; W = dry weight of starch, with the understanding that AAMA-modified starch contains acetyl groups and probably monostarch adipate groups in addition to distarch adipate groups.

2.2.16. Swelling power (SP) and solubility index (SI)

Starch (2.5 g db) was placed in tared 50-mL screw-capped centrifuge tubes. Water (10.0 mL) was added to each tube, and the contents were mixed. Tubes were placed in a boiling-water bath (without shear or shaking). After 10 min, tubes were removed, placed in an ice bath for 5 min, and then centrifuged at 3000 × *g* for 15 min. Supernatants were removed, placed in 75-mL aluminum pans, and dried 24 h at 105 °C. The centrifuge tubes containing residues were weighed. Swelling power (SP) was calculated as wet weight of residual starch/initial dry weight of starch. Solubility index (SI) was calculated as (dry weight of soluble starch/initial dry weight of starch) × 100.

2.2.17. Phosphorus analysis

Analysis of the $POCl_3$ and STMP reaction products for phosphorus was done in duplicate using ICP-MS by the Analytical Sciences Laboratory, University of Idaho, Moscow, ID, USA.

2.2.18. Nitrogen analysis

Nitrogen analysis (to determine relative amounts of protein present) was done in duplicate according to the Kjeldahl procedure by Galbraith Laboratories, Knoxville, TN, USA.

3. Results and discussion

The objective of this research was to determine if impregnating starch granules with presumed rapidly reacting reagents before adjusting the pH by addition of alkali would produce different products than those obtained using the standard/conventional procedure (adding alkali before addition of reagent). Commercial (C), laboratory-isolated (LI), and protease-treated commercial (PT) normal maize (NMS) and waxy maize (WMS) starches were used. The six starch preparations were each reacted with each of three crosslinking reagents (acetic-adipic mixed anhydride (AAMA), phosphoryl chloride ($POCl_3$), and sodium trimetaphosphate (STMP)) or each of three stabilizing reagents (acetic anhydride (AA), succinic anhydride (SA), and octenylsuccinic anhydride (OSA)), all of which, except STMP, are believed to be quite reactive. Products were characterized by determining DS values and by RVA and DSC analysis. Most results indicated differences in degrees of reaction between the two methods, but there were exceptions.

3.1. Properties of crosslinked maize starches

RVA attributes for unmodified starches and those derivatized by the standard method are given in Table 1. The same data for the starches modified by the treatment-with-reagent-first (TRF) method are given in Table 2.

Looking generally at the values in Table 1, it can be seen that the values for the commercial starches modified with the AAMA reagent using the standard method differed from those of the laboratory-isolated starches modified with the same reagent using the standard method. There are two possible reasons for the differences: (1) the starches were of different genetic backgrounds; (2) it has been found that commercial maize starches contain less protein than do laboratory-isolated maize starches (Widya, Gunawan, & BeMiller, 2010), presumably because some of the protein is removed from the external and channel surfaces by the proteolytic enzymes in commercial steepwater. So a possibility as to why LI-NMS and -WMS produced different values than did C-NMS and -WMS when the standard method was used is that the values are in part a function of protein content. Therefore, nitrogen analysis was done on the starches. The results (in %N) were as follows: LI-NMS (0.0803 ± 0.0020) > C-NMS (0.0444 ± 0) > PT-NMS (0.0394 ± 0.0002) and LI-WMS (0.120 ± 0) > PT-WMS

Table 1
RVA data for unmodified and modified normal maize starches with and without protease treatment reacted by the standard method.

Starch	Derivatizing reagent	Peak η	Breakdown	Setback	Final η
LI-NMS[a]	–	1050(13)[c]	99(6)[c]	252(21)[c]	1203(33)[c]
C-NMS[d]	–	1133(19)	185(4)	243(39)	1190(34)
PT-CNMS[e]	–	1164(14)	210(12)	313(13)	1267(20)
LI-NMS	AAMA[f]	1623(18)	631(21)	787(28)	1778(26)
C-NMS	AAMA	1362(4)	316(55)	910(31)	1956(22)
PT-CNMS	AAMA	1495(3)	439(10)	705(11)	1761(5)
LI-NMS	$POCl_3$[g]	1175(9)	229(39)	473(21)	1419(44)
C-NMS	$POCl_3$	1305(10)	414(24)	640(38)	1531(70)
PT-CNMS	$POCl_3$	1302(6)	382(30)	615(42)	1535(57)
C-NMS	–[h]	1185(16)	390(14)	405(29)	1199(29)
C-NMS	$POCl_3$[i]	1299(8)	359(17)	399(16)	1339(11)
C-NMS	–[j]	1189(8)	278(9)	431(19)	1342(9)
LI-NMS	STMP[k]	8054(57)	2973(110)	2895(108)	7976(63)
C-NMS	STMP	8582(134)	3803(108)	2674(121)	7454(54)
PT-CNMS	STMP	9149(8)	3916(303)	3083(281)	8316(27)
LI-NMS	AA[l]	1021(4)	166(29)	1026(31)	1880(30)
C-NMS	AA	1121(22)	220(10)	873(21)	1774(18)
PT-CNMS	AA	1086(5)	304(3)	1106(5)	1889(6)
LI-NMS	SA[m]	2877(16)	1599(12)	859(18)	2137(21)
C-NMS	SA	5163(122)	3986(107)	1545(189)	2722(199)
PT-CNMS	SA	3491(51)	2164(42)	908(36)	2235(58)
LI-NMS	OSA[n]	1515(23)	57(11)	683(3)	2141(13)
C-NMS	OSA	1580(4)	87(41)	745(12)	2238(39)
PT-CNMS	OSA	1772(36)	91(14)	740(6)	2371(36)
LI-WMS[o]	–	2222(36)	1251(12)	145(13)	1117(24)
C-WMS[p]	–	1791(12)	756(16)	145(13)	1179(26)
PT-CWMS[q]	–	1589(18)	609(25)	103(4)	1083(9)
LI-WMS	AAMA[f]	3531(29)	992(51)	1214(17)	3752(13)
C-WMS	AAMA	2732(1)	888(4)	661(12)	2505(9)
PT-CWMS	AAMA	2767(24)	748(42)	962(35)	2981(33)
LI-WMS	$POCl_3$[g]	2648(14)	798(18)	328(26)	2178(29)
C-WMS	$POCl_3$	1886(14)	554(26)	386(12)	1718(14)
PT-CWMS	$POCl_3$	1723(11)	522(4)	414(19)	1615(24)
C-WMS	–[h]	1460(22)	625(23)	149(12)	984(7)
C-WMS	$POCl_3$[i]	1606(8)	620(6)	175(16)	1160(9)
C-WMS	–[j]	1555(9)	703(12)	146(14)	997(15)
LI-WMS	STMP[k]	12,137(43)	3042(140)	3301(131)	12,396(25)
C-WMS	STMP	11,909(68)	3521(125)	2723(80)	11,111(60)
PT-CWMS	STMP	12,096(116)	3789(229)	2809(145)	11,115(210)
LI-WMS	AA[l]	2767(15)	1680(24)	385(20)	1472(4)
C-WMS	AA	1952(37)	722(13)	281(35)	1511(27)
PT-CWMS	AA	1864(16)	802(14)	239(6)	1301(6)
LI-WMS	SA[m]	4089(74)	2489(46)	394(61)	1994(17)
C-WMS	SA	3843(55)	2310(82)	432(17)	1965(23)
PT-CWMS	SA	3951(1)	2453(30)	563(25)	2061(3)
LI-WMS	OSA[n]	6665(45)	2844(43)	632(52)	4454(58)
C-WMS	OSA	3731(72)	1385(8)	558(59)	2903(12)
PT-WMS	OSA	4648(27)	1610(79)	815(48)	3854(84)

[a] Laboratory-isolated normal maize starch[b].
[b] Measured at 7% starch concentration (except for the OSA modification).
[c] Standard deviations in parentheses.
[d] Commercial normal maize starch[b].
[e] Protease (thermolysin)-treated commercial normal maize starch[b].
[f] Acetic-adipic mixed anhydride.
[g] Phosphoryl chloride (0.01%, pH 11.3).
[h] Starch treated at pH 11.3 without reagent.
[i] Reaction done at pH 7.0.
[j] Starch treated at pH 7.0 without reagent.
[k] Sodium trimetaphosphate.
[l] Acetic anhydride.
[m] Succinic anhydride.
[n] Octenylsuccinic anhydride (RVA analysis done at 5% starch concentration).
[o] Laboratory-isolated waxy maize starch[b].
[p] Commercial waxy maize starch[b].
[q] Protease (thermolysin)-treated commercial waxy maize starch[b].

$(0.0268 \pm 0.0016) >$ C-WMS (0.0196 ± 0.0008). The results were as expected, except for the order of PT- and C-WMS and LI-WMS containing more protein than LI-NMS. For the control (unmodified/native) NMS starches, both peak η and breakdown increased in the order LI-NMS < C-NMS < PT-NMS, indicating that the presence of surface protein inhibits granule swelling. Hamaker, Griffin, and Moldenhauer (1991) and Hamaker and Griffin (1993) proposed that the presence of proteins with disulfide bonds make swollen rice starch granules less susceptible to breakdown, either by strengthening swollen granules or by reducing granule swelling. Additional evidence that the presence of protein inhibits granule swelling was provided by Debet and Gidley (2006).

When values for peak η, breakdown, setback, and final η for C-NMS modified with the AAMA reagent using the standard method

Table 2
RVA data for unmodified and modified normal maize starches reacted by the TRF method.

Starch	Derivatizing reagent	Peak η	Breakdown	Setback	Final η
LI-NMS[a]	–	1050(13)[c]	99(6)[c]	252(21)[c]	1203(33)[c]
C-NMS[d]	–	1133(19)	185(4)	243(39)	1190(34)
LI-NMS	AAMA[e]	1120(7)	151(29)	354(48)	1323(52)
C-NMS	AAMA	1183(13)	241(24)	325(34)	1267(67)
LI-NMS	$POCl_3$[f]	1118(2)	226(53)	432(56)	1325(4)
C-NMS	$POCl_3$	1276(9)	430(20)	601(33)	1447(40)
LI-NMS	STMP[g]	8573(49)	3458(110)	2764(36)	7879(72)
C-NMS	STMP	8505(53)	3572(261)	2630(221)	7564(80)
LI-NMS	AA[h]	1163(10)	224(22)	421(33)	1360(20)
C-NMS	AA	1299(6)	463(4)	590(8)	1425(7)
LI-NMS	SA[i]	1154(12)	252(17)	463(40)	1365(25)
C-NMS	SA	1275(19)	422(26)	520(54)	1372(41)
LI-NMS	OSA[j]	1516(25)	85(8)	782(16)	2212(35)
C-NMS	OSA	1519(12)	141(8)	857(19)	2235(22)
LI-WMS[k]	–	2222(36)	1251(12)	145(13)	1117(24)
C-WMS[l]	–	1791(12)	756(16)	145(13)	1179(26)
LI-WMS	AAMA[e]	2256(7)	1219(14)	139(13)	1176(5)
C-WMS	AAMA[f]	1683(33)	673(11)	148(7)	1158(15)
LI-WMS	$POCl_3$[f]	2432(8)	1299(29)	398(30)	1532(9)
C-WMS	$POCl_3$	1505(14)	566(17)	185(21)	1124(14)
LI-WMS	STMP[g]	10,913(36)	2995(186)	3463(109)	111,380(80)
C-WMS	STMP	11,120(91)	3624(126)	2812(59)	10,308(268)
LI-WMS	AA[h]	2203(1)	1285(60)	201(59)	1118(18)
C-WMS	AA	1649(7)	679(17)	169(31)	1138(23)
LI-WMS	SA[i]	2219(25)	1240(35)	158(16)	1137(23)
C-WMS	SA	1610(25)	644(8)	127(13)	1093(12)
LI-WMS	OSA[j]	6173(35)	2403(69)	557(183)	4328(154)
C-WMS	OSA	3093(48)	839(90)	432(49)	2687(87)

[a] Laboratory-isolated normal maize starch[b].
[b] Measured at 7% starch concentration (except for the OSA modification).
[c] Standard deviations in parentheses.
[d] Commercial normal maize starch[b].
[e] Acetic-adipic mixed anhydride.
[f] Phosphoryl chloride.
[g] Sodium trimetaphosphate.
[h] Acetic anhydride.
[i] Succinic anhydride.
[j] Octenylsuccinic anhydride (RVA analysis done at 5% starch concentration).
[k] Laboratory-isolated waxy maize starch[b].
[l] Commercial waxy maize starch[b].

were compared to the same values for unmodified C-NMS, they were found to be 1.2×, 1.7×, 3.7×, and 1.6×, respectively, whereas when the TRF method was used, they were 1.0×, 1.3×, 1.3×, and 1.1×, respectively, indicating a lesser change in RVA attributes when the TRF method was used. Similar results were obtained when comparing LI-NMS modified with the AAMA reagent using the two methods. In addition, there was almost no measureable ester formation when C-NMS and LI-NMS were reacted with the AAMA reagent using the TRF method, the titratable ester being only 2% and 4%, respectively, of the values of the products made via the standard reaction (Table 3), again suggesting that very little reaction occurred when the TRF method was used.[1]

Reaction with the AAMA reagent, which also contains AA, introduces both adipyl (crosslinking) and acetyl (stabilizing) groups, making the results difficult to interpret. The fact that all four RVA attributes were substantially increased after reaction with the AAMA reagent using the standard method, and especially the fact that both breakdown and setback were increased, indicates that granules were weakened, rather than strengthened, i.e., that substitution/acetylation had a greater effect on pasting and paste properties than did crosslinking. As already stated, when LI- and C-NMS were reacted with the AAMA reagent using the TRF method, increases in the 4 RVA attributes were much smaller, indicating much less effective modification. Possible explanations for the results are that the reagent reacts with the more nucleophilic amino, imino, sulfhydryl, or aromatic hydroxyl groups on granule protein molecules when no alkali is present to ionize the hydroxyl groups of starch, resulting in formation of some amide groups, which were not accounted for by saponification and titration or that it reacted with water during the infiltration period. However, a comparison of final viscosities of LI-NMS and C-NMS reacted with the AAMA reagent revealed that they were increased substantially (1.5× and 1.6×, respectively) over the control values, when the standard method was used, but were increased only a small degree (1.1× and 1.1×, respectively) over the control values when the TRF method was used.[1]

Since it was not indicated that the standard method either produced more effective crosslinks or resulted in a greater degree of crosslinking, it may be that granules must be swollen (resulting from ionization of hydroxyl groups by alkali) before addition of the reagent in order for efficient reaction to occur. Hauber, BeMiller, and Fannon (1992) concluded that granule swelling was required for derivatization of NMS with fatty acyl chlorides, but the conclusion was based on granules reacted in a suspension in pyridine. Gray and BeMiller (2001) found that accessibility of fatty acyl amides of different chain lengths to the matrix of NMS granules in aqueous systems was controlled by the degree of granule swelling. Gray and BeMiller (2005) and Villwock and BeMiller (2005) found

[1] It needs to be remembered throughout this paper that simply subjecting a starch to the pH, time, and temperature conditions used for derivatization (without reagent) results in changes in the physical properties of the starch that can be greater than those resulting from reaction with a reagent (Sui et al., 2011) and that LI-NMS and C-NMS were from different genetic backgrounds and contained different amounts of protein and that the same was true for LI-WMS and C-WMS.

Table 3
Amounts of substitution of commercial maize and laboratory-isolated maize starches modified by both the treatment-with-reagent-first (TRF) and standard methods.

Starch	Reagent	TRF method[a]	Standard method[a]	TRF method/standard method
LI-NMS[b]	AAMA[c]	0.003(0.002)	0.069(0.003)	0.043
C-NMS[d]	AAMA	0.001(0.002)	0.046(0.000)	0.023
PT-CNMS[e]	AAMA		0.070(0.002)	
LI-NMS	$POCl_3$[f]	19.5(0.5)	12.5(0.5)	1.56
C-NMS	$POCl_3$	27(0)	17.5(2.5)	1.54
PT-CNMS	$POCl_3$		17.0(0)	
LI-NMS	STMP[f,g]	1700(0)	1950(50)	0.872
C-NMS	STMP	2200(0)	2150(150)	1.02
PT-NMS	STMP		2100(100)	
LI-WMS	AAMA	0.001(0.000)	0.068(0.001)	0.015
C-WMS[f]	AAMA	0.001(0.000)	0.051(0.001)	0.020
PT-CWMS	AAMA		0.067(0.001)	
LI-WMS	$POCl_3$[f]	14(0)	12(0)	1.20
C-WMS	$POCl_3$	8.2(0.1)	8.5(0)	0.96
PT-CWMS	$POCl_3$		11.5(0.5)	
LI-WMS	STMP[f,g]	2550(50)	2150(50)	1.05
C-WMS	STMP	2500(0)	2550(50)	0.980
PT-CWMS	STMP		2750(50)	
LI-NMS	AA	0.000(0.000)	0.137(0.004)	
C-NMS	AA	0.002(0.001)	0.059(0.006)	0.034
PT-CNMS	AA		0.285(0.008)	
LI-NMS	SA	0.017(0.000)	0.041(0.001)	0.415
C-NMS	SA	0.016(0.001)	0.045(0.001)	0.356
PT-CNMS	SA		0.057(0.000)	
LI-NMS	OSA	0.028(0.000)	0.030(0.000)	0.933
C-NMS	OSA	0.028(0.000)	0.031(0.001)	0.903
PT-CNMS	OSA		0.037(0.001)	
LI-WMS	AA	0.000(0.000)	0.245(0.004)	
C-WMS	AA	0.004(0.002)	0.129(0.003)	0.031
PT-CWMS	AA		0.321(0.000)	
LI-WMS	SA	0.014(0.000)	0.045(0.000)	0.311
C- WMS	SA	0.014(0.000)	0.052(0.000)	0.269
PT-CWMS	SA		0.062(0.001)	
LI-WMS	OSA	0.027(0.001)	0.029(0.000)	0.931
C-WMS	OSA	0.021(0.001)	0.024(0.001)	0.875
PT-CWMS	OSA		0.030(0.001)	

[a] Numbers in parentheses are standard deviations.
[b] Laboratory-isolated normal maize starch.
[c] Acetic-adipic mixed anhydride.
[d] Commercial normal maize starch.
[e] Protease (thermolysin)-treated normal maize starch.
[f] Results are given in μg/g of phosphorus and are averages of duplicate analysis.
[g] Sodium trimetaphosphate.

definitively that reaction efficiency of NMS and WMS with propylene oxide is a function of the degree of granule swelling, which was primarily controlled by pH and swelling-inhibiting salts.

Analysis of the data for C-WMS and LI-WMS modified with the AAMA reagent revealed similar general patterns of RVA attributes (Tables 1 and 2) and substitution values (Table 3) as found for C-NMS and LI-NMS, undoubtedly for the same reason. For example, when the values for peak η, breakdown, setback, and final η for C-WMS modified with the AAMA reagent using the standard method were compared to the same values for unmodified C-WMS, they were found to be 1.5×, 1.2×, 4.6×, and 2.1×, respectively, whereas when the TRF method was used, they were 0.94×, 0.89×, 1.0×, and 0.98×, respectively, and the titratable ester content was only 2% of that which resulted from use of the standard method, again indicating that very little if any reaction occurred when the TRF method was used.[1] C-NMS and C-WMS were treated with the proteolytic enzyme thermolysin (Mu-Forster & Wasserman, 1998) to remove any remaining surface protein before reaction with the crosslinking reagents. The data in Table 1 generally reveals small and inconsistent differences for these starches modified using the standard method. For example, peak η, breakdown, setback, and final η values for PT-CNMS modified with the AAMA reagent compared to those for C-NMS reacted with the AAMA reagent were 1.1×, 1.4×, 0.8×, and 0.9×, respectively, and the same comparison for PT-WMS vs. C-WMS gave values of 1.0×, 0.8×, 1.5×, and 1.2×, respectively. Neither do peak or final viscosities (Table 1) indicate a consistent pattern with respect to the amount of protein present for either the NMS or WMS products, perhaps because the LI- and commercial starches had different genetic backgrounds. Treatment with the protease resulted in a lower final η of PT-CNMS as compared to C-NMS reacted with the AAMA reagent (standard method), while the effect on C-WMS was reversed, perhaps because WMS has little matrix protein due to its lack of GBSS (Goldner & Boyer, 1989; Han & Hamaker, 2002; Yamamori, Nakamura, Endo, & Nagamine, 1994).

The data in Table 3 indicate that, when the standard method was used, removing most of the surface protein (PT-CNMS and -CWMS) generally resulted in increased incorporation of derivatizing groups when only esterification of starch molecules could be measured (reaction with the AAMA reagent, AA, SA, or OSA), indicating that some reagent reacted with protein molecules (when present), even when the standard method was used.

Crosslinking of LI-NMS and C-NMS with $POCl_3$ resulted in quite similar RVA attributes when the two methods were used, with the starches crosslinked using the TRF method Table 2) having lower final η values than did those crosslinked by the standard method (Table 1), but which values were still greater than the values for the unmodified starches.[1] Comparing peak η, breakdown, setback, and final η values of C-NMS crosslinked with $POCl_3$ using the standard method to those of unmodified C-NMS, the increases were 1.2×, 2.2×, 2.6×, and 1.3×, respectively. For C-NMS reacted with $POCl_3$

using the TRF method, the respective values were 1.1×, 2.3×, 2.×, and 1.2×, respectively,[1] indicating less, but only very slightly less, reaction when the TRF method was employed. However, when the TRF method was used, incorporation of phosphorus was 1.5× that which resulted from using the standard method (Table 3). A possible explanation for these results is that the reagent reacts with protein or starch molecules, even before the pH is adjusted to 11.3, without effecting much crosslinking. Data for C-WMS reacted with $POCl_3$ by the standard method revealed only 49% as much reaction as C-NMS reacted with the same reagent. Since C-WMS contains less protein, especially matrix protein, than does C-NMS, this is another indication of preferential reaction with protein. To further test this possibility, C-NMS was reacted with $POCl_3$ at pH 11.3 in the standard way and in a pH 7.0 buffer (using the buffer to neutralize any HCl released in the reaction), even though the C-NMS water slurry had a pH below 5 (to be discussed later). RVA results (Table 1) indicate that some reaction took place at pH 7.0, in that C-NMS reacted with $POCl_3$ at pH 11.3 produced a final η that was 1.3× that produced by the unmodified starch, while the starch treated under the same conditions without $POCl_3$, produced a final η that was the same (1.0×) as that produced by the parent starch. For C-NMS reacted with $POCl_3$, the values for treatment at pH 7.0 with and without reagent were both 1.1×. The results were more striking for C-WMS. C-WMS reacted with $POCl_3$ at pH 11.3 produced a final viscosity that was 1.5× that produced by the unmodified parent starch, while the starch treated under the same conditions without $POCl_3$ produced a final viscosity that was 0.83× that produced by the unmodified parent starch. For C-WMS reacted with $POCl_3$, the values for treatment at pH 7.0 were 1.0× (with $POCl_3$) and 0.85× (without $POCl_3$). Overall, it appears that little starch polymer molecule crosslinking occurred before pH adjustment. Because there was a greater incorporation of phosphorus when the TRF method was used with 3 of the 4 starches, but little change in pasting behavior, reaction with protein molecules without impacting behavior is indicated. Perhaps, when the TRF method was used, more non-crosslinking phosphate esters or phosphoramides (on protein molecules) were formed because of less loss of reagent due to reaction with hydroxide ions at the lower initial pH of the TRF method. Here again, ionization of hydroxyl groups effected by alkaline pH values accompanied by granule swelling may be required for efficient derivatization of starch molecules.

Reaction with STMP was different. Pasting and paste properties of starches crosslinked with STMP, whether normal or waxy maize starches, varied little when the TRF method (Table 2), as compared to the standard method (Table 1), was used. Comparing the values for peak η, breakdown, setback, and final η of starches reacted with STMP using the standard method to those of the unmodified starches, the following results were obtained: for C-NMS, 7.8×, 21×, 11×, and 6.3×, respectively; for C-WMS, 6.6×, 4.7×, 19×, and 9.4×, respectively.[1] The same comparisons for starches modified using the TRF method were as follows: for C-NMS, 7.5×, 19×, 11×, 6.4×, respectively[1]; for C-WMS, 6.2×, 4.8×, 19×, and 8.9×, respectively, indicating considerable congruency. Neither did the reaction efficiency (measured by incorporation of phosphorus) differ between the two methods (Table 3). Since there is no evidence that reaction with STMP is quite rapid[2] (as is believed to be the reaction with $POCl_3$), our results could be interpreted as suggesting that reaction efficiencies of the two methods were equalized when, in both cases, (1) there was a 3-h period with the reagent being present after pH adjustment, which should have allowed equal penetration of reagent solution into swollen granules, and (2) the dried reaction mixture was heated (Kerr, 1947; Lim & Seib, 1993; Srivastava & Patel, 1973), although it could be predicted that the ionic STMP would not penetrate as easily into ionized granules as compared to non-ionized granules, but the same might be true of hydroxide ion penetration into granules impregnated with STMP. What actually happened remains to be determined. Little reaction should have occurred before the pH was adjusted because the STMP + Na_2SO_4 solution had a pH of 6.1 before the starch was added and addition of C-NMS lowered the pH to 4.9. Lim and Seib (1993), who used the same procedure, i.e., heated dried starch impregnated with STMP, reported only limited crosslinking below pH 9.

Han and BeMiller (2008), based on RVA data, reported that $POCl_3$ crosslinking of C-NMS and C-WMS appeared to be less effective (less inhibited as revealed in RVA curves) when surface protein was removed enzymicly and tentatively interpreted the results as indicating that the reactions involved protein molecules, although perhaps indirectly. In this study, when values for amounts of substitution, peak η, final η, peak gelatinization temperature, and enthalpy of gelatinization for all products were compared, no significant correlations were found between any of the values and the amount of protein in the starch. For example, when the RVA parameter values for PT-CNMS and -CWMS were divided by the same values for C-NMS and -WMS (all derivatized using the standard method), it was found that removal of protein before crossslinking changed few of the RVA attributes by more than ±10%. Interesting findings were that (1), in every case, reaction of the PT starch with the non-crosslinking reagents (AA, SA, OSA) using the standard method gave the highest amounts of substitution (Table 3) and (2) the highest and equal amounts of measured substitution after reaction with the AAMA reagent using the standard method with both normal and waxy maize starches were given by the LI and PT starches. A possible explanation for the first observation is that a higher percentage of ester bonds, some of which could be on protein molecules, which were the only ones detected by the methods used, as opposed to amide bonds, were formed in the PT starches. In support of this, all values for the three types of WMS, which contain much less matrix protein, were greater than those of the corresponding three types of NMS after reaction with AA and SA. (Reaction with OSA will be discussed later.) Also, comparing C-NMS and PT-CNMS and C-WMS and PT-CWMS, each pair of which was from the same lot/genetic background, the apparent DS (Table 3) increased after the protease treatment and use of the standard method. We, therefore, suggest that the greater DS values found for protease-treated starches are probably due to a lesser amount of protein being present forcing more of the reaction to occur on starch molecules, thus allowing a greater DS to be determined via saponification and titration.

Table 4 gives DSC data for gelatinization and melting of retrograded pastes of starches modified by the standard method. Table 5 gives the same data for starches modified by the TRF method. The data in Tables 4 and 5 seem to support the RVA data in that the largest and most consistent differences in gelatinization properties (relative to unmodified controls) were found for the reaction with STMP. For reactions with LI-NMS and C-NMS, the reaction with STMP generally gave the largest decreases in peak temperature of gelatinization (T_p), enthalpy of gelatinization (ΔH_{gel}), and enthalpy of amylopectin melting in retrograded pastes (ΔH_R) and the largest increases in phase transition temperature ranges ($T_c - T_o$) of gelatinization and amylopectin melting in retrograded pastes, whether the standard method or the TRF method was used, perhaps indicating relatively homogeneous reactions within granules in both cases. Data for LI-WMS and C-WMS were similar (especially when the TRF method was used), but less conclusive. At least, for the NMS preparations, this is another indication that the products of reaction with STMP were similar when the two methods used in this research were used.

[2] Hirsch and Kokini (2002) indicate that reaction of WMS with STMP requires 4 h (presumably at 30 °C and pH 12), but no data is presented to support this claim.

Table 4
DSC data for unmodified and modified maize starches with and without protease treatment reacted by the standard method.

Starch	Derivatizing reagent	Gelatinization			Retrograded paste[a]		
		T_p [b]	$T_c - T_o$ [c]	ΔH	T_p [b]	$T_c - T_o$ [c]	ΔH
LI-NMS[d]	–	69.7(0.3)[e]	9.95(0.30)	16.2(1.2)	52.8(0.4)	19.0(0.6)	8.43(0.27)
C-NMS[f]	–	70.8(0.1)	10.7(0.5)	13.8(0.5)	53.3(0.2)	17.7(0.3)	8.20(0.05)
PT-CNMS[g]	–	69.4(0.1)	10.7(0.4)	14.8(0.1)	51.0(0.2)	23.4(1.2)	9.20(0.60)
LI-NMS	AAMA[h]	67.3(0.0)	10.1(0.1)	14.3(0.1)	53.1(0.5)	21.2(0.8)	5.12(0.06)
C-NMS	AAMA	67.6(0.1)	11.2(0.1)	13.8(0.1)	52.6(0.5)	23.3(0.5)	6.51(0.33)
PT-CNMS	AAMA	66.4(0.1)	11.1(0.5)	15.3(0.1)	49.2(0.7)	22.9(4.0)	5.91(0.40)
LI-NMS	$POCl_3$	69.6(0.2)	9.3(0.5)	15.3(0.4)	52.9(0.7)	19.9(0.4)	9.09(0.47)
C-NMS	$POCl_3$	70.0(0.2)	10.7(0.4)	13.4(0.6)	51.6(0.3)	21.5(0.8)	9.79(1.00)
PT-CNMS	$POCl_3$	69.7(0.0)	11.4(0.4)	13.6(0.0)	52.2(0.0)	21.2(0.1)	8.82(0.09)
LI-NMS	STMP[i]	66.2(0.3)	15.6(0.2)	13.5(0.2)	51.9(0.1)	23.3(1.0)	6.01(0.22)
C-NMS	STMP	66.6(0.4)	17.5(1.4)	13.0(0.5)	52.7(0.4)	24.0(1.2)	6.44(0.53)
PT-CNMS	STMP	66.1(0.0)	16.7(0.4)	13.0(0.2)	53.0(0.0)	22.2(1.1)	6.07(0.19)
LI-NMS	AA[j]	67.0(0.4)	10.7(0.3)	14.3(0.4)	53.2(0.2)	18.7(0.3)	6.51(0.62)
C-NMS	AA	69.3(0.1)	11.8(0.6)	13.1(0.5)	52.0(0.0)	20.5(2.4)	7.40(0.09)
PT-CNMS	AA	63.6(0.4)	12.7(0.7)	11.8(0.9)	51.6(0.4)	19.0(0.2)	4.06(0.61)
LI-NMS	SA[k]	64.9(0.6)	16.0(0.4)	14.2(0.2)	51.9(0.8)	21.4(1.4)	4.52(0.40)
C-NMS	SA	63.9(0.3)	17.6(1.3)	13.5(0.2)	53.3(0.2)	22.4(0.8)	5.61(0.39)
PT-CNMS	SA	62.7(0.1)	20.6(0.3)	10.0(0.2)	52.8(0.2)	19.3(0.1)	5.71(0.22)
LI-NMS	OSA[l]	69.0(0.1)	10.5(0.4)	13.4(0.1)	53.2(0.3)	24.2(1.2)	6.78(0.71)
C-NMS	OSA	69.6(0.2)	11.8(0.4)	11.2(0.5)	52.9(0.4)	20.4(1.2)	6.08(0.72)
PT-CNMS	OSA	68.5(0.0)	12.8(0.1)	13.0(0.4)	52.8(0.1)	21.8(0.8)	5.90(0.39)
LI-WMS[m]	–	71.0(0.1)	13.1(0.3)	23.1(1.2)	53.5(0.3)	17.3(0.6)	10.6(0.7)
C-WMS[n]	–	72.2(0.1)	12.7(0.3)	16.2(0.1)	53.2(0.1)	18.5(0.5)	10.9(0.3)
PT-CWMS[o]	–	70.1(0.1)	13.3(0.3)	18.9(0.3)	51.7(0.3)	20.8(0.1)	11.5(0.1)
LI-WMS	AAMA[h]	68.1(0.1)	11.0(0.3)	19.3(0.7)	54.4(0.4)	19.9(0.6)	2.46(0.31)
C-WMS	AAMA	68.1(0.4)	13.3(1.5)	15.3(1.1)	52.6(0.5)	23.3(0.5)	6.51(0.33)
PT-CWMS	AAMA	67.3(0.1)	11.0(0.3)	18.0(0.7)	49.2(0.7)	22.9(4.0)	5.91(0.40)
LI-WMS	$POCl_3$	70.0(0.3)	12.2(0.3)	18.4(0.7)	53.1(0.2)	19.1(0.3)	10.3(0.6)
C-WMS	$POCl_3$	70.9(0.2)	13.6(0.1)	16.3(0.1)	54.3(0.2)	17.4(0.8)	10.4(1.3)
PT-CWMS	$POCl_3$	70.3(0.1)	13.6(0.2)	18.5(0.7)	50.3(1.1)	26.8(2.4)	10.3(0.3)
LI-WMS	STMP[i]	69.2(0.1)	16.8(1.1)	12.3(1.6)	51.8(0.5)	23.9(0.3)	7.95(0.14)
C-WMS	STMP	68.1(0.3)	18.8(0.0)	13.7(0.3)	52.7(0.5)	23.0(1.1)	6.72(0.37)
PT-CWMS	STMP	67.3(0.1)	18.8(0.1)	13.6(0.3)	53.1(0.3)	23.8(0.1)	6.42(0.05)
LI-WMS	AA[j]	66.5(0.1)	10.9(0.4)	17.0(0.3)	54.4(0.6)	14.2(0.4)	1.35(0.24)
C-WMS	AA	69.1(0.3)	12.3(0.8)	15.1(0.3)	54.3(0.5)	18.5(1.7)	5.83(0.36)
PT-CWMS	AA	65.1(0.0)	12.7(0.1)	16.2(0.1)	52.5(0.1)	15.3(2.0)	1.44(0.25)
LI-WMS	SA[k]	66.9(0.3)	18.3(0.1)	18.0(0.4)	[p]	[p]	[p]
C-WMS	SA	65.5(0.0)	18.5(0.6)	14.6(0.6)	54.4(0.3)	19.7(0.2)	2.56(0.50)
PT-CWMS	SA	64.6(0.4)	20.8(0.0)	14.8(0.6)	52.7(0.2)	20.7(0.7)	3.91(0.88)
LI-WMS	OSA[l]	68.9(0.2)	12.4(0.3)	18.5(0.5)	[p]	[p]	[p]
C-WMS	OSA	62.8(0.3)	11.5(0.5)	13.3(0.3)	53.8(1.3)	17.8(1.9)	6.32(1.58)
PT-CWMS	OSA	68.7(0.3)	14.0(0.7)	14.9(1.2)	53.3(0.6)	19.6(1.4)	3.66(0.39)

[a] Amylopectin melting in retrograded paste.
[b] Peak temperature.
[c] Conclusion temperature minus onset temperature (temperature range).
[d] Laboratory-isolated normal maize starch.
[e] Standard deviations in parentheses.
[f] Commercial normal maize starch.
[g] Protease (thermolysin)-treated commercial normal maize starch.
[h] Adipic-acetic mixed anhydride.
[i] Sodium trimetaphosphate.
[j] Acetic anhydride.
[k] Succinic anhydride.
[l] Octenylsuccinic anhydride.
[m] Laboratory-isolated waxy maize starch.
[n] Commerical waxy maize starch.
[o] Protease (thermolysin)-treated commercial waxy maize starch.
[p] No endotherm obtained.

DSC data for LI-NMS and PT-CNMS and LI-WMS and PT-WMS starches exhibited considerably greater ΔH_R values in retrograded gels of starches modified with the AAMA reagent, AA, or SA (the latter two to be discussed in Section 3.2) reagents using the TRF method (Table 5) as compared to the same values for starches modified with the same reagents using the standard method, making the ΔH_R values about the same for the modified and unmodified starches (Table 4). As already stated, we believe that, when the TRF method was used, most of any reaction that occurred with these reagents occurred with protein molecules and that the starch molecules were unmodified. The equal amounts of amylopectin retrogradation for modified and unmodified starches indicated with this data support that conclusion.

In summary, data for reactions with crosslinking reagents indicate that, with AAMA as the crosslinking reagent, almost no reaction with starch occurred when the TRF method was used; with $POCl_3$, little crosslinking occurred when the TRF method was used; and with STMP, similar properties were achieved using either method, perhaps because, whether the pH was adjusted before or after the reagent was added, the time of exposure to the reagent under the alkaline condition was the same and the starches were all heated to 130 °C after being impregnated with the reagent and dried.

Table 5
DSC data for unmodified and modified maize starches reacted by the TRF method.

Starch	Derivatizing reagent	Gelatinization			Retrograded paste[a]		
		T_p [b]	$T_c - T_o$ [c]	ΔH	T_p [b]	$T_c - T_o$ [c]	ΔH
LI-NMS[d]	–	69.7(0.3)[e]	9.95(0.30)	16.2(1.2)	52.8(0.4)	19.0(0.6)	8.43(0.27)
C-NMS[f]	–	70.8(0.1)	10.7(0.5)	13.8(0.5)	53.3(0.2)	17.7(0.3)	8.20(0.05)
LI-NMS	AAMA[g]	69.7(0.0)	9.34(0.08)	16.6(0.0)	54.2(0.5)	17.4(0.7)	8.57(0.14)
C-NMS	AAMA	69.6(0.1)	10.6(0.1)	13.8(0.1)	53.0(0.7)	20.2(0.6)	9.43(0.21)
LI-NMS	$POCl_3$	69.4(0.1)	9.46(0.1)	14.9(0.13)	53.3(0.4)	19.2(1.0)	8.70(0.47)
C-NMS	$POCl_3$	70.1(0.2)	10.7(0.0)	13.0(0.5)	52.5(0.9)	20.4(0.9)	9.32(0.44)
LI-NMS	STMP[h]	66.5(0.0)	15.5(1.0)	14.5(0.1)	52.5(0.4)	23.0(0.7)	6.48(0.08)
C-NMS	STMP	66.5(0.3)	21.1(0.5)	13.6(0.3)	52.5(0.4)	23.7(0.4)	5.97(0.09)
LI-NMS	AA[i]	69.8(0.1)	9.56(0.31)	17.3(0.8)	53.4(0.1)	18.3(0.2)	9.20(0.46)
C-NMS	AA	70.3(0.5)	12.2(0.4)	16.0(0.5)	52.2(0.2)	20.2(0.4)	9.29(0.18)
LI-NMS	SA[j]	69.9(0.2)	10.2(0.4)	16.7(0.7)	52.4(0.6)	20.4(0.4)	7.65(0.05)
C-NMS	SA	70.1(0.1)	11.1(0.5)	12.9(0.4)	52.6(0.1)	19.3(0.3)	9.29(0.23)
LI-NMS	OSA[k]	69.1(0.3)	10.3(0.1)	13.8(0.4)	53.5(0.6)	22.3(0.8)	7.73(0.42)
C-NMS	OSA	69.4(0.0)	12.4(0.4)	12.4(0.1)	52.0(0.6)	16.2(0.9)	6.22(0.18)
LI-WMS[l]	–	71.0(0.1)	13.1(0.3)	23.1(1.2)	53.5(0.3)	17.3(0.6)	10.6(0.7)
C-WMS[m]	–	72.2(0.1)	12.7(0.3)	16.2(0.1)	53.2(0.1)	18.5(0.5)	10.9(0.3)
LI-WMS	AAMA[g]	70.5(0.4)	14.6(0.4)	26.8(0.8)	53.0(0.6)	18.4(0.5)	10.5(0.3)
C-WMS	AAMA	70.2(0.3)	12.6(0.3)	14.1(0.3)	52.6(0.4)	20.7(0.8)	10.7(0.5)
LI-WMS	$POCl_3$	70.1(0.1)	12.0(0.1)	19.1(0.5)	53.2(0.5)	18.5(0.5)	10.2(0.7)
C-WMS	$POCl_3$	70.7(0.1)	14.2(0.2)	16.3(0.2)	54.3(0.4)	18.0(0.2)	9.85(0.34)
LI-WMS	STMP[h]	69.4(0.2)	10.0(1.5)	12.4(0.1)	52.2(0.3)	23.5(1.0)	6.76(0.17)
C-WMS	STMP	68.3(0.3)	19.1(0.2)	13.9(0.3)	52.8(0.7)	22.8(0.5)	7.07(0.18)
LI-WMS	AA[i]	69.9(0.4)	11.4(0.6)	20.3(0.6)	54.2(0.1)	16.6(0.3)	10.3(0.8)
C-WMS	AA	70.8(0.1)	13.6(0.3)	16.3(0.2)	53.7(0.8)	18.4(1.8)	10.7(1.1)
LI-WMS	SA[j]	69.9(0.0)	12.0(0.6)	18.5(0.3)	52.4(0.4)	20.5(1.3)	10.6(0.3)
C-WMS	SA	71.1(0.1)	13.7(0.3)	16.4(0.2)	53.2(1.4)	17.0(2.0)	10.6(0.1)
LI-WMS	OSA[k]	69.0(0.1)	12.8(0.1)	17.9(0.6)	53.8(0.3)	20.4(1.0)	3.27(0.22)
C-WMS	OSA	71.6(0.2)	13.2(0.2)	15.7(0.2)	55.0(0.1)	17.2(0.2)	6.35(0.33)

[a] Amylopectin melting in retrograded paste.
[b] Peak temperature.
[c] Conclusion temperature minus onset temperature (temperature range).
[d] Laboratory-isolated normal maize starch.
[e] Standard deviations in parentheses.
[f] Commercial normal maize starch.
[g] Adipic-acetic mixed anhydride.
[h] Sodium trimetaphosphate.
[i] Acetic anhydride.
[j] Succinic anhydride.
[k] Octenylsuccinic anhydride.
[l] Laboratory-isolated waxy maize starch.
[m] Commerical waxy maize starch.

3.2. Properties of stabilized maize starches

AA, SA, and OSA also have the potential to react with nucleophilic groups of protein molecules, producing amide bonds, which would not be measured by saponification and back titration. Properties of the products of reaction with stabilizing reagents indicated that only OSA reacted with starch molecules to a significant extent when the TRF method was used.

When the values for peak η, breakdown, setback, and final η for C-NMS reacted with AA using the standard method (Table 1) were compared to the same values for unmodified C-NMS, they were found to be 0.99×, 1.05×, 3.6×, and 1.5×, respectively, whereas the same values for C-NMS acetylated using the TRF method (Table 2) were 1.1×, 2.5×, 2.4×, and 1.2×, respectively.[1] A similar pattern was obtained for products of reaction with AA using LI-NMS and the two derivatization schemes. The same values when C-WMS was reacted with AA using the standard method (Table 1) were 1.1×, 1.3×, 1.9×, and 1.3×, respectively, while the values for C-WMS acetylated using the TRF method (Table 2) were 0.92×, 0.90×, 1.2×, and 0.97×, respectively,[1] with similar results being obtained with LI-WMS. These results were supported by the measured DS results (Table 3) which show no (LI-NMS, LI-WMS) or only very little (C-NMS, C-WMS) substitution when the TRF method was used, the lack of any measured DS found with LI-NMS and LI-WMS perhaps being due to reaction with protein. When measured DS values were determined for products of the two types of NMS and WMS with AA, the patterns were similar to those obtained for reaction with the AAMA reagent (Table 3), probably for the same reason(s).

Similar patterns were seen upon reaction with SA, although the relative differences were greater when the standard method was used, as addition of the succinate ester group with its anionic charge resulted in greater granule swelling. When the values for peak η, breakdown, setback, and final η for C-NMS reacted with SA using the standard method (Table 1) were compared to the same values for unmodified C-NMS, they were found to be 4.6×, 22×, 6.4×, and 2.3×, respectively. A similar pattern was given by LI-CNMS. The same values for C-WMS reacted with SA using the standard method were 2.1×, 3.1×, 3.0×, and 1.7×, respectively, with a similar pattern being given by LI-WMS. When the reaction with SA was done using the standard method (Table 1) and C-WMS, the respective values were 2.1×, 3.1×, 3.0×, and 1.7×, with a similar pattern given by LI-WMS. When C-NMS was reacted with SA using the TRF method, the corresponding values were 1.1×, 2.3×, 2.1×, and 1.2×, respectively,[1] with a similar pattern given by LI-NMS. When C-WMS was reacted with SA using the TRF method (Table 2), the respective values were 0.9×, 0.9×, 0.9×, and 0.9×,[1] with a similar pattern given by LI-WMS, indicating no reaction with these two types of WMS. While some titratable ester was measured (Table 3) after reaction using the TRF method, it was only 36% and 42% of that measured after reaction using the standard method with

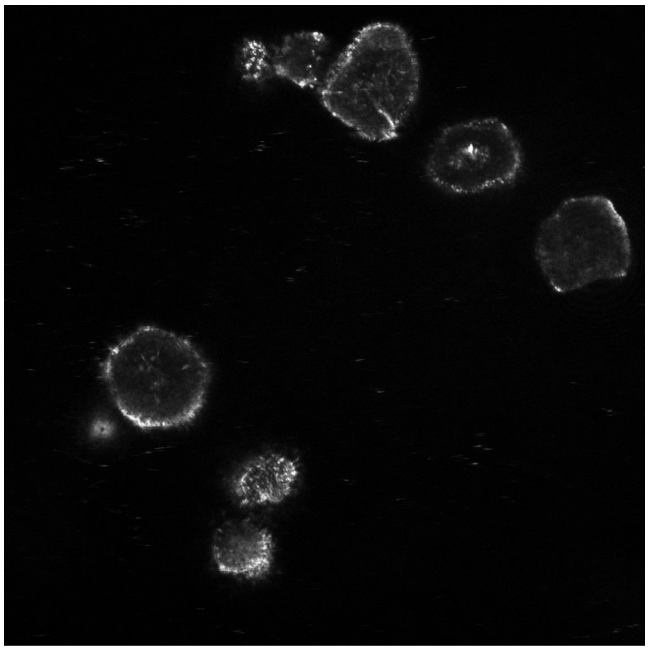

Fig. 1. R-CLSM photomicrographs of C-NMS granules reacted with SA using the standard method and examined by the procedure of Gray and BeMiller (2004).

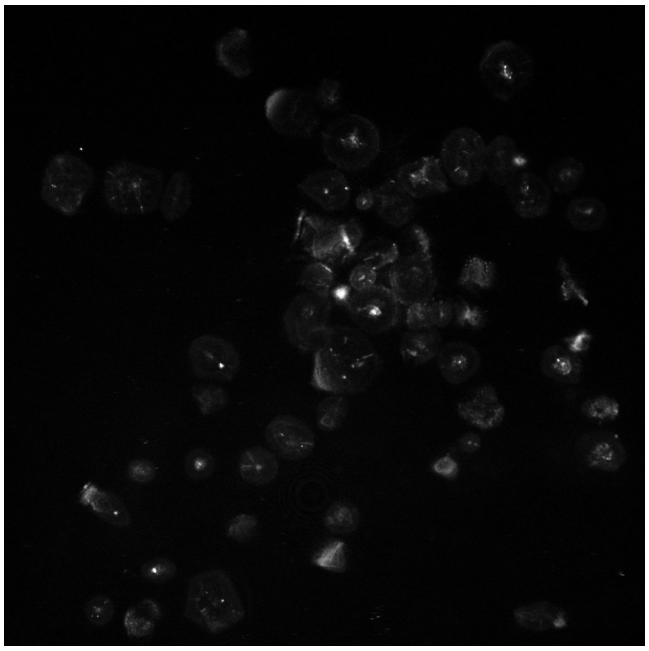

Fig. 3. R-CLSM photomicrographs of PT-CNMS granules reacted with SA using the standard method and examined by the procedure of Gray and BeMiller (2004).

C-NMS and LI-NMS, respectively, and 27% and 31% of that measured after reaction using the standard method when C-WMS and LI-WMS were the starches used.

Examination of C-NMS granules derivatized by the standard reaction by reflectance CLSM (R-CLSM) after conversion of the succinyl groups to silver salts and reduction of the silver ions to silver atoms (Gray & BeMiller, 2004) indicated reaction in channels and at specific isolated regions of the external granule surface (like that seen after reaction with $POCl_3$ in the current and previous (Gray & BeMiller, 2004) research, but even more so because of the higher DS) (Figs. 1 and 2). However, there was essentially no discernible difference between the C-NMS reacted by the TRF method as compared to that reacted by the standard method. Treatment with thermolysin prior to reaction removed almost all reaction with SA (Fig. 3), as it did for reaction with $POCl_3$. It is impossible to tell from this procedure whether the silver ions formed salts with succinate groups or protein molecules or both in the modified starches. Because treatment of the starch with the protease removed much of the silver binding, we believe that Ag^+ primarily, but not entirely, bound to protein. Furthermore, we speculate that (1) SA is rather reactive and reacts primarily with protein molecules (not detected by the back-titration method) and rather specifically with those at external and channel surfaces of granules, since those are the first protein molecules encountered by the reagent, and (2) that a high pH is not required for reaction with protein. The same pattern could be expected with reaction with other carboxylic acid anhydrides (AA, AAMA). Similar patterns were found after treatment with STMP and $POCl_3$ (not shown).

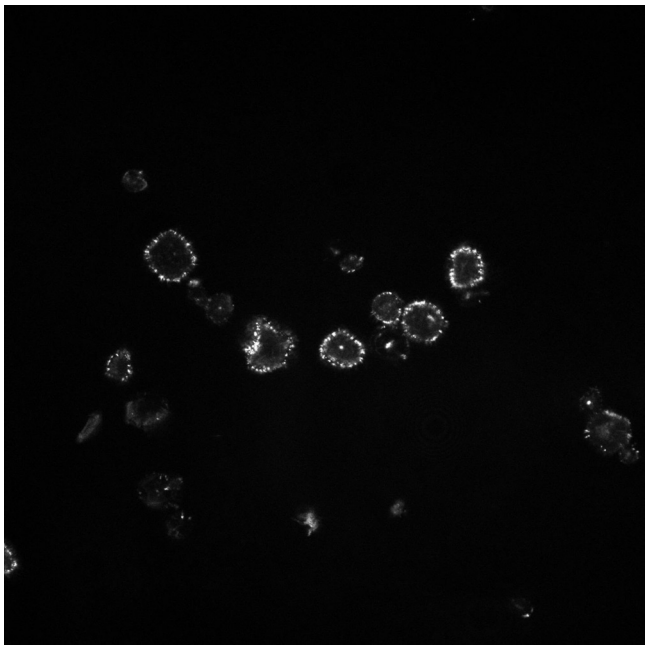

Fig. 2. R-CLSM photomicrographs of C-NMS granules reacted with SA using the TRF method and examined by the procedure of Gray and BeMiller (2004).

When RVA values for modified (standard method) PT maize starches (Table 1) were divided by the same values for the same starch modified without a prior protease treatment, only two consistent patterns were found. One was for C-NMS reacted with SA where the values for peak η, breakdown, setback, and final η were 0.68×, 0.54×, 0.59×, and 0.82×, respectively, i.e., all were reduced by 18–46%, but all were increased over the values for the controls (SA-modified PT-CNMS/unmodified PT-CNMS) by amounts of 3.0×, 10×, 2.9×, and 1.8×, respectively. This is another indication that, when surface protein is present, SA reacts with it. Addition of succinate groups to protein molecules may make them more hydrophilic, and therefore more water soluble, and remove them, allowing greater granule swelling (Debet & Gidley, 2006, 2007) and breakdown. Reaction with essentially starch alone (PT-CNMS, on whose granule surfaces little or no protein is present) could have enhanced both granule swelling and disintegration, reducing peak η and increasing breakdown. It could also be concluded that a high pH is not required for this reaction. When the same RVA attribute values for PT-CWMS reacted with SA were divided by the values for C-WMS, both of which contain much less protein than do their NMS counterparts, the results were 1.0×, 1.1×, 1.3×, and 1.0×, respectively, indicating negligible differences and another indication that

the greater amounts of protein in NMS (as compared to WMS) uses up reagent without appreciably impacting starch properties.

Another indicator of differences in products made by the two methods is determination of swelling power. The swelling power of C-NMS (determined at 100 °C) modified by reaction with SA using the standard method was 88.0 ± 0.9, while that modified using the TRF method was 70.2 ± 0.5. The lesser degree of water uptake in the latter product could be attributed to its lower apparent DS. However, it again must be remembered that only esterification of starch molecules is measured and that an increased swelling power could be due to removal of protein (Debet & Gidley, 2006, 2007).

The authors could find essentially nothing about the physical attributes of the organic reagents in water, certainly because all of them, as stated for acetic anhydride and phosphoryl chloride (Pacquette, 2009), react rapidly with water. Another source states that acetic anhydride is very soluble in water (Weast and Grasselli, 1989–1992); a much earlier paper says that the solubility of acetic anhydride in water is about 12% (Lumiere, Lumiere, & Barbier, 1906). Succinic anhydride is said to be slightly soluble in water (Pacquette, 2009) and insoluble in water (Weast & Grasselli, 1989–1992). The only other information found was the observation of Zhang et al. (2011) that, when OSA was added to an aqueous, alkaline, 35% slurry of starch in water in amounts of 0.81–5.4 (w/w%) based on the weight of starch, undissolved droplets of it could be seen. We have concluded, based on these and our own observations, the natures of oligo- and polysaccharides modified with OSA, and the structure of the OSA molecule that OSA is the least polar and least water-soluble of the reagents used. Modification with OSA appeared to be a unique process. When the values for peak η, breakdown, setback, and final η for C-NMS reacted with OSA using the standard method (Table 1) were compared to the same values for unmodified C-NMS, they were 1.4×, 0.5×, 3.1×, and 1.9×, respectively, with a similar pattern given by LI-NMS. When C-NMS was reacted with OSA using the TRF method (Table 2), the corresponding values were 1.3×, 0.8×, 3.5×, and 1.9×, respectively,[1] with a similar pattern given by LI-NMS. So in this case, there were only small differences in the effects produced by modification using the two methods. When C-WMS was reacted with OSA using the standard method, the respective values were 2.1×, 1.8×, 3.8×, and 2.5×. Reaction with LI-WMS ave a similar pattern, but larger values. Using the TRF method with C-WMS, the respective values were 1.7×, 1.1×, 3.0×, and 2.3×, with LI-WMS giving a similar pattern, but larger values. DS values obtained using the TRF method calculated as a percent of the values obtained using the standard method were as follows: for C-NMS, 90%; for LI-NMS, 93%; for C-WMS, 88%; for LI-WMS, 93%. These results, which indicate only small differences in the products made by the two methods, could be interpreted as (1) OSA being very reactive and reacting with the starch before alkali is added, (2) reaction with OSA being relatively slow so that the reagent penetrates throughout granules before reacting after alkali is added, or (3) there occurred a greater penetration of OSA into granules than occurred with other reagents, i.e., other than STMP.

With respect to the latter suggestion, Yano and Janado (1980) suggested that the internal water of gels of hydrophilic polymers is more ordered than is the bulk-phase water. Because of the ordering of water molecules within a gel pore, iceberg formation (as described by Frank and Evans (1945)) that occurs when a somewhat hydrophobic substance is added to water, is reduced so that the negative ΔS associated with dissolution in the pre-ordered water is significantly less than that associated with dissolution in the bulk-phase water (Janado, Takenaka, Nakamori, & Yano, 1980). Therefore, ΔG for the transfer of a somewhat hydrophobic solute from bulk-phase water to the ordered internal water of a hydrophilic macromolecule particle is negative, making it a favorable process. Van Steveninck, Paardekooper, Dubbelman, and Ben-Hur (1991) came to the same conclusion, viz., that there is an internal water phase in gels of hydrophilic polymers that has a solvent behavior that differs from the solvent behavior of the bulk-phase water.

The same phenomenon should be operative in starch granules in suspension, although it needs to be pointed out that this phenomenon has not been studied in hydrated starch granules and the effects of alkaline pH values and added salts as used in standard derivatization reactions are unknown. In the case of starch granules, the porous structure that results in granule hydration is not the large surface pores and channels discovered and characterized in this laboratory (Fannon, Gray, Gunawan, Huber, & BeMiller, 2003; Fannon, Gray, Gunawan, Huber, & BeMiller, 2004) that are large enough to be seen by optical microscopy (CLSM), but rather those that give granules a "microporous" structure. However, Aguerre, Suárez, and Viollaz (1989) examined the previous literature, then did their own analysis of the data, and concluded that starch granules have no intrinsic microporous structure and that water sorption by starch granules was due to their microlaminar nature, i.e., their being composed of concentric layers. They concluded that starch granules have a "parallel pore" capillary structure, i.e., one in which there are plates that are effectively infinite in length and width compared to the distance between them. They stated that water molecules are sorbed between parallel plates, promoting lattice expansion, but provided no explanation as to how water molecules access concentric layers in granules without large pores and channels, such as potato starch granules, so that they hydrate rapidly (Hennig, Lechert, & Goemann, 1976). However, the Aguerre et al. (1989) view of granule hydration would still result in an internal water phase with a solvent behavior different from that of the bulk-phase water.

Because the reactivities of OSA and SA should be similar (a supposition supported by the fact that, when DS values obtained using the TRF method were divided by those obtained using the standard method, the values fell in the order OSA > SA ≫ AA (which is probably the reverse order of their solubility in water), we attribute the fact that the extents of reaction and properties of the products were similar when the two methods were used to the less polar nature of the OSA reagent molecules, which results in them being more soluble in the ordered water in the capillaries of the porous starch granules than in the bulk external water due to an entropy difference that drives OSA molecules into hydrated granules. Further support of the concept is the report of Shogren, Viswanathan, Felker, and Gross (2000) that backscattered electron imaging of osmium-stained granules of waxy maize starch reacted with OSA revealed a uniform distribution of OS groups over granule cross-sections. To the contrary, however, Wetzel, Shi, and Reffner (2010a), using confocal FT-IR microscopy, and Wetzel, Shi, and Schmidt (2010b), using confocal Raman spectroscopy, found primarily external surface modification of OS waxy maize starch granules. Huang et al. (2010), using X-ray photoelectron spectroscopy, found that the surface of OSA-modified normal maize starch granules was "enriched with OS groups by a factor of 2 over that of the bulk granule". Zhang et al. (2011), using OS amylomaize starch and CLSM, like Huang et al. (2010), found that the OS groups were distributed throughout granules, but were in higher concentration at the granule surface, and attributed the distribution to the fact that OSA is only slightly soluble in water and, therefore, the reaction system contains both dissolved reagent and small droplets of it. They concluded that especially the reagent droplets were restricted to reaction at granule surfaces. In addition, if OSA is a rapidly reacting reagent, under the conditions of the standard method, under which hydroxyl groups at and near the granule surface would have been ionized when the reagent was added, non-exclusive reaction at the surface would still be evidence in support of the hypothesis.

When RVA values for OSA-modified (standard method) PT-CNMS (Table 1) were divided by the same values for the same starch modified with OSA without a prior protease treatment, the values for peak η, breakdown, setback, and final η were 1.1×, 1.0×, 1.0×, and 1.1×, respectively. The same comparison with C-WMS gave values that were 1.2×, 1.2×, 1.5×, and 1.3×, respectively, i.e., all were increased by 16–46%. Explanations might be that (1) when surface protein is present, reaction of OSA with it does not reduce its hydrophobicity and make it more water-soluble so that it is removed and the nature of the granules is changed, or (2) the nature of OSA drives it into the micropores of granules (bypassing surface protein), as described above. Evidence for the latter suggestion is that the DS values for OS substitution when the TRF method was used are only slightly lower than those obtained when the standard method was used.[1] Finally, ΔH_R values of gels of starches modified by OSA using the TRF method (Table 5) were much less increased over the same values for starches modified using the standard method (Table 4) (and, in the case of OSA-modified LI-WMS, decreased), the ΔH_R values were decreased as compared to the same values for the unmodified starches, especially for the waxy maize starches – indicators of amylopectin modification, even when the TRF method was used. Finally, ΔH_R values for starches modified by reaction with OSA using the TRF method (Table 5) were much less increased over the ΔH_R values for starches modified with OSA using the standard method (Table 4) (and, in case of OSA-modified LI-WMS, decreased), and the ΔH_R values were decreased as compared to the same values for the unmodified starches, especially for the WMS. These are indications of amylopectin modification, even when the TRF method was used.

DSC data in Table 5 indicate, for the most part, little differences in the various parameters for any of the stabilized starches and few trends, with the following exceptions. Of particular importance, ΔH_R values were greater for products made by using the TRF method as compared to products made using the standard method for LI- and C-NMS and -WMS derivatized with AA or SA, indicating either a less uniform pattern of derivatization or a lesser degree of starch derivatization, perhaps because of a greater degree of reaction with protein. The latter explanation is consistent with the data in Table 3. Also of importance is the fact that ΔH_{gel} and ΔH_R values were decreased the most when the TRF method was used for reaction with OSSA, indicating that reaction had occurred in this case. Another feature of Tables 4 and 5 is that ΔH_{gel} was greatest for the control LI starches and for the LI products, i.e., those with the greatest protein content, whether the standard or the TRF method was used, except for products of reaction with STMP.

Unquestionably, reaction with SA using the two methods resulted in the greatest differences in RVA parameters and reaction with OSA using the two methods resulted in the least differences. In summary of the data for reactions with relatively rapidly-reacting stabilizing reagents, determination of indicators of amounts of reaction of LI- and C-NMS and LI- and C-WMS revealed that the TRF method resulted in only slightly less derivatization as compared to the standard method for reactions with OSA (0.97–0.93×), much less derivatization for reactions with SA (0.27–0.42×), and very much less derivatization for reactions with AA (ca. 0–0.03×). DSC data showed little differences in properties of the products made by the two methods, with the exception that ΔH_R was greater for products made by the TRF method as compared to those made by the standard method for products of derivatization with AA or SA, indicating either a less uniform pattern of derivatization or a lesser degree of derivatization of starch molecules. DS data supports the latter suggestion. It is possible that, when the TRF method was used with these reactive reagents, any reaction that occurred was with protein molecules

4. Conclusions

It is concluded that little or no crosslinking occurred when the maize starches were reacted with $POCl_3$ using the TRF method, even though there was a greater incorporation of phosphorus in the case of LI- and C-NMS and LI-WMS. The latter is possibly due to reagent reacting with more nucleophilic groups on protein molecules, which does not require a high pH and therefore results in less loss reagent by hydrolysis, a suggestion supported by several pieces of indirect evidence. Reaction of $POCl_3$ with protein molecules resulted in relatively small effects on pasting and paste behaviors.

In the case of reaction with STMP, there was essentially no difference in reaction efficiency when the order of reagent and catalyst addition was reversed. This fact is likely due to the fact that STMP has less tendency to react with water alone, so no reaction occurred, at least until after the pH is adjusted and perhaps until after the dried mixture was heated, a conclusion supported by RVA and DSC data that show little difference in product properties when the two 5

In the case of reaction with the AAMA reagent using the standard method, it is concluded that substitution/acetylation had a greater effect on pasting and paste properties than did crosslinking.

Reaction of the four carboxylic acid anhydrides (the AAMA reagent, AA, SA, and OSA) resulted in some reagent molecules reacting with protein molecules, regardless of which of the two methods was used, but especially so when the TRF method was used because a high pH is not necessary for reaction with protein.

In the case of reaction of either NMS or WMS with OSA, there was little difference in the properties of the products of modification using the two methods because (we believe) reagent molecules were driven into the structured internal water of hydrated granules, bypassing any surface protein. Whether this happened before or after the pH was adjusted to 8.5 was not determined. The facts that the OSA-modified starches prepared using the standard method did not have substantially higher DS values and that reaction with OSA using the TRF method decreased the enthalpy of amylopectin melting in retrograded gels, especially so for waxy starch preparations, are additional indications of this phenomenon. It is also concluded that none of the other reagents were non-polar enough to exhibit this phenomenon.

The overall conclusion is that the method of adding the reagent before adjusting the pH offers no advantage in terms of either efficiency or enhanced product characteristics in the case of reaction with $POCl_3$, STMP, or any of the carboxylic acid anhydrides. A possible explanation is that granules must be somewhat swollen via ionization of hydroxyl groups for effective reagent solution penetration and reaction to occur.

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