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Three classes of starch granule swelling: Influence of surface proteins and lipids

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Abstract

The role of non-carbohydrate surface components of granular starch in determining gelatinisation behaviour has been tested by treatment of native starches with a range of extractants. Resulting washed starches were analysed for (bio)chemical, calorimetric and rheological properties. Sodium dodecyl sulphate (SDS) was the most efficient extractant tested, and resulted in major changes to the subsequent rheological properties of wheat and maize starches but not other starches. Three classes of starch granule swelling behaviour are identified: (i) rapid swelling (e.g. waxy maize, potato), (ii) slow swelling that can be converted to rapid swelling by extraction of surface proteins and lipids (e.g. wheat, maize), and (iii) limited swelling not affected by protein/lipid extraction (e.g. high amylose maize/potato). Comparison of a range of extractants suggests that all of protein, lipid and amylose are involved in restriction of swelling for wheat or maize starches. Treatment of starches with SDS leads to a residue at comparable (low) levels of SDS for all starches. ^{13}C NMR analysis shows that this SDS is present as a glucan inclusion complex, even for waxy maize starch. We infer that under the conditions used, glucan inclusion complexation of SDS is equally likely with amylopectin as with amylose.

Keywords: Starch granule; Gelatinisation; Starch proteins; Starch lipids; Starch rheology; Amylopectin inclusion complexes

1. Introduction

All starch granules swell when heated in the presence of water. This process requires the prior loss of at least some of the ordered structures within the native granule, and is often regarded as the final stage in the process of gelatinisation. Despite the central importance of the swelling process in many technological applications of starches, there is limited understanding of the factors that control its rate and extent. The botanical/genetic origin of starches is recognised to be an important determinant of granular swelling properties. Characteristic swelling profiles are found for common starches (Fig. 1) that only show minor variation with cultivar or growth conditions. It is particularly striking that for, e.g. wheat, maize, waxy maize, tapioca, and potato starches, temperatures of structural disorganisation (as monitored by loss of birefringence or by differential scanning calorimetry) are relatively similar, yet swelling profiles show major differences. This illustrates the fact that starch swelling rate and extent cannot be predicted directly from knowledge of the thermally induced loss of granular order.

Gelatinisation of starch granules in excess water involves a series of structural disorganisation processes accompanied by water ingress. The thermal dissociation of crystallites based on double-helical segments of amylopectin removes many of the constraints restricting granule swelling. In some cases, e.g. potato, tapioca and waxy maize starches, this is the trigger that allows rapid swelling of granules, as conveniently monitored by viscosity development (Fig. 1). Expansion is so extensive, that the resultant swollen granules are relatively fragile, breaking down under the shearing conditions in the viscometer and resulting in a peak in viscosity development. For other starches, e.g. maize and wheat, swelling is delayed to higher temperature and is slower to develop. A third category of starches includes those with high amylose contents that have elevated temperatures of crystallite dissociation, preventing the onset of swelling below ca. 90 °C and that show slower granule expansion subsequently, e.g. high amylose maize (Fig. 1).

If granules were composed of only discrete molecules of amylose and amylopectin, it would be predicted that heating to above (amylopectin) crystallite/double helix melting

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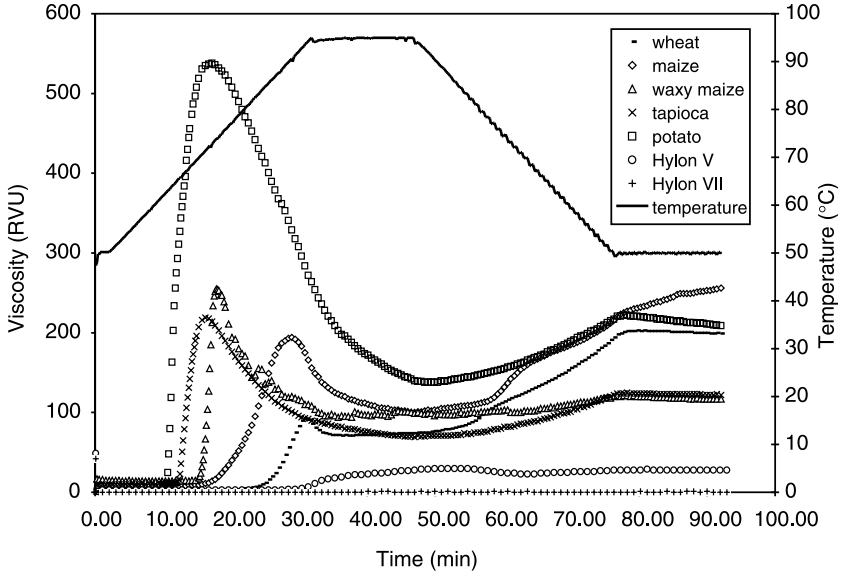

Fig. 1. RVA traces for 10% (w/w) suspensions of wheat, maize, waxy maize, tapioca, potato and high amylose maize ('Hylon V' and 'Hylon VII') starches.

temperatures should lead to complete molecular dissolution. As this does not happen for any common native starch, other factors must come into play. One potential mechanism for restraining the rate and extent of swelling is the presence of non-polysaccharide material at or near the surface of the granule. Both lipids and proteins are known to be associated with both the surface and the interior of granules (Baldwin, 2001; Morrison, 1995). The 'surface' material is defined as the readily extractable material, i.e. extracted by methods not apparently destructive to the granule (mild extractions at room temperature). Readily extracted material typically consists of protein and/or lipid components of the seed, tuber, etc. carried through the extraction process, as well as distinct granule-associated minor components. Their amount is highly dependant on the extraction procedure, and they are characteristically different from proteins and lipids found inside starch granules. Isolation of this 'internal' material requires more disruptive methods such as stringent extractions at higher temperatures, chaotropic agents or starch degrading enzymes.

Surface proteins in starches are often dominated by storage proteins, particularly endosperm storage proteins for cereals. These have a low molecular weight ($\leq$30 kDa) in wheat (Schofield & Greenwell, 1987) and maize (Mu-Forster & Wasserman, 1998). Some proteins seem to be present both on the surface and inside granules, such as a 30 kDa wheat protein (Lowy, Sargeant, & Schofield, 1981; Rayas-Duarte, Robinson, & Freeman, 1995). Neutral lipids are the main component of surface lipids (Vasathan & Hoover, 1991), consistent with the composition of storage lipids, e.g. endosperm lipids for cereals. The levels of free fatty acids (FFA) and lysophospholipid (LPL) is boosted by starch granule associated surface lipids for cereals (Morrison, 1995).

The nature of protein/starch granule interactions is not well characterised. It is believed that most surface proteins are simply adsorbed onto the surface of the starch granule (Baldwin, 2001). In soft wheat starch, the interaction of the 15 kDa starch granule surface protein friabilin with starch may be mediated by surface bound polar lipid (glycolipids and phospholipids) (Greenblatt, Bettge, & Morris, 1995). Surface lipids from the endosperm are loosely associated or absorbed into the surface layers of the granules, while 'true' starch lipids are complexed with amylose (Morrison, 1995).

There is an apparent correlation between swelling behaviour, shear sensitivity and lipid/protein content of starches. Starches that swell rapidly on heating tend to be more shear sensitive and contain less protein and lipid than starches displaying a more controlled swelling (Fig. 1 and Table 1). Among the main commercial starches, cereal starches (wheat, maize, barley, rice) contain more lipids (0.6–1%, w/w) than tuber (potato, 0.05%), root (tapioca, 0.1%), legume (less than 0.6%) and waxy mutant cereal starches. This is also true for the protein content: 0.25–0.6% for cereal starches compared with 0.06% for potato and 0.1% for tapioca. For two wheat starches differing in phospholipid content yet with similar amylose content and similar amylopectin crystallinity, higher phospholipid content was associated with a lower peak viscosity, indicative of lower swelling (Lin & Czuchjowska, 1998). In general, FAM (the unleachable portion of free amylose AM) is positively correlated with swelling, whereas LAM (lipid bound AM) has a strong inhibitory effect (Tester & Morrison, 1990).

Table 1
Typical amylose, lipid and protein contents for frequently studied starches

Starch source	Amylose (%)	Lipids (%)	Proteins (%)
Wheat	28	0.7	0.4
Maize	27	0.8	0.4
Waxy maize	0	0.2	0.2
Tapioca	21	0.2	0.1
Potato	20	0.1	0.06

Data summarised from Morrison (1995) and Vasathan and Hoover (1991) and references therein.

A range of extraction conditions can be used to remove lipids and/or proteins from granule surfaces with varying degrees of efficiency. Although extracted lipids and proteins have been analysed chemically, only limited work has been done to characterise the properties of surface-extracted granules. Evidence that depletion of surface proteins and lipids can influence swelling properties is provided by a range of studies: partial defatting or partial protein removal result in enhanced rate and extent of granule swelling (Bowler, Towersey, Waight, & Galliard, 1985; Eliasson, Carlson, Larsson, & Miezis, 1981; Han & Hamaker, 2002; Maningat & Juliano, 1980; Melvin, 1979; Oates, 1991; Ohashi, Goshima, Kusuda, & Tsuge, 1980; Seguchi, 1986; Seguchi & Yamada, 1989; Tester & Morrison, 1990). Nierle, El Baya, Kersting, and Meyer (1990) showed that treatment with sodium dodecyl sulphate (SDS) or (to a lesser extent) aqueous ethanol resulted in a more rapid swelling of wheat starch at lower temperatures compared to native granules. Previous work by Gough, Greenwell, and Russell (1985) also pointed to a similar result.

In order to clarify the general influence of surface proteins and lipids on starch granule swelling, we now describe a study encompassing a range of starches and a number of extraction regimes. Elemental analysis has been used to quantify the efficiency of extraction, and SDS-PAGE has been used to identify extracted and non-extracted proteins. Rheological properties have been characterised using a standardised set of conditions. Structural and thermal properties of native and extracted starches have been monitored by ^{13}C NMR and DSC, respectively. Taken together, the data obtained point to a key role for surface proteins and lipids in the swelling of some, but not all, starches.

2. Experimental

2.1. Materials

Most starches were obtained from commercial sources: wheat starch from ABR, Corby, UK; potato starch from Roquette, Lestrem, France; waxy, normal and high amylose maize (Amioca, normal, Hylon 5 / 7, respectively) and tapioca starches from National Starch, Bridgewater, USA. High amylose potato starch was obtained from line 208 as described by Schwall et al. (2000). Proteinase K, EC 3.4.21.14, a wide spectrum protease from the fungus *Tritirachium album*, was obtained from Sigma.

Protein molecular weight (M_w) markers for electrophoresis ('Rainbow') were obtained from Amersham International, Little Chalfont, UK.

2.2. Selective starch extractions

Starches were incubated with a range of solvents for the selective extraction of surface components, followed by three water washes and freeze–drying. Solvents included water, sodium chloride (NaCl; 0.5 M), proteinase K (PK; 10.7 tyrosine units/mg) and its sodium acetate buffer control (buffer; 0.05 M, pH 7.4), hexane, water-saturated-butanol (WSB), 3-[(3-cholamidopropyl) dimethyl-ammonio] 1-propanesulphonate (CHAPS; 2%, w/v) and sodium dodecyl sulphate (SDS; 2%, w/v).

For extractions with water, sodium chloride, CHAPS and SDS, the following method was used: starch (100 or 10 g) was suspended in solvent (250 or 25 ml, respectively) (40%, w/v) at room temperature and stirred using a magnetic stirrer for 30 min (55 min for CHAPS). The suspension was then centrifuged (9000*g* or up to 13,000*g* if necessary for efficient separation, 15 min) and the supernatant removed. The pellet was then washed three times by resuspension in water and centrifugation. The pellet was finally resuspended in water and freeze–dried. The first supernatant was kept for further analysis and stored at 4 °C. Starch was also washed three times in SDS by incubating three times (in succession) with fresh SDS for 30 min, then washing three times with water and freeze–drying.

For extractions with WSB and hexane, starch (2×5 g, 20%, w/v) was suspended in solvent (25 ml) at room temperature in Teflon screw-cap 50 ml centrifuge glass tubes and sparged in N_2 prior to incubation. The mixtures were stirred using a magnetic stirrer for 30 min (WSB) or 16 h (hexane). (As the fatty acid moiety of most starch lipids is C_{18} and C_{16} (Vasathan & Hoover, 1991), and as the solubility of lipids in hexane decreases with increasing chain length of the fatty acid moiety, their dissolution in hexane is likely to be slow, hence the longer incubation time). The suspension was then centrifuged (1209*g*, 15 min) and the supernatant removed. Starch was then resuspended in 10 ml fresh hexane (hexane extraction only) and centrifuged. All pellets were then washed three times by resuspension in water (3×30 ml) and centrifugation, and finally resuspended in water and freeze–dried. The WSB or hexane supernatants were pooled and evaporated under reduced pressure at 40–50 °C and redissolved in 1 ml of chloroform.

2.3. Protease digestion

Protease activity of proteinase K was assayed at 10.7 tyrosine units/mg in phosphate buffer (0.067 M, pH 7.4), and absence of contaminating amylolytic activity verified. One tyrosine unit hydrolyses casein pH 7.4 to produce colour equivalent to 1.0 μmol (181 μg) of tyrosine per minute at 40 °C. The absence of starch depolymerising activity was demonstrated by measuring the viscosity of a solution of waxy maize starch incubated with a range of levels of proteinase K over 24 h. The time required for the solution to flow between two graduations on a 0.1 ml pipette was measured. The deliberate addition of very low levels of amylolytic activity reduced flow times very quickly. Only those batches of protease which showed no difference in flow times from controls over 24 h were used for subsequent experiments.

For incubations with starch, proteinase K was dissolved (0.16%, w/v) in 0.05 M sodium acetate buffer pH 7.4 containing sodium azide (2 mg%, w/v). Starch (10 g) was suspended in 25 ml of buffer/active enzyme (40%, w/v) at pH

7.4 and incubated at 40 °C for 25 h. Suspensions were then centrifuged (2000*g*, 15 min) and the starch washed three times by resuspension in 25 ml of water and centrifugation, and finally resuspended in water and freeze dried.

2.4. Physical characterisation of starches

Viscosity development of starch suspensions on heating was monitored by a Rapid Visco Analyser (model RVA-3CR, Newport Scientific, Warriewood, Australia). The 25 g suspension of starch in water was held at 50 °C for 2 min, heated to 95 °C at 1.5 °C/min, held at 95 °C for 15 min, cooled to 50 °C at 1.5 °C/min, then held at 50 °C for 15 min. Starch suspensions were typically 10%, w/w (wwb) unless specified otherwise. High amylose potato starch (8%, w/w suspensions) was analysed using a faster profile (held at 50 °C for 1 min, heated to 95 °C at 12.2 °C/min, held at 95 °C for 2.5 min, cooled to 50 °C at 11.8 °C/min, and held at 50 °C for 2 min). Stirring speed was 900 rpm for the 10 initial seconds, then 160 rpm throughout.

For differential scanning calorimetry (DSC), a Perkin-Elmer DSC 7 calorimeter was used. Sample (2–4 mg of powder) was weighed accurately in aluminium pans and 12 μl of de-ionised distilled water was then mixed in (i.e. maximum 25%, w/w of starch). After sealing, the pans were heated at 10 °C/min from 10 to 95 °C with an empty pan as reference. The thermograms were analysed using the Perkin-Elmer software programmes (Thermal Analysis Software 7). Data were normalised on the basis of sample dry weight (all powders were assumed to contain 10% moisture). Gelatinisation enthalpy, onset and peak temperatures were recorded.

^{13}C cross-polarisation and magic angle spinning NMR spectra of dry starches (Gidley & Bociek, 1985) were recorded at room temperature at 75.48 MHz on a Bruker MSL-300 instrument using a double bearing probe. A single contact time of 1 ms, a spectral width of 30 kHz and line broadenings of 10–20 Hz were routinely used. Spinning rates were typically 4 kHz and ^{1}H decoupling fields of ~80 kHz (20 G) were employed. Typically, 1 K time domain points were collected during a 52 ms acquisition time with a transform size of 8 K: a recycle delay of 3 s was used.

2.5. Chemical characterisation of starches

Amylose/amylopectin ratios were determined using the method of Morrison and Laignelet (1983).

The amounts of protein and lipid removed by extraction processes were estimated from elemental analysis. Starch protein content was evaluated by the measurement of nitrogen content. Lipids associated with starch are mostly phospholipid (wheat starch) or a mixture of free fatty acids and phospholipid (maize starches). As an index of lipid removal, phospholipids were estimated by trace phosphorus analysis.

Nitrogen content was determined using an Antek 720 Chemiluminescence Nitrogen detector by Butterworth Laboratories, Teddington, Middlesex TW11 8LG, UK.

Carbon, hydrogen, nitrogen and sulphur were measured simultaneously by a Carlo Erba EA 1108 Elemental Analyser (Fisons Instruments, UK). The results were used primarily to estimate sulphur content (e.g. from residual sodium dodecyl sulphate), as nitrogen determination was found to be not as sensitive to small changes in protein content as the chemiluminescence method.

For phosphorus analysis, starch samples (0.1 g) were digested with concentrated nitric acid (1 ml) at 140 °C for 2 h. After cooling and dilution with water, phosphorus present in the extract was determined using Atomic Emission Spectrometry (AES).

2.6. Electrophoresis (SDS-PAGE) of starch granule proteins

Protein extracts from starches were analysed by SDS-PAGE according to Laemmli (1970), using the mini-Protean II dual slab gel apparatus (Bio-Rad Corporation). Laemmli buffer (typically 1 ml) (2% SDS + 10% (v/v) glycerol + 1% dithiothreitol + 0.01% bromophenol blue + 0.3125 M Tris–HCl adjusted to pH 6.8) was added to starch residues (typically 150 mg). After mixing, the mixtures were placed in a boiling water bath for 5 min (2 min for standards), cooled to room temperature and centrifuged at 11,600*g* for 10 min. The supernatants were loaded onto the gels, typically 20–30 μl for most starches, 4–5 μl for protein-rich high amylose maize starches. The gels consisted of 5 and 10% polyacrylamide stacking and separating gels, respectively. The gel was run at a constant voltage of 180 V for about 45 min. Proteins were visualised by silver staining as described by Morrissey (1981). The gels were dried at 70 °C under vacuum in between transparent sheets for about 1 h on a gel drier then photographed.

3. Results

3.1. Effect of SDS extraction on physical properties of starch

Sodium dodecyl sulphate (SDS) is the most frequently used reagent for extraction of proteins and lipids from more hydrophilic substrates such as starch, so was used to compare effects on starches of different botanical origin. Following SDS extraction, the visual appearance and birefringence of granules at room temperature was not changed detectably for any starch. However, the effect of prior SDS extraction on the viscosity profile on heating in excess water was found to be highly dependent on the starch source (Fig. 2). SDS extraction converted slow swelling wheat and maize starches to rapid swelling types (Fig. 2a and b), with similar profiles to starches from, e.g. waxy maize or tapioca (Figs. 1 and 2c and d). The effect was dramatic, with a reduction in the viscosity onset temperature (large for wheat, moderate for maize), a more rapid viscosity development and a large increase in peak viscosity. These effects are indicative of increased swelling rate and extent. The lower final paste viscosity (Fig. 2a and b) is likely to be due to increased shear disruption of the more highly swollen granules.

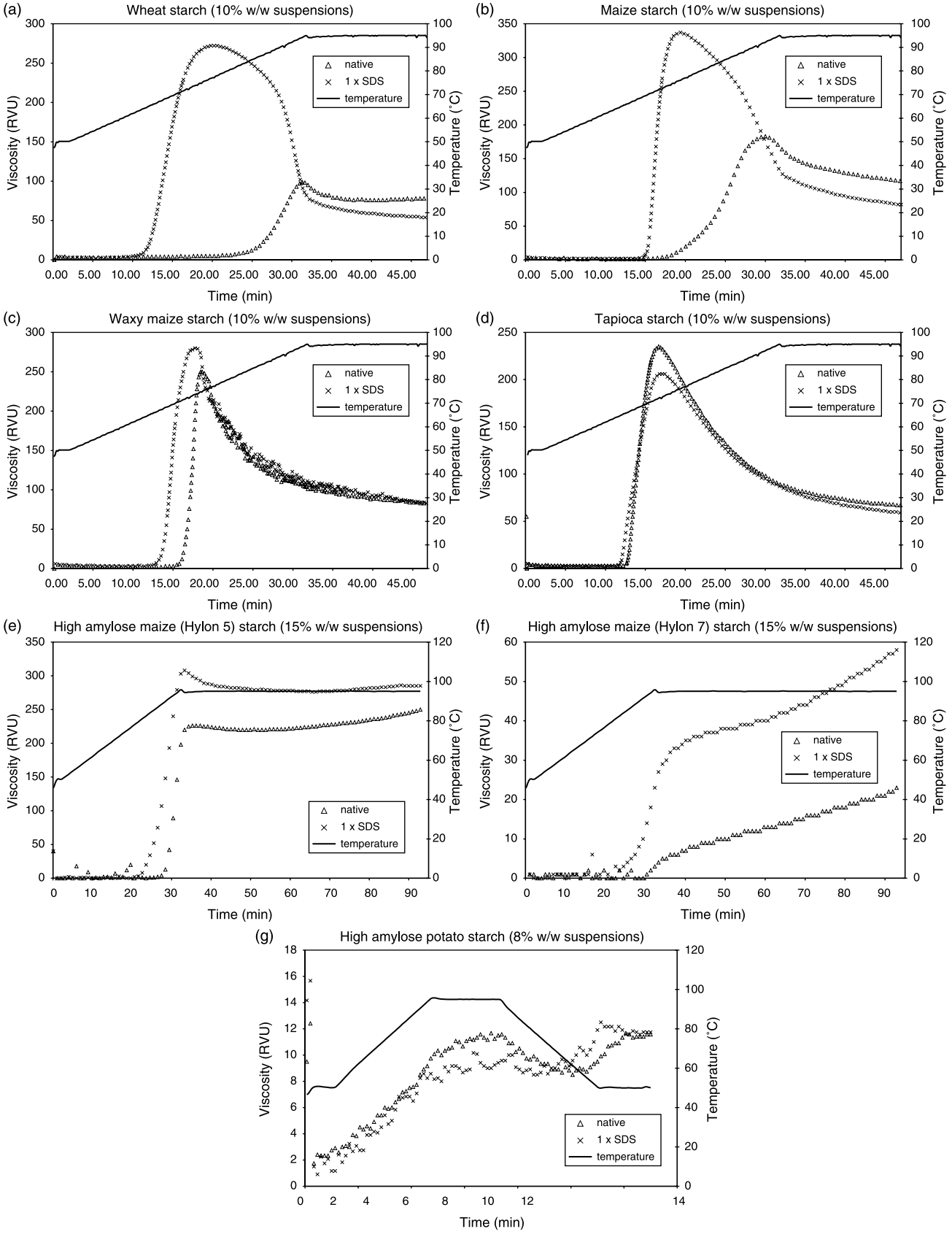

Fig. 2. RVA traces for starch suspensions before (native) and after (1×SDS) extraction once with 2% (w/v) SDS at room temperature and subsequent extensive water washing. Data are shown for starches from (a) wheat, (b) maize, (c) waxy maize, (d) tapioca (10% suspensions), (e and f) high amylose maizes ('Hylon V', 'Hylon VII') (15% suspensions) and (g) high amylose potato starches (8% suspensions).

The rapid swelling starches waxy maize and tapioca (Fig. 2c and d) did not exhibit altered viscosity profiles following extraction with SDS, with only a slightly earlier onset observed for waxy maize starch. Limited swelling high amylose maize or potato starches were also affected only slightly by SDS extraction (Fig. 2e–g). The viscosity of the high amylose maize starches was raised slightly, indicating a slightly enhanced swelling, while the profile of high amylose potato starch remained essentially unchanged. Potato starch gave a slightly changed profile after SDS extraction, with lower viscosity onset temperature and peak viscosity when washed with SDS (not shown). However, water washing alone disrupted potato starch, causing a lower viscosity onset temperature (not shown) as described previously (Muhr, Blanshard, & Bates, 1984). For all other starches, water washing had no detectable effect on subsequent viscosity profiles.

Starch gelatinisation behaviour, assessed by differential scanning calorimetry, was not altered significantly by SDS washing, except for potato (Table 2). Potato showed lower onset temperatures after SDS washing and to a lesser extent after water washing. The different response to pre-gelatinisation treatments of potato starch compared with other starches has been observed previously with respect to, e.g. freeze–drying (Muhr et al., 1984).

3.2. Chemistry of SDS extraction

Starch protein and phospholipid content were evaluated by measurement of nitrogen and phosphorus contents, respectively, knowing that phospholipid also contributes to nitrogen content in the ratio phospholipid $N = 0.4 \times P$ (Sulaiman & Morrison, 1990). Nitrogen and phosphorus content, therefore protein and phospholipid content, increased with amylose content in the maize starch series, as previously reported by Morrison (1995) (Table 3). The higher level of phosphorus in native wheat starch compared to maize (Table 3) reflects its different lipid composition, with a higher proportion of phospholipids relative to free fatty acids (>90% compared to 40/60 PL/FFA in maize) (Morrison, 1995). The high phosphorus content of potato starch is indicative, not of lipid, but of endogenous phosphate esters (Kim, Wiesenborn, Orr, & Grant, 1995). As expected, potato, tapioca and waxy maize starches contain much less protein and phospholipid than the other starches. The results for wheat starch are consistent with previous data for commercial starches; the surface components of 100 g of native wheat starch can be described as 9 g of LPL nitrogen and 22 mg of protein nitrogen corresponding to 250 mg of protein (Sulaiman & Morrison, 1990).

Residues of SDS washing are depleted in phosphorus (for phospholipid-containing cereal starches) and nitrogen (Table 3). Little phosphorous or nitrogen was extracted from tapioca, potato or high amylose potato starches, while larger amounts were removed from wheat, waxy maize, maize and high amylose maize starches.

Gel electrophoresis (SDS-PAGE) was used to characterise proteins associated with starches both before and after extraction with SDS. Native starches show protein profiles characteristic of the botanical origin (Fig. 3). Wheat shows a complex protein profile, ranging in molecular weight from a few kilo dalton to over 100 kDa as described previously by Darlington et al. (2000), Rahman et al. (1995), Schofield and Greenwell (1987) and Zhao and Sharp (1996). Maize starch proteins appear less complex, with the storage protein zein as a low molecular weight protein doublet (22–24 kDa), the waxy (granule bound starch synthase 1) protein at about 60 kDa and a few higher molecular weight proteins. Waxy maize shows a much fainter profile, and lacks the waxy protein. These proteins have been described by Echt and Schwartz (1981), Goldner and Boyer (1989), Han and Hamaker (2002), Mu, Mu-Forster, Bohonko, and Wasserman (1998), Mu-Forster et al. (1996) and Sano (1984). Hylon 7 starch contains more protein and required dilution (5×) for comparison, but had a profile broadly similar to that of maize starch. Potato and tapioca show a faint profile with mostly high molecular weight protein bands (69 kDa and above), as reported previously (Smith, 1990; Vos-Scheperkeuter, De Boer, Visser, Feenstra, & Witholt, 1986).

Table 2
Gelatinisation parameters (DSC) of various starches before and after SDS (1×) or water (4×) washing

Starch	Treatment	DSC endotherm		
		Onset temperature (°C)	Peak temperature (°C)	Enthalpy (J/g starch, wet basis)
Wheat	Native	53.4	58.7	10.5
	1×SDS	54.4	59.6	9.9
Maize	Native	67.5	71.6	13.4
	1×SDS	65.9	71.2	13.2
Waxy maize	Native	67.6	73.1	15.9
	1×SDS	65.4	71.9	16.4
Tapioca	Native	65	69	14.8
	1×SDS	65.4	69.2	16.2
Potato	Native	60.3	64.9	17.4
	4×H_2O	56.4	61.4	17.5
	1×SDS	52.7	58.1	16.3

Table 3
Residual nitrogen, phosphorous and sulphur contents before and after SDS washing

Starch	Native or SDS treated	Nitrogen content (mg/100 g)	Phosphorus content (mg/100 g)	Sulphur content (%)
Wheat	Native	76	59	0
	SDS	35	43	0.3
Maize	Native	58	19	0
	SDS	36	16	0.2
Waxy maize	Native	24	2	0
	SDS	7	1	0.3
Tapioca	Native	8	5	0
	SDS	2	4	0.2
Potato	Native	14	65	0
	SDS	6	71	0.2
High amylose maize 5	Native	59	29	n.d.
	SDS	41	23	
High amylose maize 7	Native	106	35	0
	SDS	59	27	0.2
High amylose potato	Native	31	244	n.d.
	SDS	20	251	

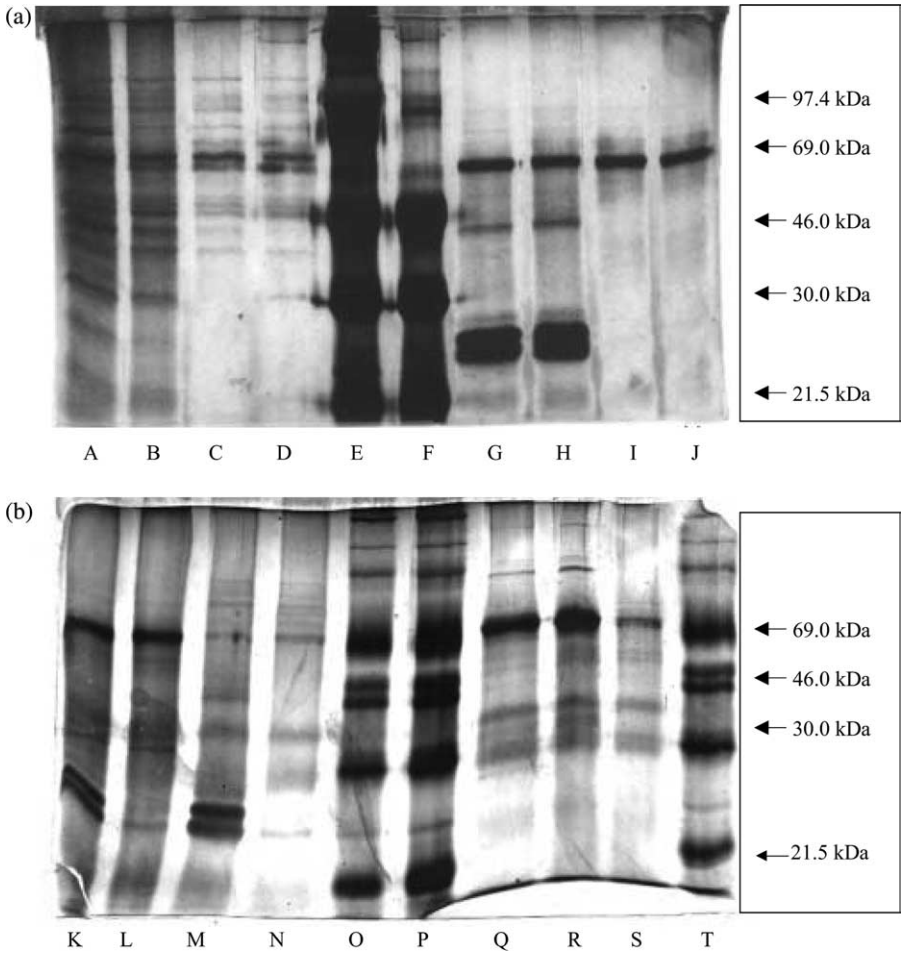

Fig. 3. SDS-PAGE analysis of starch-associated proteins before and after extraction with water or 2% (w/v) SDS at room temperature. All extractions were followed by extensive water washing. (a) Wheat starch before extraction (lane A) and after extraction once with water (lane B), once with SDS (lane C) or three times with SDS (lane D). Standards of high (lane E) and low (lane F) molecular weight (7 μg of each protein). Maize starch before extraction (lane G) and after extraction once with water (lane H), once with SDS (lane I) or three times with SDS (lane J). (b) Hylon 7 starch before extraction (lane K) and after extraction three times with SDS (lane L), waxy maize starch before extraction (lane M) and after extraction three times with SDS (lane N), standards of high molecular weight with 0.2 μg of each protein (lane O) and 0.5 μg of each protein (lanes P and T), potato starch before extraction (lane Q) and after extraction three times with SDS (lane R), tapioca starch before extraction (lane S).

All SDS-extracted starches, except for potato and tapioca, showed a much fainter electrophoretic profile for proteins below 60 kDa, indicative of a large reduction in low molecular weight (surface) protein content (Fig. 3). All maize starch residues have lost zein. The protein profile of potato and tapioca, which shows only faint high molecular weight bands, is apparently unaltered. Proteins extracted by SDS were also detected by electrophoretic analysis (not shown). In all cases, results were as expected from a comparison of starch-associated proteins before and after SDS extraction (Fig. 3), e.g. zein found for all maize extracts.

^{13}C NMR of starches shows identical spectral details over the range 50–120 ppm (particularly in the anomeric region at ca. 100 ppm) before and after SDS extraction for, e.g. maize (Fig. 4a vs b) and waxy maize (Fig. 4c vs d). This shows that starch double helix content was not significantly altered by the SDS treatment (Gidley & Bociek, 1985). However, additional intensity was observed for SDS-treated samples at ca. 30 ppm in the region normally associated with lipids, and similar in chemical shift with a dry mix of starch + SDS (Fig. 4e) or SDS alone (Fig. 4f).

Sulphur was also found by elemental analysis after SDS washing, implying the retention of SDS in extracted granules. 0.2–0.3% sulphur or about 2.5 ± 1.0% (w/w) of SDS was found for all starches, whether waxy or not.

Expansion of the 20–40 ppm region of the NMR spectrum shows peaks at 31–33 ppm for native maize (Fig. 5a), SDS-treated maize (Fig. 5b) and SDS-treated waxy maize (Fig. 5d), but not native waxy maize (Fig. 5c). The signal at 31–32 ppm has been shown to be due to the major alkyl carbons from lipid complexed with amylose (Morrison, Law, & Snapea, 1993; Morrison, Tester, Snape, Law, & Gidley, 1993), but has not previously been reported for waxy starches or amylopectin. SDS alone (Fig. 5f) and a dry mix of SDS + starch (Fig. 5e) have the major alkyl carbon signal at 33–34 ppm characteristic of non-complexed lipid (Morrison, Law et al., 1993; Morrison, Tester et al., 1993). We therefore conclude that residual SDS is present in the form of

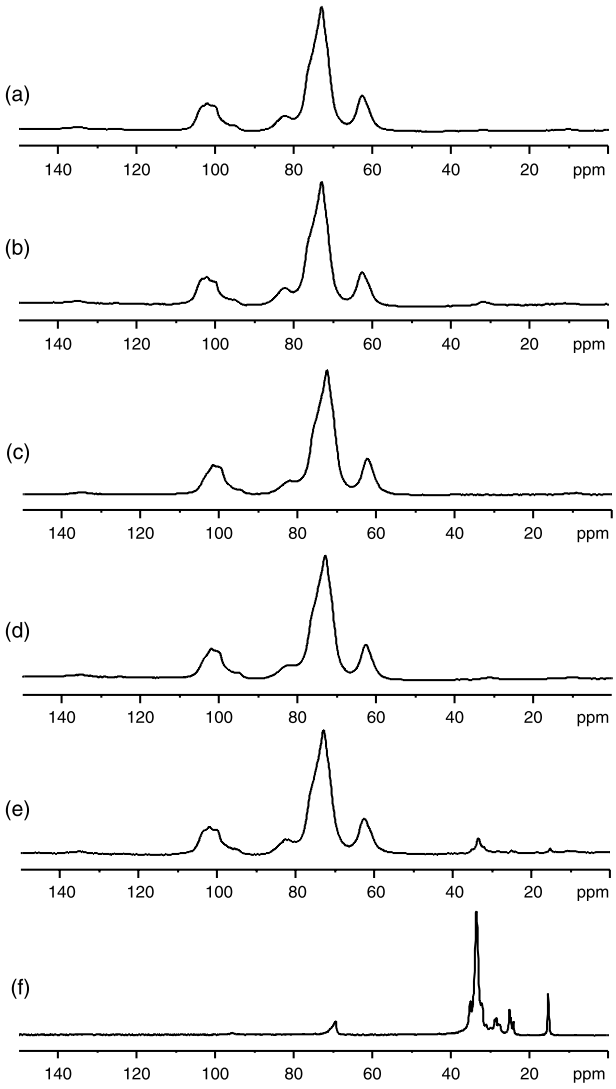

Fig. 4. ^{13}C CP/MAS NMR spectra of (a) native maize starch, (b) maize starch after extraction with SDS, (c) native waxy maize starch, (d) waxy maize starch after extraction with SDS, (e) dry mix of maize starch and SDS, and (f) dry SDS.

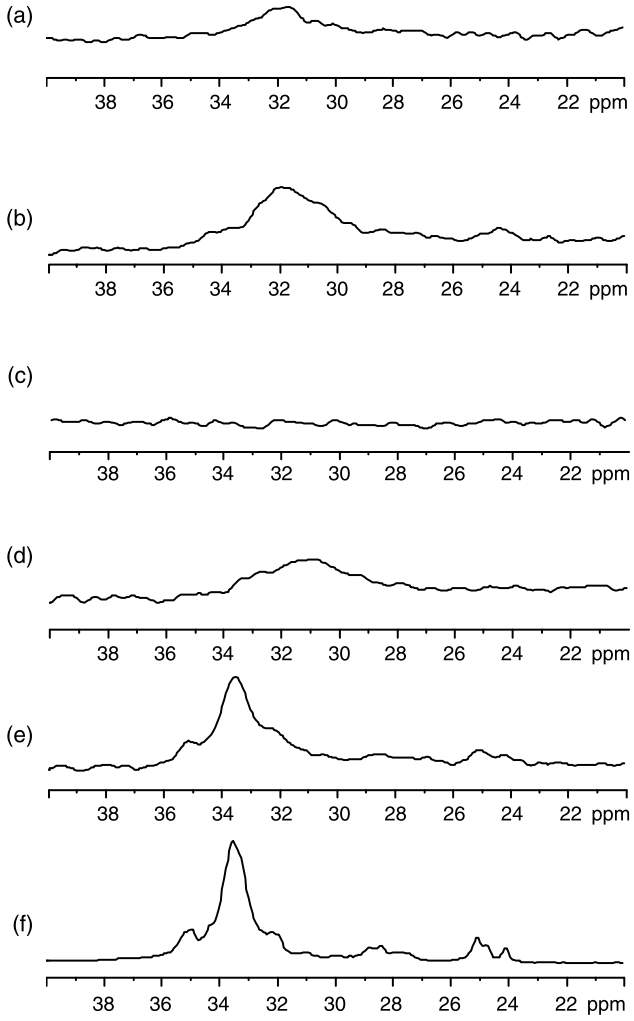

Fig. 5. Expansion of 20–40 ppm region for spectra shown in Fig. 4(a)–(f).

inclusion complexes for both maize (31–32 ppm signal intensity increase in Fig. 5b cf. Fig. 5a) and waxy maize starch. Results for potato and tapioca starches are similar to those for waxy maize with no detectable alkyl carbon signal before SDS treatment, and a signal at 31–32 ppm after extraction. Although both gelatinisation and swelling properties are known to be affected significantly by the addition of SDS (Svensson, Autio, & Eliasson, 1998), the similar (low) levels of SDS found in all extracted starches and the great range of subsequent swelling/viscosity effects suggests that the presence of a small amount of SDS in samples is not systematically affecting physical properties. SDS is a relatively alkaline extraction agent, so it could be argued that this pH rise may lead to a partial gelatinisation, however the negligible effect of SDS extraction of the gelatinisation rheology of, e.g. tapioca starch (Fig. 2d) argues against a systematic effect of pH elevation. Similarly, gelatinisation in the presence of SDS causes significant changes in gelatinisation parameters detected by DSC (Svensson et al., 1998), but systematic changes were not observed for extracted starches in this study (Table 2).

3.3. Effects of other extractants on wheat, maize and waxy maize granule swelling

Additional extraction conditions were carried out to identify the agents(s) critical for the restriction of swelling in wheat and maize starches. Water and salt were expected to only extract the more hydrophilic surface proteins, such as albumins and globulins (Osborne, 1907). Proteinase K, a powerful wide spectrum protease, was chosen to attempt exhaustive surface protein extraction. Water saturated butanol (WSB) at room temperature is deemed an efficient surface lipid extractant, which does not extract many prolamins (Morrison & Milligan, 1982). Sequential proteinase K and WSB extraction was also carried out. Hexane, less polar than WSB, was aimed at part of the surface lipids without extraction of the highly hydrophobic surface proteins such as zein. The detergent CHAPS was

expected to extract surface material but not be able to complex with amylose or amylopectin.

The comparative effectiveness of extractants in removing proteins and lipids from wheat and maize starches was assessed from elemental analyses for nitrogen and phosphorous (Table 4). For both wheat and maize starches, protease treatment and SDS washing were the most efficient at extracting nitrogen/protein. Other treatments led to intermediate levels.

SDS and proteinase K+WSB extracted the most phospholipids from wheat starch, with the other treatments leading to intermediate levels. For maize starch, these two treatments were the only ones to extract significant levels of phospholipids (Table 4).

The water washed residues of wheat and maize starches show an apparently unchanged protein profile (Fig. 3) and only very small changes in nitrogen and phosphorous content (Table 4). All other reagents extracted both phosphorous and nitrogen from wheat starch (Table 4), suggesting a close association between phospholipid and protein. In particular, proteinase K, which acts specifically on proteins, also removed significant amounts of lipid from wheat starch (Table 4). Further, evidence for association of lipids and proteins is given by the minor reduction in phosphorous (lipid) for WSB, but a much increased level of extraction following proteinase K treatment for both wheat and maize starches. The detergent CHAPS was the least effective protein or phospholipid extractant. In contrast, proteinase K+WSB was nearly as efficient as SDS.

Table 4
Residual nitrogen and phosphorus after a range of extractions

Starch	Treatment	Nitrogen (mg/100 g)	Phosphorus (mg/100 g)
Wheat	Native	76	59
	H_2O	74	62
	NaCl	46	56
	Proteinase K	36	53
	Hexane	46	58
	WSB	48	56
	CHAPS	56	57
	1×SDS	35	43
	3×SDS	34	39
	Proteinase K+WSB	35	44
Maize	Native	58	19
	H_2O	56	18
	NaCl	44	19
	Proteinase K	33	19
	Hexane	41	19
	WSB	42	19
	CHAPS	48	18
	1×SDS	36	16
	3×SDS	34	15
	Proteinase K+WSB	34	16
Waxy maize	Native	24	2
	Hexane	16	n.d.
	WSB	17	n.d.
	CHAPS	25	n.d.
	1×SDS	7	1
	3×SDS	6	n.d.

For both wheat and maize starches, the various extraction processes enhance starch granule swelling, the effect ranging from negligible (water), to slight/intermediate (NaCl, proteinase K, hexane, WSB and CHAPS), to large (proteinase K+WSB) to dramatic (SDS) (Fig. 6). Increased swelling appears to correlate broadly with the more efficient removal of minor components, as assessed by elemental analyses (Table 4). The relative effect of the various extractants is slightly different between wheat and maize starches, e.g. NaCl enhances swelling more than lipid solvents for maize starch, but the reverse is true for wheat starch (Fig. 6a and b). More obviously (Fig. 6a and b), reduction in swelling onset temperatures is only seen for SDS treatment of wheat but is apparent for several treatments of maize starch.

Despite extracting as much nitrogen as SDS from wheat and maize starches, protease treatment has a limited effect on the RVA profile. Protease control treatment (acetate buffer) extracts less nitrogen than protease, yet causes a similar slight increase in swelling (data not shown). Extraction of the residual phospholipid by WSB following protease treatment enhances swelling further. Consistent with the limited effect of SDS extraction on waxy maize starch (Fig. 2c), other extraction conditions result in essentially unaltered RVA profiles (Fig. 6c).

4. Discussion

4.1. SDS extraction discriminates between three types of starch swelling behaviour

Starches from different botanical origins have characteristic swelling profiles (Fig. 1), which can result in different rheological properties and application performance. SDS extraction was found to have a dramatic effect on the swelling of standard wheat and maize starches in agreement with results of Gough et al. (1985) and Nierle et al. (1990), but not on the swelling of tapioca, waxy maize or high amylose maize or potato starches. Following SDS extraction, viscosity profiles for wheat and maize starches were similar to those for the same concentration of tapioca or waxy maize starch. High amylose starches had a restricted swelling/viscosity profile both before and after SDS extraction. This wide range of starches can therefore be grouped into three categories:

(i) Starches that swell rapidly at temperatures above that required for thermal gelatinisation (e.g. tapioca, waxy maize, potato).
(ii) Starches that have restricted swelling above gelatinisation temperatures due to the restraining action of surface lipids/proteins (e.g. wheat, maize).
(iii) Starches that have highly restricted swelling not related to surface lipids/proteins (e.g. high amylose maize and potato).

SDS was the most efficient of the several protein and lipid extractants assessed in this study. The absence of significant amounts of proteins characteristic of starch surfaces (Fig. 3,

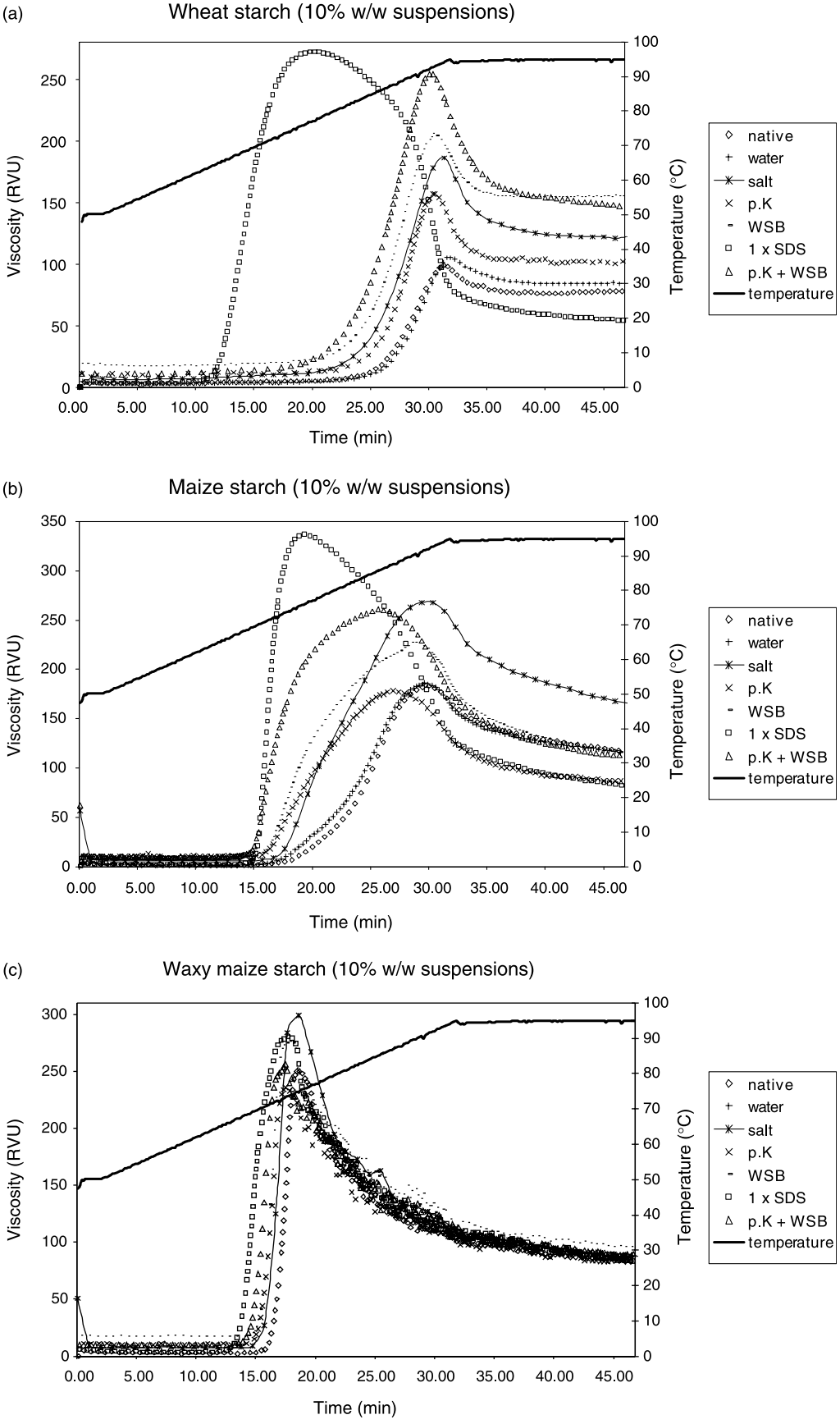

Fig. 6. RVA traces for 10% (w/w) suspensions of (a) wheat, (b) maize, and (c) waxy maize starches after different extraction treatments as shown. P.K, proteinase K; WSB, water-saturated butanol; P.K+WSB, sequential proteinase K then water-saturated butanol.

e.g. zein in maize) suggests that SDS may remove all surface proteins. This would be consistent with previous work which showed that wheat starch extracted with 1% SDS + mercaptoethanol gave no staining with a protein dye (amido black) indicating that starch surface protein had been removed (Nierle et al., 1990; Seguchi & Yamada, 1989; Seguchi & Yoshino, 1999). About 1% SDS only was just as efficient according to Nierle et al. (1990), but not to Seguchi (1986) who detected some residual staining. Similarly, washing with 1% SDS decreased the fluorescence of maize starch granules dramatically, as fluorescent protein-fuchsin dye complexes were removed (Wasiluk, Fulcher, Jones, & Gengenbach, 1994).

Following SDS treatment, comparable amounts of SDS remain associated with maize and waxy maize starches (Table 3). However, ^{13}C NMR data show that the lipid signal for SDS-treated maize starch is more intense than that for SDS-treated waxy maize starch (Fig. 5b and d). This suggests the presence of additional lipids in the SDS-treated maize starch, which we ascribe to amylose–lipid complexes present within the granule, i.e. resisting SDS extraction under ambient conditions.

Except for potato starch, gelatinisation temperature and enthalpy, as assessed by DSC, were not affected by SDS washing, as previously observed for SDS (Gough et al., 1985) and SDS + mercaptoethanol washed wheat starch (Nierle et al., 1990). This indicates that the changes brought about by SDS washing are not due to an alteration of amylopectin crystallite structure. This is confirmed by the lack of change in the solid state ^{13}C NMR carbohydrate signals. The destabilisation of potato starch observed following not only SDS extraction, but also water washing and freeze–drying has been reported previously (Muhr et al., 1984).

4.2. SDS forms inclusion complexes with amylopectin

Small amounts of residual SDS remaining after extensive water washing are present in complexes with amylose and/or amylopectin, as shown by ^{13}C NMR (Figs. 4 and 5). This order at the molecular level is not accompanied by detectable differences in the DSC behaviour between 0 and 95 °C. The melting of the complexes either occurs at a higher temperature, is masked by the gelatinisation endotherm, or is non-cooperative in nature. It is interesting that comparable amounts of SDS are retained following the treatment of waxy maize compared with other starches. This suggests that under the extraction conditions used, complexation with amylopectin is not only possible but may be more prevalent than complexation with amylose. We propose that under aqueous room temperature (20–25 °C) conditions, SDS is able to form inclusion complexes with glucan chains that are at the surface of the granule, irrespective of whether they originate from amylose or amylopectin.

The formation of SDS–amylopectin complexes has been detected by potentiometric titration (Gudmunsson & Eliasson, 1990; Huang & White, 1993; Wulff & Kubik, 1992), surface tension measurements (Svensson, Gudmunsson, & Eliasson, 1996), and also inferred by DSC from the reduction of amylopectin retrogradation enthalpy upon addition of SDS (Lundqvist, Nilsson, Eliasson, & Gorton, 2002). However, in all these studies, amylopectin had been solubilised prior to complexation. Granular SDS–amylopectin complexes have not been detected as a transition by DSC, but have been inferred from the decrease in gelatinisation enthalpy observed in addition of SDS to waxy maize starch (Eliasson, 1994; Evans, 1986). The decrease in enthalpy is thought to result from the formation of additional SDS–amylopectin complexes from excess SDS as starch becomes available/exposed on heating, and the exothermic effect of complex formation is superimposed on the melting transitions of AP crystallites, resulting in the reduction of gelatinisation enthalpies. SDS-washed waxy maize starch, after dry heating for 3 days at 125 °C, showed a small endotherm at around 40 °C on heating in excess water, and a small exotherm at 34 °C on subsequent cooling (data not shown). These transitions are consistent with the melting of an SDS–amylopectin complex on heating, followed by its reformation on cooling. Dry heating may have annealed the complex, making it more homogeneous and more cooperative on heating, i.e. detectable by DSC.

The fact that SDS binds to all starches during extraction procedures, whereas subsequent effects on swelling/viscosity are limited primarily to wheat and maize starch, argues against a role for complexation in changing swelling/viscosity behaviour. Other monoacyl lipids, which also complex with starch, have the opposite effect to SDS, i.e. they delay the gelatinisation process, as assessed by DSC (Eliasson, 1994). Complexation of the surfactant lauric acid (same alkyl chain length as SDS) also postpones pasting to higher temperatures (Harbitz, 1983). In contrast, gelatinisation in the presence of SDS results in a lower onset temperature (Svensson et al., 1998). We suggest that the predominant effect of SDS on gelatinisation and swelling of starch is due to extraction of surface lipids and proteins, and that this effect outweighs any consequences of complexation which tend to act in the opposite direction.

4.3. Lipid, protein and amylose are all necessary to restrict swelling in wheat and maize starches

Investigation of a range of extraction conditions for wheat and maize starches allows some conclusions to be drawn concerning the factors controlling subsequent swelling/viscosity behaviour. Results of note (Fig. 6, Table 4) include:

- Extraction conditions targeting lipids (hexane, water-saturated butanol) also removed significant amounts of protein for both wheat and maize.
- Proteinase K removed as much protein as SDS but had only a relatively minor effect on swelling/viscosity behaviour.
- Prior treatment with proteinase K resulted in a greater removal of lipids by water-saturated butanol, and significantly increased swelling as evidenced from peak viscosities.
- For wheat starch, salt or buffer (and all other extractants) removed not only some protein, but also significant amounts of lipid.

Taken together this data suggests a close relationship between lipids and proteins on the surface of starch granules, both with respect to extractability and also consequences on post-gelatinisation viscosity. Association of lipids and proteins on the surface of starch granules has been suggested previously. The removal of both protein and lipid by protease (pronase) treatment was observed in wheat starch by ESCA and SDS-PAGE (Russell, Gough, Greenwell, Fowler, & Munro, 1987) and by protein specific dye binding (Seguchi & Yamada, 1989). Increased swelling was observed after protease (bromelain) treatment of mung bean starch (Oates, 1990, 1991), but not after chymotrypsin treatment of rice starch (Hamaker & Griffin, 1990).

Further evidence for a close association between lipid and protein in wheat has emerged from extensive studies on the mechanism of endosperm texture. Grain softness in wheat affects milling performance and has been correlated with the presence of 'free' polar lipids in the endosperm, (i.e. phospholipids and glycolipids) (Morrison, Law, Wylie, Coventry, & Seekings, 1989). Softness is also correlated with friabilin, a family of 15 kDa proteins, on the starch granule surface (Greenwell & Schofield, 1986) and in the whole endosperm (Jolly, Rahman, Kortt, & Higgins, 1993). Furthermore, occurrence of bound polar lipids at the wheat starch granule surface (glycolipids and phospholipids) follows the same pattern as friabilin occurrence. The association of (at least) some of the friabilin/puroindoline polypeptides with the surface of soft wheat starch granules may be mediated by those bound polar lipids present at the surface of starch granules (Greenblatt et al., 1995). Puroindolines are suspected to bind lipids via tryptophan rich domains. Ionic, hydrogen and hydrophobic bonds contribute to the stability of puroindoline–polar lipid complexes (Dubreil, Compoint, & Marion 1997).

Evidence that surface protein alone is not sufficient to restrict swelling also comes from a comparison between waxy maize and (normal) maize. In both cases, SDS extraction removes similar amounts of protein based on N analysis (Table 3) and electrophoresis shows the absence of the surface protein, zein (Fig. 3). However, SDS extraction has a dramatic effect on maize starch swelling but only a minor effect on waxy maize (Fig. 2). It may be tempting to ascribe this difference to the absence of amylose in waxy maize, but it may equally well be due to a low level of lipid in the waxy starch. As has been demonstrated by Morrison (1995) and Morrison, Tester et al. (1993), amylose and lipid contents are tightly coupled in cereal starches. Phospholipid levels are very low in waxy maize (Fig. 3), but hplc/tlc analysis of neutral lipids suggests a similar level of free fatty acids in the maize and waxy maize samples studied (data not shown). Whether there is a specific role for amylose or not, it seems reasonable to invoke molecular scale association between the lipid/protein components involved in swelling/viscosity control and glucan chains of the swelling granule. In wheat starch, phospholipid removed by protease treatment is probably associated with protein, while the residual phospholipid is presumably associated with glucan. In maize starch, it was not possible to extract phospholipid without extracting protein, while the reverse was possible. This suggests that, unlike wheat, phospholipid is only associated with glucan. We therefore propose that the relatively restricted swelling of native wheat and maize starches is due to a combination of lipid, protein and glucan features. It is possible that these features are present in a molecular complex, perhaps similar to the one described for starch, whey proteins and fatty acids by Zhang, Maladen, and Hamaker (2003). These authors showed a major effect on starch rheology that required the presence of all three components, with amylose playing a key role.

Based on the different relative responses to extraction treatments for wheat and maize starch (Fig. 6a and b), it seems likely that specific chemical differences in protein/lipid components have a significant effect on their extractability with consequent effects on granule swelling.

4.4. Lipid and protein effects on swelling are secondary in high amylose starches

SDS treatment had a limited effect on the viscosity behaviour of limited swelling high amylose maize starch (Fig. 2e and f), and none on high amylose potato starch (Fig. 2g), despite extracting more surface lipid and protein than from the slow swelling starches (wheat and maize), where it has a dramatic effect. Even after SDS treatment, swelling was severely restricted over the temperature range of 50–95 °C. However, the elevated gelatinisation temperature of these starches gives a limited window for the study of viscosity under standard RVA conditions. Peak gelatinisation temperatures (from DSC) are 80–85 °C (50% amylose maize) and 90–95 °C (70% amylose maize and high amylose potato). For other starches, swelling typically commences at or soon after the peak gelatinisation temperature. This is consistent with the swelling/viscosity profile for 50% amylose maize (Fig. 2e) which shows a modest increase in viscosity at around 90 °C, but interestingly, no increase in viscosity on holding at 95 °C. SDS pre-treatment had very little effect on this behaviour (Fig. 2e).

This suggests that for high amylose starches, swelling behaviour is dominated by the carbohydrate composition, and that lipid and protein only have a small secondary effect. The inference is that swelling is inhibited with high amylose content. We propose that this involves initial swelling of granules at temperatures determined by melting of ordered double-helical structures (Cooke & Gidley, 1992). A limited swelling could then allow entry of sufficient water to facilitate the formation of double helices of greater length than in the native granule. These helices are expected to have a higher melting temperature of ca. 5 °C per glucan unit (Safford et al., 1998). Once formed in sufficient amounts, helices would then effectively restrain subsequent expansion swelling and viscosity development.

The role of surface components (linear glucan + protein + lipid) is therefore important in granular swelling (rate and extent), but only for some starches. Two of the most commonly used starches (maize and wheat) fall into this category. If the

levels of lipids and/or proteins and/or linear glucans on the surface of granules are relatively low, then there is little effect of removing them (e.g. waxy maize, potato and tapioca starches). If the glucan composition is such that swelling facilitates formation of more thermally stable (longer) double helices, then subsequent swelling is proposed to be limited irrespective of surface lipids and/or proteins (e.g. high amylose maize and potato starches).

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