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The degree of starch phosphorylation is related to the chain length distribution of the neutral and the phosphorylated chains of amylopectin

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Abstract

The chain length distribution of the amylopectin neutral and phosphorylated α-glucan chains of isoamylase (EC 3.2.1.68)-debranched starch in dependence of starch phosphorylation has been analyzed using high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Neutral chains of moderately phosphorylated starches showed polymodal distributions with main peaks at dp (degree of polymerisation) 14–15 and 50, respectively. Highly phosphorylated starches specifically showed an increasing proportion of chains with mean dp 19. In the extremely highly phosphorylated curcuma (*Curcuma* spp.) starch, this chain population dominated the chain length profile. The sub-population of the corresponding phosphorylated chains from highly phosphorylated starches showed longer retention times than moderately phosphorylated starches on HPAEC. Separation of the phosphorylated chains using anion-exchange chromatography at neutral pH demonstrated a partial separation of chains containing Glc3*P* residues and Glc6*P* residues, respectively, as well as a separation of chains containing one or several phosphate groups. Major determinants for the observed elution characteristics of phosphorylated chains using HPAEC are suggested to be a combination of chain length (as determined by gel-permeation chromatography), multiple phosphorylation of the chains (as determined from their content of Glc6*P* and reducing ends) and finally, the presence of the phosphate group on either the C-3 or the C-6 position on the glucose moieties (as determined by ^{31}P NMR). The results demonstrate that general rules exist in the relationship between starch phosphorylation and chain length distribution of starches.

Keywords: Amylopectin; Chain length distribution; Starch phosphorylation; Starch structure

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1. Introduction

Amylopectin and amylose are the two major α-D-glucan polymers of starch granules in higher plants and algae. Amylose is an essentially linear molecule with a mass of 10^5–10^6 kDa mainly consisting of α-(1→4)-linked α-D-Glc*P* backbone and very few α-(1→6) branching points [1]. In contrast, amylopectin has a molecular mass of about 10^7 kDa, a flat/elliptical shape [2], and contains frequent α-(1→6) branching points unevenly distributed along the molecule [1,3,4], thus generating 9 nm chain clusters [5]. The polymodal distribution of linear unit chains of enzymically debranched amylopectin has been suggested to directly reflect the three dimensional cluster [4,6,7]. Recent electron microscopic investigations of α-amylase eroded starch granule fragments [8,9] suggest amylopectin to be arranged in super helical structures that may be formed by co-operative processes.

Amylopectins from most plant sources contain small amounts of glucose moieties (0.1–1%) with phosphate groups that are monoesterified to O–6 or O–3 of the glucosyl unit. Generally, amylopectins from tuber and root starches have the highest degree of phosphorylation [10]. These starches also exhibit a B-type X-ray diffraction pattern [11], arising from a crystallinity form that is partly characterized by less densely packed double helical segments than in the A-type pattern. The latter type is found in starches showing a low degree of starch phosphorylation e.g. cereal starches. The presence of phosphate groups has a major impact on the rheological properties of isolated starch, causing clearer gels and higher viscosity, which is advantageous for many industrial applications [12,13]. Approximately 60–70% of the phosphate groups are bound as a rather acid-stabile monoester at O–6, whereas 30–40% are linked at O–3 as a more acid-labile monoester. A trace of phosphate is also found at O–2 [14]. The vast majority of the phosphate groups are located in the longer unit chains and only a small, but distinct, population is found in shorter chains [15]. Restrictions of phosphate distribution have been demonstrated by structural analyses of potato amylopectin. One phosphate group is found per unit chain in a moderately phosphorylated starch and no phosphate groups are located on the non-reducing end [16] or closer than 9 glucosyl units from an α-(1→6) branching point [15]. However, the position of phosphate groups in relation to the clusters has not yet been revealed.

In spite of a generally low degree of starch phosphorylation, starch phosphate constitutes an important part (30–50%) of the total phosphate content of potato tubers [17,18]. However, the degree of starch phosphorylation is subject to large genetic variation. The phosphate content in 24 randomly selected starches from different potato varieties spanned from 8 to 24 nmol glucose 6-phosphate (Glc6*P*) mg^{-1} starch [19]. Potato plants grown at low temperature show an increased degree of starch phosphorylation [7] whereas plants grown under phosphate deprivation have a lower starch-phosphate content [20] implying the phosphorylation mechanism to be rather flexible.

Net biosynthesis of starch is accomplished by an enzyme machinery involving ADP-glucose pyrophosphorylase, starch synthase, starch branching enzyme [21], and possibly a set of starch hydrolyzing enzymes, e.g. debranching enzyme [22,23]. The phosphate groups have been shown to be incorporated during starch biosynthesis [24] but the enzyme system involved in starch biophosphorylation is, however, still enigmatic. However, by using the anti-sense technique towards several starch granule bound proteins and enzymes involved in starch metabolism, and by over-expression of starch/glycogen synthesizing enzymes in potato tubers, various effects have been demonstrated both on the phosphorylation level and starch fine structure [25–28]. Together with the structural information available on potato [15,16,29], these data suggest a positive correlation between the chain length of the branched α-glucan and the amount of covalently bound phosphate on these chains.

The methodology described in this study facilitates automated analysis of both the neutral and the phosphorylated chains of amylopectins from various sources. The characterization, made on the starches in this study, demonstrates that general structural rules regarding starch phosphorylation, in relation to chain length distribution, can be established. This is of special importance in relation to the specific needs for structural characterization on the molecular level of e.g. genetically modified starches or "exotic" starches. Subtle differences in the molecular structure of starch often lead to drastic alterations in the physical properties of the prepared gels. Thus, rheological investigations must be performed on structurally well-characterized starches in order to provide a correlation between specific structural features and rheological properties.

The aim of this work was to investigate a possible correlation between the degree of starch phosphorylation and the chain length distribution of neutral and phosphorylated amylopectin chains using high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Special emphasis was made on the analysis of extremely highly phosphorylated starch isolated from *Curcuma spp*. [30,31]. The investigation reveals relationships between the distribution of neutral and phosphorylated chains and between the degree of phosphorylation and the chain length distributions.

2. Results and discussion

Determination of the pulsed amperometric detector response in relation to chain length.—To quantitatively assess the chain length distributions, a detector response curve for the Dionex PAD was determined for different chain lengths [32]. Known amounts of homo-disperse chains from dp 6 to dp 20 and of a narrow distribution of chains with mean dp 40 were injected and the resulting peaks integrated. The data were fitted to a hyperbolic curve (Fig. 1) which was used to correct values from integrated single peaks obtained in subsequent separations of neutral oligosaccharides.

The degree of phosphorylation.—Starches isolated from a number of potato cultivars, as well as from cassava, arrowroot, curcuma, rice, mung bean and sorghum, were analysed for their content of starch bound phosphate expressed as nmol Glc6*P* mg^{-1} starch which has been shown to be linearly correlated to the content of Glc3*P* [19]. The Glc6*P* content spanned from 0.9 nmol mg^{-1} starch in sorghum to 60.0 nmol mg^{-1} starch in Curcuma (Table 1). The content of phytic acid and of total phosphate in the potato tubers was also determined. No correlation was found between the content of phytic acid and total phosphate compared to starch bound phosphate (results not shown) suggesting that the in vivo concentrations of these substances are not directly important for starch phosphorylation.

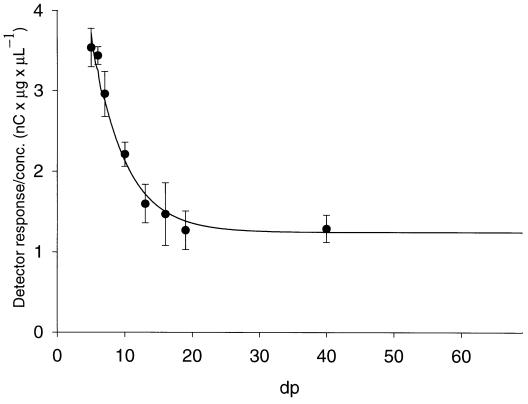

Fig. 1. The response curve for the PAD detector. Known amounts of linear chains were injected and the resulting peaks were integrated. The data were fitted to an exponential function which was used to convert the peak areas obtained in the following neutral chain length distribution experiments into absolute amount. Error bars denote SE of 3 determinations.

The chain distribution of phosphorylated and neutral amylopectin chains.—The chain distribution of the neutral and phosphorylated linear α-glucan chains obtained from rice, arrowroot, potato (cv Dianella) and curcuma starch is shown in Fig. 2. All samples show a polymodal distribution with at least two main chain populations. The distributions of the neutral chains showed maxima at dp 14–15, dp 19 and dp 50 (Fig. 2, left) and very few chains were detected below dp 6. Similar distributions have been obtained in other investigations [32,33]. The highly phosphorylated starches become enriched in a component of dp 19, which broadens the main peak of these starches. The dp 19 component constitutes the dominant peak in curcuma starch, the most highly natural phosphorylated starch available to date. Starches isolated from cassava, mung bean and sorghum show a relatively low degree of phosphorylation (Table 1) but exhibit the same general relationships in the distribution pattern of the neutral chains with respect to the degree of phosphorylation as described above (data not shown).

Table 1
The degree of phosphorylation of starches

Source	Degree of phosphorylation (nmol Glc6*P* mg^{-1} starch)
Potato tuber (cv Godiva)	14.6
Potato tuber (cv Dianella)	17.0
Potato tuber (cv Kaptah)	18.1
Potato tuber (*Solanum phureja*)	28.6
Potato tuber (cv 90-BKG22)	32.6
Potato tuber (cv 87-BDN56)	33.1
Cassava root	2.5
Arrow root	4.6
Curcuma root	60.0
Sorghum seed	0.9
Rice seed	1.0
Mung bean	3.5

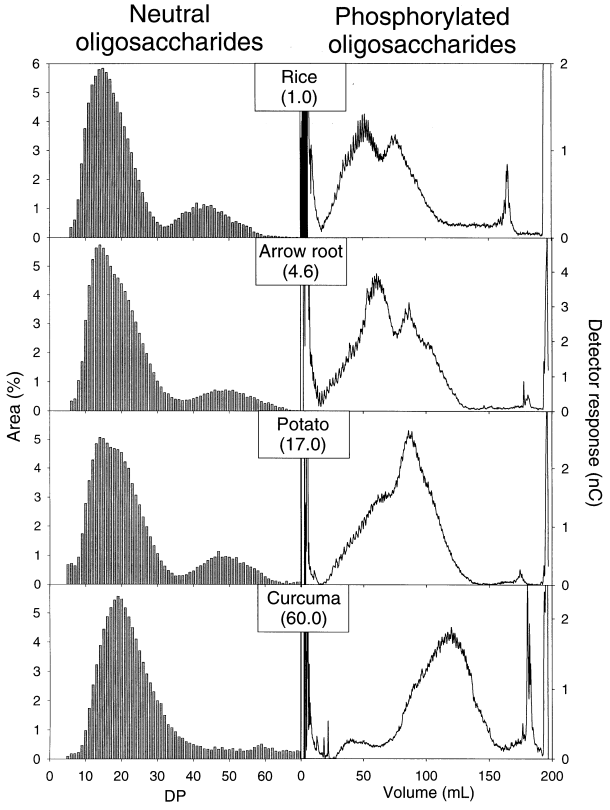

Fig. 2. The distribution of linear neutral (left) and phosphorylated (right) α-glucan chains from starches with different degrees of phosphorylation as determined by HPAEC-PAD. Peaks from chromatograms of neutral chains were integrated and normalized to 100%. The profiles for phosphorylated chains were normalized to the same total area. Numbers in parenthesis denote the degree of phosphorylation (nmol Glc6*P* mg^{-1} starch).

A comparison of the different profiles of phosphorylated chains reveals two prominent features: (1) an increased degree of phosphorylation results in a pronounced decrease in chains eluting at 62 mL and an increase in chains eluting at 87 mL; (2) at yet higher phosphorylation levels, the whole profile is shifted to 120 mL (Fig. 2, right). This demonstrates that a high degree of starch phosphorylation is manifested either by the occurrence of longer α-glucan chains, by the occurrence of multiply phosphorylated chains or by variations in the positions of the phosphate group in the chains or within the individual glucose unit. Each of these alternatives is investigated in further detail as described below.

By comparing chain length distribution patterns for a range of potato starches, the same main correlations with respect to the degree of phosphorylation as described above were found. The highly phosphorylated potato starches contained increased proportions of the dp 19 component and the elution of the phosphorylated chains were somewhat retarded (Fig. 3). All the tuberous starches in Table 1 showed the same dependencies with respect to the degree of phosphorylation as shown in Fig. 3, but for clarity only two extremes were included in the figure. Moreover, as also observed with curcuma neutral chains (Fig. 2) the highly phosphorylated starch is depleted in chains of dp 45–50 (Fig. 3B). This may indicate that the pool of dp 45–50 is phosphorylated and consequently depleted in the fraction containing neutral chains.

Whereas the separation of the neutral α-glucans by HPAEC proceeds according to chain length, the chromatographic behavior of the phosphorylated glucans is more complex because of additional influence by the presence as well as the position of the phosphate groups in the α-glucan chains. To analyze this problem, phosphorylated chains were treated with β-amylase before HPAEC. β-Amylase hydrolyses maltose units from the non-reducing end of the chains but cannot pass a phosphate group. The resulting products will therefore be shortened phosphorylated chains in which the phosphate group is situated one or two glucose units from the non-reducing end [16]. As seen in Fig. 4, the β-amylase-treated chains elute earlier than the full-length chains and the elution profile is still polymodal. Provided that the ability of the phosphate groups in the β-amylase-treated chains to interact with the column matrix was not altered compared to their presence in the full length chains, these results imply that the chain length is an important determinant of the elution characteristics. All the potato amylopectin samples shown in Table 1 produced similar bimodal profiles (results not shown).

Gel-permeation chromatography of phosphorylated chains.—The supposed relationship between the degree of phosphorylation and the chain length of the phosphorylated chains as suggested by HPAEC analyses was further investigated for arrowroot, potato and curcuma by Sephacryl-200 gel-permeation chromatography (see Experimental). Using this technique, the chains derived from the highly phosphorylated starches were found to elute first from the column implying that they contain longer chains (Fig. 5). This observation substantiates that the elution profiles obtained in the HPAEC experiments at least partly reflect the chain length distributions of the phosphorylated chains.

Anion-exchange chromatography of phosphorylated chains at neutral pH.—The possible occurrence of several phosphate groups on the same unit chain could be another important factor, which would affect the HPAEC retention times of the phosphorylated chains. Multiple phosphorylation sites could thus explain the long retention times observed with the highly phosphorylated starches (Figs. 2 and 3). Based on the following statistical calculations, glucan chains with multiple phosphorylation sites would be predicted to exist. Highly phosphorylated potato amylopectin (Table 1) contains approximately one phosphate group per 100 glucose units provided that the amylose content is 20% and Glc3*P* constitutes 30% of the phosphorylated glucose moieties. The main pool of phosphorylated unit chains has a mean dp of 56 ([15], and see above). Because this population constitutes about 40% of the unit chains [34] an equal distribution of the phosphate

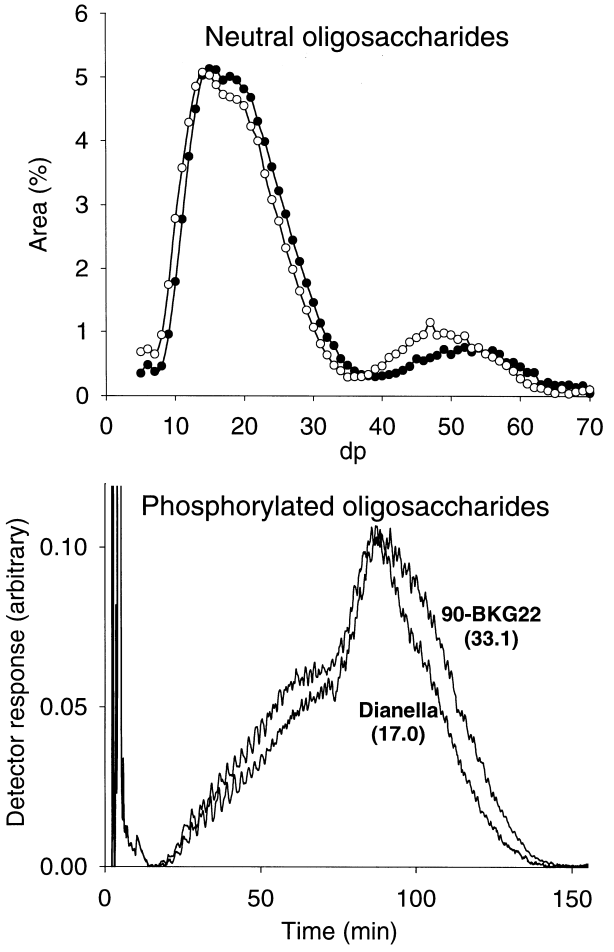

Fig. 3. HPAEC profiles of neutral and phosphorylated linear α-glucans from potato tuber starches with different levels of starch phosphorylation. Dianella (17.0 nmol mg^{-1} starch) (○), 90-BKG22. (33.1 nmol mg^{-1} starch) (●). Peaks from chromatograms of neutral chains were integrated and normalized to 100%. To permit easy comparison, elution profiles of phosphorylated chains were normalized to equal area between 20 and 150 mL elution volume.

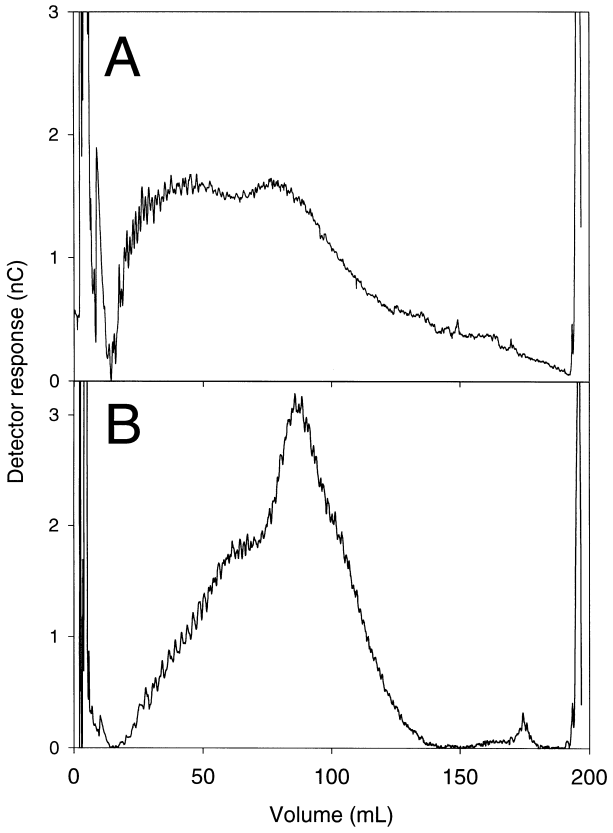

Fig. 4. The effect of β-amylase treatment on the HPAEC profile of linear phospho-oligosaccharides from potato tuber (cv Dianella). A: β-amylase treated phospho-oligosaccharides. B: untreated phospho-oligosaccharides.

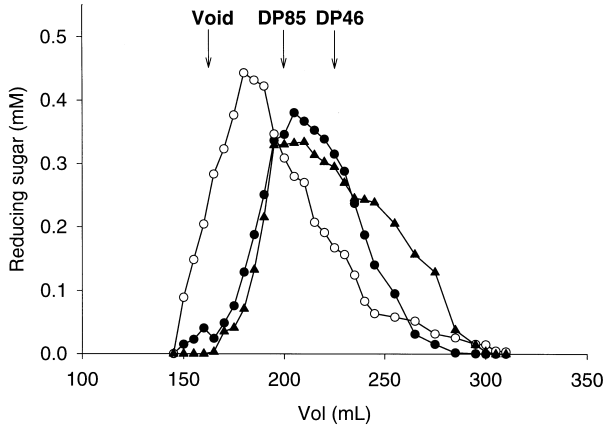

Fig. 5. Gel-permeation chromatography of phosphorylated oligosaccharides prepared from arrowroot (▲), potato tubers (90-BKG22) (●), and curcuma (○). Markers for void volume (blue dextran) and dp 85 and dp 46 (see experimental) are indicated in the figure.

groups would give 1.4 phosphate groups per phosphorylated chain. For curcuma starch this value would be even higher. These different types of chains can be separated by anion-exchange chromatography where the unit charge of each chain, as defined by the number of phosphate groups, determines the strength by which the chain is bound to the anion-exchange resin. The binding strength is expected to be less affected by the position of the phosphate group on the glucan chain or on the glucose residues (Glc6*P* or Glc3*P*, respectively).

Anion-exchange chromatography at neutral pH separated the phosphorylated chains from curcuma into three main populations, C1, C2 and C3, and the phosphorylated chains from potato into two populations, P1 and P2 (Fig. 6A). Iodine staining of P1 and P2 showed the λ-max for their iodine/α-glucan complexes to be 573 and 583 nm, respectively. This demonstrates that the longer chains elute later. More detailed analysis of the chain length distributions and the degrees of phosphorylation showed that C1 and P1 was composed of short chains (dp 48 and dp 43, respectively) containing only 0.64 and 0.52 Glc6*P*/chain, respectively (Table 2). This indicates that these chains contained phosphate groups linked to the more labile 3 position of the glucose moiety that were lost during the analytical acid hydrolysis step. P2, C2 and C3 contain longer chains and show phosphorylation values greater than 1 Glc6*P*/chain (1.37–1.70). The results demonstrate that a fraction of the isolated α-glucan chains is multiply phosphorylated. In order to test the hypothesis that C1 and P1 populations contain Glc3*P* residues, the chains were hydrolyzed enzymatically using α-amylase (Termamyl, Novo Nordisk, Denmark) and subjected to ^{31}P NMR analysis as described by Bay-Smidt et al. [19]. The ^{31}P NMR spectrum of curcuma starch before separation on DEAE-Sepharose is shown in Fig. 6B. The signal at δ 1.0 is assigned to Glc6*P* in internal parts of the glucan chains and a signal at δ 1.75 corresponds to Glc3*P* [19,35]. A minor signal at δ 1.4 corresponds to a differently positioned Glc6*P* residue probably residing at the non-reducing end of the chain [19,35]. Accordingly, the signal at δ 1.0 splits into a triplet and the signal at δ 1.75 to a doublet when proton decoupling is not applied (Fig. 6B). In C1, a signal at δ 1.0 is recovered corresponding to Glc6*P* residues (Fig. 6B). A signal at δ 1.75 corresponding to Glc3*P* is found in pools C1 and C2, demonstrating that chains eluting early contain Glc3*P* residues. Pool C3 mainly shows signals corresponding to Glc6*P* residues at δ 1.0 and δ 1.4. However, the minor orthophosphate signals at δ 0.7, most probably originating from hydrolysis of the rather labile phosphate group on Glc3*P* in the course of the high temperature, acid and alkaline treatment [19], suggests that pool C3 also contains small amounts of Glc3*P*-containing chains. Thus, for curcuma, the α-glucan chains, eluting early from the DEAE column (C1), are a mixture of short and singly substituted Glc6*P*- or Glc3*P*-containing chains. The middle peak (C2) contains phosphorylated chains with Glc3*P* and/or Glc6*P* residues and the last pool (C3) mainly contains longer chains with one or several Glc6*P* residues. The results demonstrate that the structural basis for the apparent separation by anion-exchange chromatography according to chain length at neutral pH is not because of the long chains per se but because these chains have a higher probability of being multiply phosphorylated and thus are more retarded on the column. However, as the number of Glc6*P* residues per chain is about 1.5, these pools should also contain chains that have only one phosphate group, suggesting that the position of the phosphate groups on the chains is also important for the interaction with the functional groups on the column matrix. The Glc3*P*-enriched chains may elute earlier than the Glc6*P*-containing chains either because of steric restrictions of their phosphate groups to interact with the column or because these chains are shorter and therefore more frequently contain only one phosphate group. A plausible explanation is that the phosphate groups on the Glc3*P* residues are somewhat shielded by α-helical secondary structures of the chains in which these phosphate groups point inwards [36] and consequently elute earlier from anion-exchange columns.

The relation between chain separation using DEAE chromatography and HPAEC was investigated by subjecting the different DEAE pools to a HPAEC separation after desalting as described above and freeze-drying. As shown in Fig. 6C, the α-glucan pools elute in the same order in the HPAEC as in the DEAE experiment, suggesting similar mechanisms for separation in the two systems. The appearance of two sharp peaks in the HPAEC chromatogram for curcuma pool 3 was always obtained for these curcuma samples and may indicate the presence of chains containing one

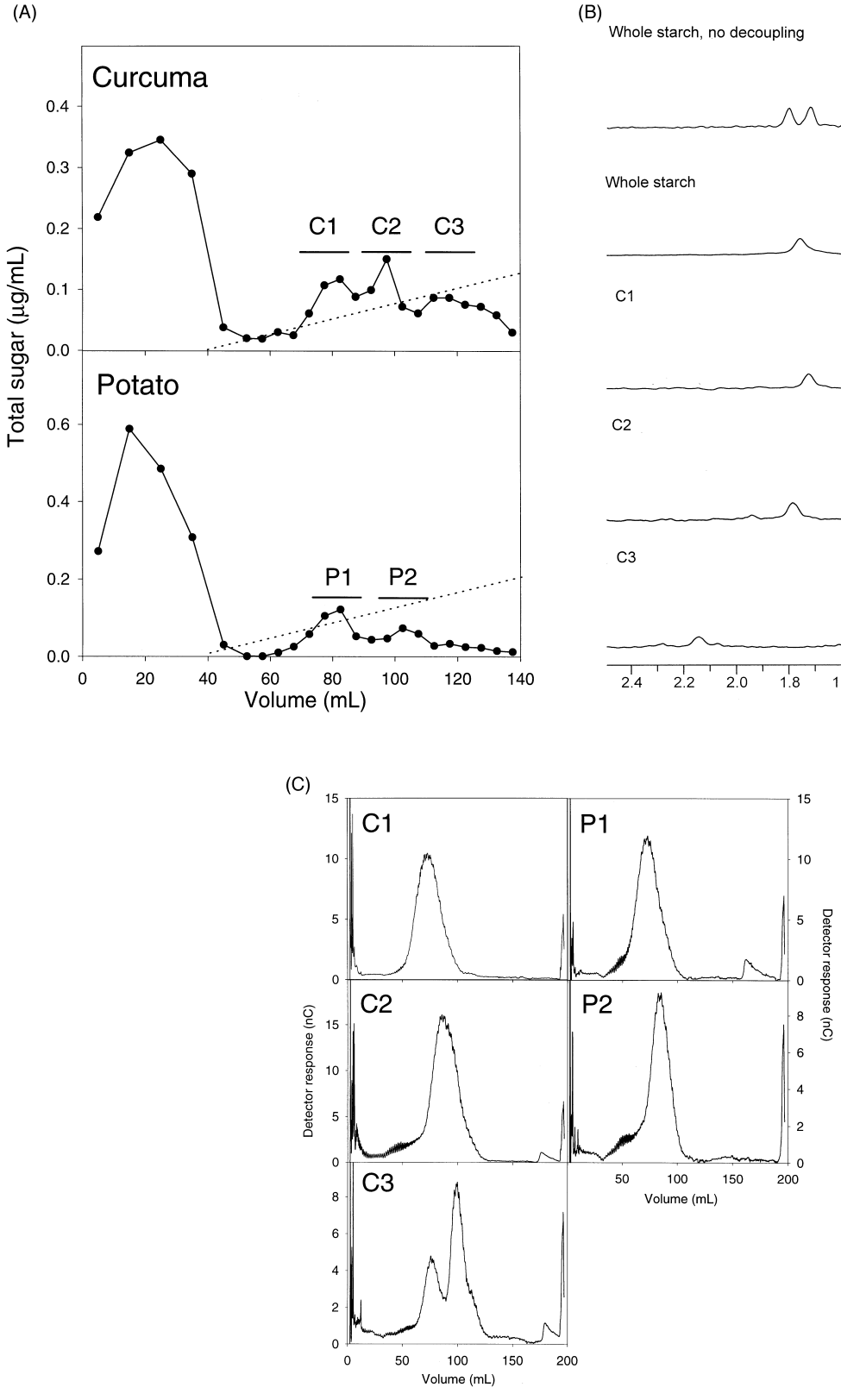

Fig. 6. A. DEAE Sepharose chromatography of phosphorylated oligosaccharides from curcuma and potato (cv Dianella). C1-C3 and P1, and P2, respectively are fractions pooled for HPAEC analysis. Dotted lines denote 0–50 mM NaCl linear concentration gradient. B. ^{31}P NMR spectra of C1-C3 pools in Fig. 6A. Spectra denoted "whole starch, no decoupling" and "whole starch" are Termamyl-treated curcuma starch recorded without and with proton decoupling applied, respectively. C. HPAEC-PAD of the combined fractions obtained by DEAE chromatography in Fig. 6A.

Table 2
DP and number of Glc6*P* residues per chain in DEAE fractions as indicated in Fig. 6A

Sample	dp	Glc6*P*/ reducing end
Curcuma pool 1 (C1)[a]	48	0.64
Curcuma pool 2 (C2)[a]	86	1.70
Curcuma pool 3 (C3)[a]	94	1.41
Potato pool 1 (P1)[a]	43	0.52
Potato pool 2 (P2)[a]	58	1.37

[a] As denoted in Fig. 6A

or two Glc6*P* residues, respectively, as almost no Glc3*P* was detected in this pool. The position of the phosphate groups on either the 6 or the 3 position on the glucose residues is probably also important for the elution characteristics as discussed above and may give rise to broadening of the HPAEC profiles in C1 and C2.

The obtained results suggest a nearly random distribution of both the Glc3*P*-containing chains and the Glc6*P*-containing chains within a long-chain population of amylopectin. It can therefore be generally concluded that amylopectin is phosphorylated in the amorphous part of the amylopectin as short chains are mainly present in single clusters and consequently involved in crystalline double helices [4]. However, Glc3*P* residues, but not Glc6*P* residues, have steric access to the interior part of a double helix [36] and therefore long as well as short Glc3*P*-containing chains may form part of crystalline regions in amylopectin.

3. Experimental

Preparation of starches.—Different cultivars of potato tubers (*Solanum tuberosum* and *Solanum phureja*) were grown outdoors in pots and starch was isolated from freshly harvested tubers using a fruit juicier (Moulinex 753) and subsequently purified as described elsewhere [19]. Starch was isolated from mung bean (*Phaseolus cocinus*) and sorghum (*Sorghum bicolor*) seeds and from curcuma (*Curcuma spp.*) and cassava (*Manihot esculenta*) roots by disintegration of the dry tissues in 2 volumes of distilled water at 0°C for 10 min using a Philips HR 2871/A food processor. The starch in the slurry was separated from cell debris by passage through a 50–70μ mesh, sedimented at 0°C in 2 L distilled water and finally dried by sequential washes in EtOH and acetone.

Preparation of linear neutral and phosphorylated chains obtained from debranched amylopectin.—Starch (5 mg) was solubilised in 100 μL 0.5 M NaOH and then diluted with 1.5 mL water. The starch solution was neutralised with HCl and adjusted to pH 4.0 with HOAc. Isoamylase (0.5 units, Megazyme, Sydney, Australia) was added and the mixture was incubated for 2 h at 40°C. The reaction mixture was neutralised with NaOH and the pH was adjusted to 7.5 with 5 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl). The reaction was stopped by boiling for 5 min and denatured protein removed by centrifugation. Neutral unit chains of amylopectin and amylose were recovered by passing the debranched starch over a DEAE-Sepharose® (Pharmacia, Uppsala, Sweden) column (1.5×3 cm) equilibrated with 5 mM Tris-HCl, pH 7.5. Phosphorylated unit chains were eluted from the column with 100 mM NaCl, 10 mM HCl. Separation of phosphorylated unit chains at neutral pH (5 mM Tris-HCl, pH 7.5) was achieved by elution with a 0–50 mM NaCl linear concentration gradient (80 mL, 1 mL min^{-1} flow rate) and 5 mL fractions were collected. For these experiments, 15 mg debranched starch was used, and the volumes for sample preparation and debranching described above were scaled up accordingly. β-amylase treatment was performed at 37°C for 2 h in 500 μL reaction mixtures containing phosphorylated chains, prepared as described above, 50 units of β-amylase (EC 3.2.1.2, Megazyme, Sydney, Australia), and 50 mM Na-citrate, pH 6.0.

High performance anion-exchange chromatography (HPAEC) of linear oligosaccharides.—Neutral and phosphorylated linear oligosaccharides were separated using a Dionex DX 500 system equipped with an S-3500 autosampler, a GP40 pump, an ED40 pulsed amperometric detector (PAD) and a CarboPac PA-100 column. Samples of 30 μL neutral oligosaccharides or 100 μL phosphorylated oligosaccharides prepared as described above were injected and separated using a 1 mL min^{-1} flow rate, isocratic 150 mM NaOH, and the following NaOAc concentration gradient profiles: neutral oligosaccharides: 0–5 min linear gradient of 0–110 mM NaOAc; 5–130 min convex gradient (curve 4) of 110–350 mM NaOAc. Phosphorylated oligosaccharides: 0–5 min linear gradient of 0–170 mM NaOAc; 5–190 min linear gradient of 170–500 mM NaOAc. Amylose in the neutral chain preparation had an extremely high affinity and was

washed off the column after the gradient with 1 M NaOAc without NaOH. Preparative separation of linear neutral chains containing >80% of homodisperse chains ranging from dp 6 to dp 20 obtained from debranched potato amylopectin was performed using a semi-preparative CarboPac PA-1 column operated at 5 mL min^{-1} flow rate and the same program as used above for neutral oligosaccharides. Collected fractions were passed over a cation-exchange column (Dowex 50W, H^+;Fluka) to remove Na^+ ions. The samples were freeze dried to remove water and HOAc and finally stored dry at −20°C.

Gel-permeation chromatography.—Phosphorylated chains were fractionated by gel-permeation chromatography using a 830×26 mm Sephacryl-200 (Pharmacia Biotech, Uppsala, Sweden) column equilibrated with 10 mM NaOH and operated at room temperature with a flow rate of 1 mL min^{-1}. Samples (3–5 mg) or linear markers (see below) were solubilised in 500 μL 200 mM NaOH and applied on the column. Fractions (5 mL) were collected and assayed for total sugar content (see below). Linear α-glucan markers for gel-permeation chromatography with dp 46 ± 11 and 85 ± 20 (interval indicates 90% of the chains) as determined by HPAEC-PAD using maltohexaose as standard, were prepared by elongation of maltohexaose using rabbit muscle phosphorylase-*a* (Sigma). The reaction mixtures (50 mL), buffered by 100 mM Na-citrate pH 7.0, containing 25 mM Glc1*P*, 1 mM AMP and maltohexaose as primer, were incubated at 37°C for 20 h. The final mean chain lengths in the different samples were controlled by the concentrations of maltohexaose as follows: 160 μM maltohexaose and 4 mg phosphorylase-*a* gave dp 46, and 80 μM maltohexaose and 8 mg phosphorylase-*a* gave dp 85. The products were recovered by precipitation with 75% MeOH and dried with acetone.

Additional assays and chemicals.—The degree of starch phosphorylation was determined as nmole Glc6*P* mg^{-1} starch as previously described [19]. For most starches, Glc3*P* is in the range of 30–40% of the total starch phosphate [10,19] why this methodology provides a good measure of the total starch phosphate content of starch. Total sugar was analysed using the phenol–H_2SO_4 method as described [37], and reducing-end analysis was performed according to a modified Park-Johnson method [38]. Linear malto-oligosaccharides of dp 2–7 were purchased from Sigma.

^{31}P nuclear magnetic resonance.—^{31}P NMR spectra were recorded at 101 MHz using a Bruker AC250P NMR instrument. Chemical shifts are referred to external 85% H_3PO_4 subsequently inserted. The pH of the desalted samples was adjusted to 4.5 and the sample temperature was 300 K. A 90° pulse angle, a relaxation delay of 2 s and CPD-decoupling were used and several thousand transients were accumulated. Lorentz/Gauss resolution enhancement was applied to the spectra run without CPD-decoupling. Otherwise, exponential multiplication using a line broadening of 2 was used.

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