Running head: Phosphorylation of A- and B-type crystallized maltodextrins

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The two plastidial starch-related dikinases sequentially phosphorylate glucosyl residues at the surface of both the A- and B-allomorph of crystallized maltodextrins but the mode of action differs.

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Abbreviations: DP degree of polymerization; GWD glucan, water dikinase; MD maltodextrins; MD<sub>cryst</sub> crystalline maltodextrins; MD<sub>sol</sub> solubilized maltodextrins; PWD phosphogluca, water dikinase
Abstract:
In this study, two crystallized maltodextrins were generated that consist of the same oligoglucan pattern but differ strikingly in the physical order of double helices. As revealed by X-ray diffraction, they represent the highly ordered A- or B-type allomorph. Both crystallized maltodextrins were similar in size distribution and birefringence. They were used as model substrates to study the consecutive action of the two starch-related dikinases, the glucan, water dikinase and the phosphoglucan, water dikinase. The glucan, water dikinase and the phosphoglucan, water dikinase selectively esterify glucosyl residues in the C6 and C3 position, respectively. Recombinant glucan, water dikinase phosphorylated both allomorphs with similar rates and caused complete glucan solubilization. Soluble neutral maltodextrins inhibited the glucan, water dikinase-mediated phosphorylation of crystalline particles. Recombinant phosphoglucan, water dikinase phosphorylated both the A- and the B-type allomorph only following a prephosphorylation by the glucan, water dikinase and the activity increased with the extent of prephosphorylation. The action of the phosphoglucan, water dikinase on the prephosphorylated A- and B-type allomorphs differed. When acting on the B-type allomorph, by far more phosphoglucans were solubilized as compared to the A-type. However, with both allomorphs the phosphoglucan, water dikinase formed significant amounts of monophosphorylated phosphoglucans. Thus, the enzyme is capable of acting on neutral maltodextrins. It is concluded that the actual carbohydrate substrate of the phosphoglucan, water dikinase is defined by physical rather than by chemical parameters. A model is proposed that explains, at a molecular level, the consecutive action of the two starch-related dikinases.

Introduction:
In terms of quantity starch is one of the most prominent photosynthesis-derived products. The global starch production by land plants has been estimated to be approximately 2,850 million tonnes per year (Burrell 2003). Starch is highly relevant for nutrition of animals and humans but it is also used for many industrial applications, such as additives in paper or textile industry and in pharmacy as well. In addition, starch appears to be increasingly important as a photosynthesis-based renewable energy source that can be converted into technologically relevant products such as bioethanol or hydrogen (Hannah and James 2008; Zhang et al. 2008).
Native starch is formed as a water-insoluble particle designated as granule that is thought to comprise two types of polyglucans, amylopectin and amylose. The latter is an almost unbranched \(\alpha\)-1,4-glucan and usually is the minor constituent of the starch particle accounting for 10 to 35% of the total starch dry weight (Ball 2000). However, in some mutants the relative amylose content is strongly diminished resulting in an essentially amylose-free starch (such as in the waxy mutant of *Zea mays* L.) or, alternatively, it is increased forming up to 70% of the starch mass (e.g. in the amylose extender mutant from maize; Gérard *et al.* 2001). Nevertheless in wild type starches amylopectin typically is the major constituent that also is essential for the molecular organization of the glucans within the entire starch granule (Ball and Morrell 2003). Like glycogen, amylopectin is a branched \(\alpha\)-glucan with 4-6% of the interglucose linkages being \(\alpha\)-1,6-bonds (Ball 2000) but, as opposed to glycogen, the branching points occur as intramolecular clusters. Due to the length distribution of the side chains and the clustering of the branching points, neighbouring glucan chains are capable to form highly ordered double helices (Smith 2001; Zeeman *et al.* 2002).

As revealed by X-ray diffraction analysis, two major native starch structures are known that differ in the arrangement of the double helices. The A-type allomorph which is typical of wild-type cereal starches but also occurs in lower plants is more compact, as compared to the B-type, and consists of flat layers of double helices. By contrast, in the B-type allomorph six double helices are thought to surround a central cavity that is filled with water molecules. The B-type allomorph is found in starch synthesized by dicotyledal storage organs, such as potato tubers, in some high-amylose starches from cereal mutants (Gallant *et al.* 1997; Gérard *et al.* 2001) and in assimilatory starches from *Solanum tuberosum* L. and *Arabidopsis thaliana* L. as well (Hejazi *et al.* 2008). Legume starches are believed to represent another allomorph that is designated as C-type. However, actually this allomorph is a mixture of both the A- and the B-type crystallites within a single native starch particle rather than a third distinct type of the double helical arrangement (Bogracheva *et al.* 2001; Imberty *et al.* 1991).

It should be noted, that both the A- and the B-type allomorph of native starch granules often contain, as a minor constituent, an additional crystal structure designated as V-type. Unlike the A- and B-allomorph, the V-type is assumed to arise from single amylose helices some of which are complexed with endogenous granular lipids. When estimated for the dry state, the V-type crystal structure accounts only for a few percent of the total starch granule crystallinity (Lopez-Rubio *et al.* 2008).

The physical structure of the native starch particle is likely to have important biochemical implications as it affects the performance of carbohydrate-active enzymes and, thereby, the
transition of carbohydrates from the solid to the soluble phase. This conclusion has been
reached by in vitro experiments demonstrating that the pancreas α-amylase hydrolyzes A-type
starch faster than the B-type counterpart (Gérard et al. 2001).

Another metabolically important feature of amylpectin is the occurrence of covalent
modification by phosphate esters that are found in a small proportion of the glucosyl residues.
Most frequently phosphorylation occurs at the C6 position of the glucosyl residue but C3 and,
to a minor extent, C2 can also be esterified (Hizukuri et al. 1970). Recently evidence has been
presented that the esterification of the C6 and the C3 position of glucosyl residues differ in
their structural effect on the neighbouring interglucose bonds (Hansen et al. 2009).

Phosphorylation at C6 is mediated by the recently identified α-glucan, water dikinase (GWD;
EC 2.7.9.4) that utilizes ATP as dual phosphate donor and three distinct acceptors two of
which are sequentially used. The enzyme transfers the terminal phosphate group to water
(thereby forming orthophosphate) and the β-phosphate group first to a conserved histidin
residue within the catalytic domain of the monomeric GWD and, subsequently, to the C6
target of the glucosyl residue to be phosphorylated (Ritte et al. 2002; 2006). Phosphorylation
at C3 is catalyzed by a second dikinase, designated as phosphoglucan, water dikinase (PWD;
EC 2.7.9.5; Ritte et al. 2006). The amino acid sequence of the catalytic (C-terminal) domain
of PWD shares similarity with that of GWD and, in principle, the PWD-mediated catalysis
follows the same mode of action as GWD, including the transient autophosphorylation at a
conserved histidin residue (Kötting et al. 2005; Baunsgaard et al. 2005). However, PWD
deviates from GWD in the amino acid sequence of the N-terminal domain, especially in the
carbohydrate-binding region. PWD possesses a single carbohydrate-binding module (CBM)
that has been grouped into the family CBM20 (Machovič and Janaček 2006a; 2006b). By
contrast, the N-terminal domain of GWD contains two putative carbohydrate-binding motives
similar to those of an α-amylase that presumably is located in the chloroplasts (Yu et al.
2005). However, the structure of these motives is still not known and, therefore, a sequence-
based prediction of the actual carbohydrate target is not yet possible.

GWD- and PWD-deficient Arabidopsis mutants possess to some extent similar but not equal
phenotypes: Leaves of GWD-deficient lines (that contain essentially unchanged levels of
functional PWD) have starch levels that are at least five-times higher than those of the wild
type and remain high even after prolonged darkness. Growth of the entire plant is strongly
compromised. The phenotype of PWD-deficient mutants (that express functional GWD) is
less severe as growth is only slightly diminished and transitory starch levels are elevated but
not as strongly as in the GWD-deficient lines. Mutants lacking functional PWD can degrade
transitory starch but net degradation occurs at a lower rate as compared to wild type plants (Kötting et al. 2005). These data clearly indicate that in vivo PWD cannot substitute GWD and glucosyl 6-phosphate residues are involved in a more strict control of the starch turn-over as compared to the C3-phosphate esters.

When considering the metabolic function(s) of the starch phosphorylation it should be noted that phosphorylation occurs both during net starch synthesis and degradation although the rates of phosphorylation are likely to be different (Nielsen et al. 1994; Ritte et al. 2004). It is, therefore, reasonable to assume that starch phosphorylation exerts an important role in the entire transitory starch metabolism rather than being functional only during the degrading process (and, consequently, the starch-related dinkines cannot, in a strict sense, be considered as ‘starch-degrading enzymes’).

Depending on the botanical source, the degree of starch phosphorylation varies strongly. In potato tuber starch approximately 0.1 to 0.5 % of the glucosyl residues are phosphorylated (Ritte et al. 2002) and this value is considered to be indicative of a high level of phosphorylation. By contrast, cereal starches contain a by far lower relative phosphate content that often is close to the limit of detection (approximately 0.002%; Glaring et al. 2006). These differences could, in principle, be due to different rates of phosphorylation, as catalyzed by the two-starch-related dinkines, and this assumption seems to be supported by the observation that, in general, starches of the B-type allomorph appear to have a higher degree of phosphorylation as compared to those of the A-type allomorph. If so, the dinkines may preferentially act on the B-type allomorph. Alternatively, the phosphorylation catalyzed by the two dinkines could be balanced by counteracting phosphatases, such as SEX4. This plastidial enzyme has been shown to act as a (phospho)glucan phosphatase that is involved in the leaf starch metabolism (Kötting et al. 2009). If antagonistic enzyme activities are taken into consideration, the actual level of starch phosphorylation is determined by the rate of both phosphorylation and the subsequent hydrolysis of phosphate esters and, consequently, does not necessarily reflect the action of the starch-related dinkines.

Recently, crystallized maltodextrins (MDcryst) have been prepared that, by using X-ray diffraction, were identified as being the B-type allomorph and possess a highly ordered structure (which exceeds that of native starch granules). MDcryst have been applied as a substrate for a recombinant GWD from Solanum tuberosum L. Using a carefully optimized assay, the rate of phosphorylation was by far higher than that observed with any other carbohydrate substrate, such as native starch granules or starch-derived polysaccharides. By contrast, solubilization by heat treatment of the MDcryst almost completely abolished the
activity of GWD. Phosphorylation resulted in the formation of singly, doubly and triply phosphorylated glucans and favoured the solubilisation of both neutral glucans and phosphoglucans (Hejazi et al. 2008). Recombinant PWD also phosphorylated MD\textsubscript{cryst} provided the crystalline maltodextrins had been prephosphorylated by GWD and were not solubilized by heat treatment (Hejazi et al. 2008).

Because of the high phosphorylation rates and the phosphorylation pattern obtained, MD\textsubscript{cryst} are a suitable model carbohydrate that mimics phosphorylation-relevant features of highly ordered regions within the native starch granule. It allows to study the action of the two starch-related dikinases and the transition of carbohydrates from the solid to the soluble state without any other starch-related enzyme being required.

Until now only the B-type allomorph of the MD\textsubscript{cryst} has been applied as substrate of the two dikinases. Using native starch granules as a target, the rates of phosphorylation as obtained with recombinant GWD varied largely within the B-type allomorph (Hejazi et al. 2008) and, therefore, it is reasonable to assume that additional but largely unknown features of the native starch granule also strongly affect the action of GWD. This implies that any preference or specificity of the starch-related dikinases for a given allomorph can be analyzed most convincingly if MD\textsubscript{cryst} preparations representing both the B- and the A-type allomorph are available.

In this communication we used two MD\textsubscript{cryst} preparations that are indiscernible in their oligoglucan patterns but differ in the physical arrangement of the double helices and represent the highly ordered A- or B-type allomorph. By using these two MD\textsubscript{cryst} preparations, we analyzed the action of the two starch-related dikinases. The size distribution of the MD\textsubscript{cryst} particles has been determined using the Coulter Counter and surface properties of both allomorphs were monitored by scanning electron microscopy. Thermal stability of the two allomorphs was analyzed by measuring the temperature dependency of light scattering. Finally, the phosphorylation-dependent solubilization of both allomorphs and the transition of (phospho)glucans into the soluble state have been studied.

Results

Physical and (physico)chemical properties of the A- and B-type allomorph of crystalline maltodextrins
In a previous study we used commercially available maltodextrins to prepare MD_{cryst} representing the B-type allomorph (Hejazi et al. 2008). However, we were unable to prepare any A-type MD_{cryst} when using the same starting material and following the procedure described by Gidley and Bulpin (1987). Therefore, no experiments could be designed that allowed to elucidate any possible allomorph preference of the two starch-related dikinases. As we assumed that a more homogeneous starting material could facilitate the crystallization of the two allomorphs, we established a procedure enabling us to select a relatively narrow size range of linear \(\alpha\)-glucans. Many commercially available MD are dominated by small size constituents (DP 1 to 4) that do not (co)crystallize. In addition, they usually contain medium size dextrins (whose DP range from 6 to approximately 40) and, as very minor constituents, larger glucans with a DP exceeding 40. In principle, complexity of a given MD preparation can be diminished by conventional fractionating techniques, such as the precipitation within a narrow range of ethanol-water mixtures. However, when applied in a preparative scale the efficiency of these techniques is often limited as less abundant larger dextrins tend to coprecipitate with highly abundant smaller ones.

To overcome these limitations, a two-step fractionation procedure was established that precedes the actual crystallization of the A- and B-type allomorph. It includes, firstly, the crystallization of a commercially available MD preparation that results in the B-type allomorph and, more importantly, quantitatively excludes maltodextrins with a DP of less than 5. Secondly, following solubilization of the crystallized dextrins by heat treatment they were subjected to a fractionated precipitation between 60 and 70 % [v/v] ethanol. Under the conditions established, precipitation with 60% [v/v] ethanol reliably removes higher molecular weight glucans but medium size maltodextrins are retained in solution even when performed in a preparative scale. Finally, the ethanol concentration is raised to 70% [v/v] and the MD that are insoluble between 60 and 70% [v/v] ethanol were used as starting material for either of the two crystallization procedures described by Gidley and Bulpin (1987). In the following, the crystallization starting with a 30% [w/v] MD solution at 4°C is designated as procedure I, the other one (50% [w/v]; 37°C) as procedure II.

The MD_{cryst} obtained by either procedure were characterized by various analytical techniques. The results are compiled in Fig. 1 and Table 1. Firstly, aliquots of the two MD_{cryst} suspensions were solubilized by heat treatment and the oligoglucan patterns were determined by HPAEC-PAD. For both preparations the MD patterns were indiscernible. They consist of DP ranging from 5 to approximately 30 (Fig. 1A). It should be noted that the sensitivity of the pulsed amperometric detection tends to decrease with increasing size of the carbohydrates.
and, therefore, the signal intensities do not strictly reflect the relative abundance of the various maltodextrins. Nevertheless, the conclusion is safe that the two crystallization procedures do not cause any noticeable chemical diversification of the MD_{cryst} preparations. Secondly, the size distribution of the two MD_{cryst} particles was determined using a Coulter Counter (Fig. 1B). The particles obtained by procedure I possessed a slightly smaller size and a relatively wider size distribution than those prepared by procedure II. For the former, the most frequently observed particle diameter was 7 to 8 µm whereas the corresponding value for the latter was 12 to 13 µm. For both MD_{cryst} preparations the maximum particle size was indiscernible. Importantly, both MD_{cryst} preparations are sufficiently stable when suspended in buffer and agitated using exactly the conditions as applied for the dikinase assays (see below). Stability was ensured by monitoring the size distribution following the resuspension of both MD_{cryst} preparations in the reaction buffer (lacking any recombinant dikinase) and continuous agitation (Fig. 1B).

Both the MD_{cryst} prepared either by procedure I or II were analyzed by X-ray diffraction using a NanoStar instrument that allows carbohydrate particles to be analyzed in a fully hydrated state (for details see Hejazi et al. 2008). For comparison, native starch granules from potato tubers (B-type) and from wild type maize kernels (A-type allomorph) were also analyzed using the same conditions. The X-ray diffraction patterns clearly show that the MD_{cryst} prepared by procedure I resembles native starch granules from potato tubers and represent the B-type allomorph. By contrast, the MD_{cryst} obtained by procedure II is clearly the A-type allomorph which is similar to the native starch from wild type maize kernels (Fig. 1C). We also isolated native starch from leaves of green house grown wild type maize plants and analyzed the transitory starch under exactly the same conditions. These maize starch particles exhibited the B-type allomorph as does potato leaf starch (data not shown). When applying the method of Nara and Komiya (1983), for both MD_{cryst} preparations a degree of crystallinity indiscernible from unity was observed. By contrast, the two native starch preparations possess a lower degree of order (Fig. 1C and Table 1). A similar observation had been made for a previously described MD_{cryst} preparation representing the B-type allomorph (Hejazi et al. 2008). However, the latter preparation contained a wider DP range covering values from 6 to approximately 40 (Hejazi et al. 2008).

Based on the X-ray diffraction patterns, the crystallite size of the two MD_{cryst} preparations and both native starch granules as well was determined using the Scherrer equation (Table 1). Both MD_{cryst} preparations possess a crystallite size larger than that of the two native starch granules.
Light microscopical analyses using polarized light are consistent with the data shown in Fig. 1B and 1C. Both the A-type (Fig. 1D) and the B-type (Fig. 1E) allomorph of the MD_{cryst} possess birefringence and exhibit a characteristic polarization cross. Size distribution of the A-type particles is more homogeneous than that of the B-type.

The thermal stability of the two MD_{cryst} preparations was studied in more detail by monitoring the temperature-dependent light-scattering. Under continuous stirring, a diluted suspension of the two MD_{cryst} preparations was subjected to a controlled heating (5°C per min) and scattering at 532 nm was continuously monitored. During solubilization of the MD_{cryst} particles, the light scattering decreases by several orders of magnitude (Fig. 1F) and, therefore, a temperature can defined that reflects 50% loss of scattering (T_{50}). For the A- and B-type allomorph, the T_{50} value is 79 and 61°C, respectively. It should be noted that similar differences have been observed for native starch granules: B-type granules tend to be disrupted at lower temperatures as compared to A-type granules (Bograchev et al. 1997).

When analysing several independently prepared A-type MD_{cryst} we consistently observed an increase in scattering that takes place in a relatively narrow range of elevated temperatures at or immediately before the onset of the disintegration of the particles and is maximal at approximately 75°C (Fig. 1F). As revealed by light microscopic evaluation, this phenomenon (that never has been observed with the B-type MD_{cryst} allomorph) is due to a significant degree of aggregation of the A-type MD_{cryst} particles at higher temperatures (data not shown). Presumably, another observation is more relevant for the enzymological studies described below: The graphs shown in Fig. 1F clearly rule out that the preparation of the A-type allomorph is, to any noticeable extent, cross-contaminated with that of the B-allomorph and vice versa. The same conclusion is derived from the X-ray patterns (Fig. 1C). However, due to the largely different T_{50} values, the temperature-dependent light scattering permits a more sensitive test for the homogeneity of each of the two crystalline MD preparations. Several independent preparations of each allomorph were obtained by using the two crystallization procedures. The chemical and physical features of the various MD_{cryst} preparations were very similar to those shown in Fig. 1 and Table 1.

In summary, the data shown in Fig. 1 and Table 1 clearly demonstrate that the two MD_{cryst} preparations possess indiscernible chemical features but differ in structural properties, i.e. the physical arrangement of double helices. Therefore, in biochemical studies they can be used as model carbohydrate particles that mimic structural features of native starch granules representing either the A- or the B-type allomorph.
Recombinant stGWD phosphorylates both the A- and the B-type of crystalline maltodextrins

The two MD_{cryst} allomorphs were used to determine the initial rates of the C6-phosphorylation as catalyzed by recombinant stGWD. The GWD standard assay was applied but varying amounts of the A- or B-type allomorph (up to 12 mg each) were added. Reaction mixtures were incubated for 10 min under continuous agitation. Subsequently, the MD_{cryst} preparations were solubilized by heat treatment and the total $^{33}$P content of the (phospho)maltodextrins was monitored by phosphor imaging following thin-layer chromatography. The assay conditions (such as the continuous agitation in the reaction buffer) did not alter the size distribution of the MD_{cryst} (cf. Fig. 1B). Recombinant stGWD did phosphorylate both the A- and the B-type allomorph and the phosphorylation rates obtained were similar. For both the A- and the B-type allomorph, the rates increased with increasing amounts of MD_{cryst} but saturating levels of the maltodextrin particles were not achieved (Fig. 2). When the stGWD-catalyzed reaction was terminated after 5 min using otherwise identical assay conditions, essentially the same results were obtained (data not shown).

In the next series of experiments, the $^{33}$P content of the insoluble and the soluble (phospho)glucans was determined separately. At 10 min intervals, aliquots of the reaction mixtures were withdrawn and centrifuged. The supernatant was immediately analyzed by thin layer chromatography and phosphor imaging. The pellet was washed with water, solubilized by heat treatment and then processed as described before. The total reaction time was extended to 60 min using otherwise the conditions defined for GWD standard assay. For both the A- and the B-type allomorph, the ratio between the insoluble and the soluble $^{33}$P-label changed during incubation: During the first 30 min, the majority of the radioactivity was recovered in the insoluble state but during the second half of the incubation period the $^{33}$P content of the soluble fraction increased more strongly than that of the pellet (Fig. 3A and B). These data clearly show that the GWD-mediated phosphorylation of both the A- and the B-type allomorph of MD_{cryst} initiates solubilization of the (phospho)glucans.

For a more detailed analysis of the GWD action on both allomorph types, the phosphorylation patterns in the soluble and insoluble (phospho)glucans were determined. In addition, the total amount of the solubilized glucosyl residues was determined enzymatically following acid hydrolysis. After 30 or 60 min incubation, aliquots of the soluble (phospho)glucans were subjected to HPAEC-PAD. Following the separation into singly, doubly and triply...
phosphorylated glucans, the label of the (phospho)glucans was quantified by liquid scintillation counting. Likewise, insoluble (phosphor)glucans were solubilized by heating. Subsequently, they were chromatographed and quantified as described above. The degree of phosphorylation was confirmed by MALDI-MS (for details see Hejazi et al. 2008). The data obtained for 60 min reaction period are compiled in Table 2. (Phospho)glucans released from the both the A- and B-type MDcryst consisted mostly of monophosphorylated glucans but doubly phosphorylated (phospho)glucans and, although as a very minor constituent, triply phosphorylated were clearly detectable. For the A-type-derived soluble (phospho)glucans, the abundance of the monophosphorylated maltodextrins were slightly higher than that for the B-type-derived compounds. In the insoluble fraction of both the A- and the B-type allomorph, singly phosphorylated dextrins were dominant whereas incorporation into the doubly phosphorylated compounds accounted for lesser than 1% of the total label (Table 2). For both allomorphs, triply phosphorylated glucans were below the limit of detection. The phosphorylation patterns obtained after a phosphorylation period of 30 min are similar to those shown in Table 2 but, as expected, the incorporation of phosphate is lower (data not shown).

During the GWD action on both the A- and the B-type allomorph, neutral maltodextrins were released in addition to the phosphoglucans but quantitatively the two allomorphs differed. From the B-type fourfold more neutral maltodextrins were converted into the soluble state as compared to the A-type allomorph (Table 2). Similarly, more neutral maltodextrins were detected during separation by HPAEC-PAD (data not shown).

The experiments described above were restricted to the initiation of the GWD-dependent solubilization process and, therefore, it remained uncertain whether or not the GWD action results in an almost complete solubilization of both allomorphs. For the following reasons this is an important point to be clarified: Physical properties, as revealed by X-ray diffraction and temperature-dependent light scattering, reflect structural features of the entire MDcryst particles and, therefore, they do not exclude the possibility that the surface of the particles may possess structural features deviating from those of the interior parts of MDcryst. If so, these surface-related features are difficult to detect as they contribute little to the signal intensities derived from the entire particles. However, they could be essential for biochemical reactions, such as the action of GWD. Therefore, we tested for a massive solubilization of MDcryst using unlabeled ATP and monitored the release of glucans into the soluble phase. Over an extended period of time, the reaction mixtures were separated into an insoluble and a soluble fraction. The latter was subjected to an exhaustive acid hydrolysis and the glucose formed was
quantified by a coupled enzymatic assay. Preliminary experiments clearly indicated that
during prolonged incubation the activity of recombinant stGWD is not retained (see below).
Therefore, at each time point the reaction mixtures were centrifuged and, following the
removal of the supernatant, the pelleted MD_{cryst} were resuspended in a freshly prepared
incubation buffer having the same composition as the previous one but containing a new
sample of recombinant stGWD. The volume of the reaction mixtures remained unchanged
over the entire incubation period. As a control samples were treated identically but GWD was
omitted.

During 12 h of GWD action, the majority of both the A- and the B-type allomorph the MD_{cryst}
was converted into the soluble state. This conversion was strictly dependent on the presents of
GWD (data not shown). Because of the massive phosphorylation-dependent solubilization, it
can be excluded that the action of GWD is restricted to the initial surface area of either of the
MD_{cryst} preparations (Table 3).

The morphology of the A- and B-type MD_{cryst} particles during the action of the two
starch-related dikinases

This conclusion is supported by scanning electron microscopical analyses of the
A- and B-type allomorph of the MD_{cryst}. Analyses were performed before and during the
action of GWD. Consistent with the size distribution (as revealed by the Coulter Counter) and
the light-microscopic evaluation (Fig. 1) both allomorphs occurred as a relatively
homogeneous population of particles but the morphology of both types differed. A-type
particles possess a more complex particle structure and a more uneven surface (Fig. 4 A and
B). By contrast, the B-type particles are more evenly formed and have a smooth surface (Fig.
4 C and D). During the action of stGWD the entire structure of both types of particles is
altered but, depending on the allomorph, the alterations differ. In the entire population of the
type A-allomorph particles the surface is gradually smoothened (Fig. 4 E and F). This clearly
indicates that the stGWD-mediated solubilization is not restricted to distinct areas of the
particle surface but rather takes place at the entire surface of the particles and proceeds
towards the interior part of the particles. By contrast, the GWD action on B-type allomorph
particles resulted in structural diversification that takes place at the particle surface. The
typical heterogeneity of the surface of the MD particles consists of structures that are
vertically oriented towards the particle surface. These structures that are never observed with
the A-type allomorph are shown in Fig. 4 G and H. It should be noted that these alterations require both a catalytically active stGWD and the presence of ATP. When either ATP was omitted or the recombinant GWD had been inactivated by heat treatment the structure of both types of particles remained unchanged during the entire incubation period (data not shown). Based on kinetic studies it appears that the structural alterations as observed with the B-type allomorph reflect transitory states within the solubilization process (data not shown). Nevertheless, the phosphorylation-dependent transition of crystalline maltodextrins to the soluble state occurs at the entire surface of both the A- and the B-type allomorph.

The activity of recombinant stGWD is inhibited by soluble maltodextrins

The decrease of the stGWD activity during the massive solubilization of MDcryst is due, at least in part, to an inhibitory effect that is exerted by soluble maltodextrins on the recombinant enzyme. This conclusion was reached in experiments in which a fixed amount of MDcryst (B-type allomorph) was incubated with a constant amount of stGWD and varying amounts of soluble maltodextrins. Two soluble maltodextrins were applied. First, soluble maltodextrins were obtained by heat treatment of MDcryst (B-type). These soluble maltodextrins possess exactly the same size distribution as the insoluble MDcryst acting as GWD substrate (see Fig. 1A). The other soluble MD applied was a commercial sample (ICN; see Materials and methods). The size distribution of this MD sample, as revealed by HPAEC-PAD, was dominated by DP 1 to 11 and, therefore, clearly deviated from that of the MDcryst-derived MD. The two MD preparations were similarly efficient in inhibiting stGWD despite the difference in size distribution. As a control, varying amounts of a commercially available sample of soluble dextrans from *Leuconostoc mesenteroides* were added to the reaction mixture. The dextran sample consists of α-1,6-linked oligoglucans the majority of which has a DP ranging from 1 to 17 (data not shown). The soluble dextrans exerted a far less inhibitory effect on GWD (Table 4).

The action of recombinant atPWD on both the A- and B-type allomorph of crystalline MD
The phosphorylation at the C3-position was studied by using recombinant atPWD (EC 2.7.9.5). Both the A- and the B-type allomorph of the MD\textsubscript{cryst} served as carbohydrate substrates. Preliminary \emph{in vitro} experiments clearly indicated that any PWD activity requires a prephosphorylation of both allomorphs by GWD. In order to selectively monitor the esterification catalyzed by PWD, prephosphorylation was performed using unlabeled ATP and \([\beta^{33}\text{P}]\text{ATP}\) was exclusively used for the subsequent phosphorylation by atPWD. In order to vary the degree of prephosphorylation, both the A- and the B-type crystalline maltodextrins were prephosphorylated for varying periods of time. At intervals, stGWD was inactivated by adding of SDS. Subsequently, the prephosphorylated crystalline maltodextrins were incubated for 10 min with recombinant atPWD (250 ng each) and \([\beta^{33}\text{P}]\text{ATP}\). Following the inactivation of atPWD by adding EDTA (final concentration) the reaction mixtures were separated into a soluble and insoluble fraction by centrifugation. Both fractions were heated and aliquots of the two solutions were analyzed by phosphor imaging following thin-layer chromatography.

In both the A- and the B-type allomorph of the MD\textsubscript{cryst}, the activity of PWD increased with increasing prephosphorylation catalyzed by stGWD (i.e. with the length of the prephosphorylation period). However, in the A-type allomorph, approximately 80% of the PWD-catalyzed \(^{33}\text{P}\) incorporation remained in the insoluble state. By contrast, phosphorylation by atPWD of the prephosphorylated B-type allomorph resulted in solubilization of approximately two thirds of the \(^{33}\text{P}\)-label in the soluble state (Fig. 5).

For a more detailed analysis of the distribution of the \(^{33}\text{P}\)-label within the soluble and pelletable maltodextrins, in each of the four (phospho)maltodextrin samples mono-, di- and triply phosphorylated \(\alpha\)-glucans were separated and the \(^{33}\text{P}\) contents were quantified and, in addition, the total amount of glucosyl residues of the soluble maltodextrins was determined enzymatically. For monitoring the \(^{33}\text{P}\) distribution neutral maltodextrins were separated from (phospho)glucans by HPAEC-PAD. The (phospho)glucans were separated into mono-, doubly and triply phosphorylated maltodextrins as described previously (Hejazi \textit{et al}. 2008). The data are compiled in Table 5.

Four observations can be made: Firstly, due to the PWD action on prephosphorylated B-type MD\textsubscript{cryst} approximately threefold more glucosyl residues are converted into the soluble form as compared to the A-type allomorph. Secondly, the ratio of the \(^{33}\text{P}\) labeling of the soluble and insoluble MD differs depending on the A- or B-type allomorph. The soluble maltodextrins derived from the A-type MD\textsubscript{cryst} contain less than 10% of the \(^{33}\text{P}\)-labeled glucosyl-3-phosphate residues whereas approximately 90% of the \(^{33}\text{P}\)-label is recovered in the pellet.
fraction. By contrast, following the PWD action on the B-type allomorph most of the $^{33}$P label is observed in MD$_{sol}$. Thirdly, the phosphorylation patterns of the soluble phosphoglucans differ depending upon the A- and B-type allomorph: In the A-type derived MD$_{sol}$ monophosphorylated glucans are predominant. By contrast, the soluble (phospho)glucans derived from the B-type allomorph were dominated by triply phosphorylated glucans accounting for 52% of the total label of MD$_{sol}$. Fourthly, in the A-type derived insoluble MD more than 70% of the phosphoglucans exist as mono- and triply phosphorylated dextrins whereas in the B-type derived pelletable (phospho)glucans monophosphorylated glucans are more prominent (Table 5).

It is important to note that in all samples analyzed a significant proportion of the $^{33}$P-label was recovered as monophosphorylated chains. This implies that atPWD, although its catalytic activity depends on a prephosphorylation of MD$_{cryst}$ by GWD, is capable to utilize neutral α-glucan chains as a substrate and, at the level of the individual glucan chains, activity of atPWD is not restricted to (phospho)glucans. Thus, when considering the interaction of stGWD and atPWD these data strongly suggest that the preceding GWD-mediated phosphorylation is essential for PWD as it alters physical properties of MD$_{cryst}$ such as the arrangement of (double) helices. For the PWD-mediated phosphorylation the alterations of physical properties of MD$_{cryst}$ appear to be by far more important than any chemical modification of single α-glucan chains.

Furthermore it should be noted that both the doubly and triply phosphorylated glucan chains, as separated by HPAEC-PAD, may consist of a mixture of chemically heterogeneous compounds. In principle, doubly phosphorylated α-glucan chains can include phosphoglucans that carry a phosphate ester both in the C6- and the C3 position or, alternatively, two C3 esters. If phosphate esters occur both on the C6 and the C3 position the latter is labeled with $^{33}$P. If, however, the phosphoglucan chain contains two C3 phosphate esters at least one of both needs carry $^{33}$P. Similarly, the triply phosphorylated glucan chains, in principle, consist of a mixture of up to three phosphoglucan chains that contain at least one $^{33}$P-labeled C3 ester but up to two C6-bound phosphates. Thus, the triply phosphorylated glucans may contain three C3 or two C3 plus one C6 or one C3 and two C6 phosphate esters per molecule.

Discussion

In this communication we have used an *in vitro* assay in order to analyse the action of two starch-related dikinases. The assay comprises (at least) three distinct phases. One phase is
soluble and the products of the catalysis of GWD (or of PWD) finally enter this phase. The second phase is the surface of the crystalline maltodextrins and in this phase the GWD-mediated α-glucan phosphorylation takes place. As shown in Fig. 3 and 4, the second phase is dynamic and gradually moves towards the centre of the crystalline particles as the phosphorylation-dependent solubilization proceeds. The third phase (the size of which gradually decreases) is the interior space of the crystalline particles. The α-glucans in this phase are not immediately accessible to the dikinase and, therefore, they do not participate in the phosphorylation (and the subsequent solubilization) unless the surface of the particles has progressively moved.

As both the phosphorylation and the solubilization of the crystalline maltodextrins require the action of only one enzyme (i.e. GWD) it is likely that, in a spontaneous process, the phosphate esters formed locally increase the hydration of the (phospho)glucan chains and thereby favour solubilization at room temperature. Heat treatment results in a complete solubilization of both allomorphs without any enzymatic action being required.

In this study both the A- and the B-type allomorph of crystalline maltodextrins were included. Both allomorphs were derived from the same maltodextrin preparation and, following crystallization, their maltodextrin patterns are indiscernible. This implies that (at least when using a relatively narrow MD size distribution) the two crystallization procedures chosen to not exert any size-dependent selectively. Consequently, the two maltodextrins allowed us to experimentally test for any allomorph specificity or preference of GWD.

In in vitro experiments stGWD phosphorylates both the A- and the B-type allomorph of MDcryst and the rates obtained with both allomorphs exceed those obtained with native starch granules (Hejazi et al. 2008). In fact, stGWD appears to phosphorylate the A-type even with a higher rate than the B-type (Fig. 2). For a detailed evaluation of the two rates obtained, the physical features of the two crystalline maltodextrin preparations should be compared (Fig. 1). Both particle preparations possess a similar but not identical size distribution. Therefore, equal amounts of both crystalline particles added to a reaction mixture are expected to result in a similar but not necessarily equal total surface area. As a further complication, the surface of the A-type particles is less even than that of the B-type (Fig. 4). Both with the A- and the B-type allomorph no substrate saturation has been achieved (Fig. 2). Therefore, the initial phosphorylation rates are measured under similar but not necessarily identical conditions. The recombinant stGWD applied in the in vitro assays is derived from Solanum tuberosum L. which synthesizes the B-type allomorph both as transitory and reserve starch (Hejazi et al. 2008) and, therefore, under in vivo conditions the enzyme is unlikely to ever act on an A-type
allomorph starch. The high activity of stGWD observed with both allomorphs of MD$_{cryst}$ clearly rules out any obvious allomorph specificity or preference exerted by GWD phosphorylation. By contrast, the total amount of maltodextrins that are solubilized due the action of GWD the allomorphs differ significantly. Solubilization is fourfold higher with the B-type allomorph as compared to the A-type. Thus it appears that the phosphorylation-dependent transition into the soluble phase is more efficient with the B-type allomorph than with the A-type (Table 2 and 3). Presumably, this difference is reflected by the lower thermal stability of the B-type allomorph (cf. Fig. 1).

Both the A- and the B-type allomorph of native starch granules contain highly ordered and less ordered regions as well. Probably, the crystalline maltodextrins that have been used in this study are suitable model substances only if those metabolic processes are to be studied that take place at the ordered regions of the granule. By contrast, the biochemistry of the less ordered regions appears to be different and may even not to include the action of GWD. However, even the highly ordered areas of the native starch granules differ in an important feature from the crystalline maltodextrins. Phosphorylation and hydration of the chains within the highly ordered regions of the starch granules do not result in a transition of the (phospho)glucans into the soluble phase as they remain covalently bound via $\alpha$-1,6-interglucose bonds and, therefore, further enzyme activities are required for solubilization.

The data presented in this *in vitro* study clearly demonstrate that a efficient glucan phosphorylation catalyzed by GWD (and by PWD as well) does not require occurrence or vicinity of any $\alpha$-1,6-linterglucose bonds. Obviously, these results do not necessarily exclude the possibility that, during native starch granule biosynthesis, the occurrence of branching points might be affected by phosphorylated sites.

The carbohydrate substrate of GWD appears to be defined by physical properties rather than by chemical features. As GWD phosphorylates both the A- and the B-type allomorph with similar rates it is tempting to speculate that the vicinity of two $\alpha$-glucan double helices may be an essential structural feature of the carbohydrate substrate of GWD.

The data shown in Fig. 2 are consistent with *in vitro* experiments in which native starch granules from *Chlamydomonas reinhardtii* were phosphorylated by stGWD (data now shown). Both under normal photoautotrophic growth conditions and under mineral deficiency (N- or P deficiency) the starch formed by *Chlamydomonas* is the A-type allomorph (data not shown). Furthermore, the genome of *Chlamydomonas* contains at least 4 genes that putatively encode starch-related dikinases. It is, therefore, reasonable to assume that initial steps of the mobilization of the highly ordered regions of the native starch granules are essentially the
same, irrespective of the A- or B-type allomorph but the processes downstream the phosphorylation at the C6 position differ between both allomorphs. Furthermore, the low stationary level of phosphate esters that is frequently observed in A-type starch granules (0.002%) is likely to be caused by the regulation of the starch-related dikinases and/or that of counteracting enzymes, such as the SEX4 laforin-type phosphatase (Kötting et al. 2009).

The data presented in Table 4 clearly indicate that the recombinant stGWD is inhibited by soluble maltodextrins. This effect is, however, difficult to be analyzed in more detail as the two soluble maltodextrins preparations used consist of a complex mixture of maltodextrins. Therefore, it is not inconceivable that, depending on the size of the maltodextrins, the inhibitory effect varies and some of the constituents of the soluble maltodextrins may even be ineffective.

Recombinant atPWD did phosphorylate both the A- and the B−type allomorph of MD_cry. Provided a GWD-mediated prephosphorylation at the C6-position of some glucosyl residues (Table 5) and, therefore, PWD acts downstream of GWD. A similar conclusion has been reached by in vitro assays using native starch granules (Baunsgaard et al. 2005; Kötting et al. 2005). However, the data presented in Table 5, provide a mechanistical explanation of the strict dependency of PWD on a preceding phosphorylation. Due to the atPWD action, monophosphorylated α-glucans are formed in addition to doubly and triply phosphorylated MD. This implies that atPWD acts on neutral glucans and strongly suggests that the preceding phosphorylation at the C6-position generates the carbohydrate substrate of atPWD by altering physical rather than chemical features of MD_cry. Therefore, we propose that the actual carbohydrate substrate of PWD is a transition state from a highly ordered to a less ordered structure which is defined by the physical order of glucan chains and/or glucan double helices (Fettke et al. 2009). This conclusion is supported by the high rate of atPWD-mediated phosphorylation that has been observed with native starch granules from some mutant plants even without any preceding GWD-mediated phosphorylation (Fettke et al. 2009). Presumably in these cases starch-related mutations results in structural alterations at the starch granule surface that are similar to those achieved in wild type starches as a consequence of the GWD-mediated phosphorylation.

Unfortunately, the transition state that represents the carbohydrate substrate of PWD is difficult to be analyzed. In the case of the B-type allomorph of MD_cry it is tempting to speculate that the structural changes that are visible during the stGWD-mediated
solubilization are related to this transition state (Fig. 4 G and H) but this speculation is
difficult to prove or disprove experimentally.

Materials and methods

Plant materials

Wild type maize plants were grown in a local green house.

Chemicals and enzymes

$[^33P] \text{ATP}$ (3,000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig,
Germany). Maltodextrins were obtained either from Sigma-Aldrich (Munich, Germany;
Product No. EC 232-940-4) or from ICN Biomedicals GmbH (Dextrin Maltose Product No.
960048; Eschwege, Germany). Dextrans from \textit{Leuconostoc mesenteroides} were purchased
from Fluka (Product No. 2326775; Hamburg, Germany). Native starch granules from maize
kernels (Product No. EC 232-679-6) were obtained from Sigma-Aldrich (Munich, Germany)
and native potato tuber starch (Product No. 102954) was purchased from ICN. Polyglucans
were quantified using the starch kit from Roche Diagnostics (Mannheim, Germany). PEI-
Cellulose F thin layer plates were purchased from Merck (Darmstadt, Germany).
Recombinant GWD from \textit{Solanum tuberosum} L. and PWD from \textit{Arabidopsis thaliana} L.
(At5g26570) were heterologously expressed and purified as previously described (Ritte \textit{et al.}

Fractionation and crystallization of maltodextrins

Fractionation. For the crystallization procedure I and II described below the same
maltodextrin fractionation method was applied. Commercial maltodextrins (Sigma-Aldrich,
see above) were freed from low molecular weight constituents by crystallization according to
Hejazi \textit{et al.} (2008). The resulting B-type crystalline maltodextrins ($\text{MD}_{\text{cryst}}$) were
resuspended in water to give a final concentration of 5% [w/v], dissolved by heating (15 min
at 95°C) and were then cooled to room temperature (RT). Subsequently, ethanol was slowly
added to give a final concentration of 60% [v/v]. The precipitated maltodextrins were
removed by centrifugation (10 min at 8,000 g; RT) and were discharged. In the supernatant the ethanol concentration was slowly increased to 70% [v/v] and the precipitated maltodextrins were collected by centrifugation (as above). The pelleted maltodextrins were washed five times with ice-cold 70% [v/v] ethanol and were then dried by lyophilization. Finally, the dried maltodextrins (MD) were dissolved in water by heating (see below) and were then used for the preparation of A- and B-type crystalline maltodextrins.

Crystallization. Procedure I: A solution containing 30% [w/v] MD was prepared by heating (15 min at 120°C using an autoclave) and was then kept at 4°C over night. Crystalline material was collected by centrifugation (10 min at 800 x g; RT). The pelleted maltodextrins were washed five times with water (centrifugation as above). Finally, sodium acid was added (final concentration 0.05%) and the suspension was stored at 4°C until use. Procedure II: A solution containing 50% [w/v] MD was prepared by heating (as above) and was then kept over night at 30°C. Crystalline maltodextrins were collected, washed by centrifugation and stored as in procedure I. Lyophilization of the crystalline maltodextrins tended to diminish the stability of the particles. Type B-allomorph MD\textsubscript{cryst} particles were more labile than the A-type allomorph.

Assay of the activity of recombinant GWD and PWD

GWD (standard assay). This assay was used to measure the initial rates of the GWD-catalyzed glucan phosphorylation. Except were stated reaction time was restricted to 5 or 10 min. Within 10 min, the rate of phosphorylation was constant. In a final volume of 50 µL, the standard reaction mixture contained 4 mg A- or B-type MD (as stated), reaction buffer (consisting of 50 mM Hapes-KOH pH 7.5, 6 mM MgCl\textsubscript{2}, 1 mM EDTA, 2 mM dithioerythritol [DTE]), 20 µg BSA, 25 µM ATP including 2 µCi [\(\beta\)-\textsuperscript{33}P]ATP. As a control, GWD was inactivated by heat treatment prior to the addition to the assay mixture. Phosphorylation was started by the addition of recombinant stGWD (250 ng except where stated). The reaction mixture was incubated for 10 min (except where stated) at 30°C under continuous agitation. Incubation was terminated by heating for 5 min at 95°C under agitation and centrifugation (10 min at 10,000 x g). Subsequently, 2 µL of the supernatant was spotted onto PEI-cellulose plates and subjected to thin layer chromatography (see above). In some experiments the standard conditions were modified as stated.

GWD (modified assays). In some experiments the standard assay was modified. For scanning electron microscopy, both the A- and the B-type MD were incubated with
recombinant stGWD. In a final volume of 50 µL, the reaction mixture contained A- or B-type MD\textsubscript{cryst} (100 µg each), reaction buffer (as in the standard assay), 50 µM unlabeled ATP, 400 µg mL\textsuperscript{-1} BSA, and 100 µg recombinant stGWD. As controls, either ATP was omitted (but functional stGWD was added) or heat-inactivated stGWD was added (but ATP was included). The total incubation time of the reaction mixtures was 3 h (30°C under continuous agitation). However, at 60 min intervals the entire reaction mixtures were centrifuged (5 min at 10,000 x g; RT) and the pelleted MD\textsubscript{cryst} were resuspended in a freshly prepared reaction mixture including freshly diluted stGWD. Finally, the reaction was terminated by adding SDS (final concentration 2% [w/v] and centrifugation (as above). For massive GWD-mediated solubilization of MD\textsubscript{cryst}, the same assay was applied, except the amount of crystallized maltodextrin and stGWD was 40 µg, respectively. At intervals, the soluble and the insoluble maltodextrins were separated by centrifugation for 5 min at RT. The pelleted maltodextrins were treated as above and the soluble maltodextrins were hydrolyzed in 0.7 N HCl for 2 h at 95°C. Following neutralization with 0.7 N NaOH, glucose was quantified enzymatically using the starch kit.

**PWD (standard assay).** PWD activity was measured using A- or B-type MD following a prephosphorylation using unlabeled ATP and GWD. Prephosphorylation was performed using the assay mixture described above except that [\(\beta\textsuperscript{-33P}\)]ATP was omitted and the time of the prephosphorylation varied as stated. Prephosphorylation was started by the addition of 250 ng recombinant stGWD. Following incubation (as indicated) prephosphorylation was terminated by adding SDS to give a final concentration of 2% [v/v] and the resulting mixture was centrifuged (10 min at 10,000 x g). The insoluble prephosphorylated MD\textsubscript{cryst} was freed from SDS by washing with water (five times, 10 min each). Subsequently, aliquots (4 mg each) of the prephosphorylated (but unlabeled) MD\textsubscript{cryst} were resuspended in the reaction mixture described above (including 2 µCi [\(\beta\textsuperscript{-33P}\)] ATP; final volume 50 µL) and used as substrate for PWD. Labeling was started by adding recombinant atPWD (250 ng). A reaction mixture containing heat-inactivated atPWD served as a control. Reaction mixtures were incubated for 5 or 10 min at 30°C under continuous agitation and were then inactivated by adding a final concentration of 20 mM EDTA [w/v]. Samples were separated into a soluble and insoluble fraction by centrifugation (10 min at 10,000 x g) and the insoluble fraction was resuspended in 50 µl water. Both fractions were heated for 5 min at 95°C and 2 µL of the solution was spotted onto PEI-cellulose plates and subjected to thin layer chromatography (see above).
Analytical techniques

**Light microscopy.** Crystalline MD were analyzed by polarization light microscopy using an AX 70 microscope from Olympus (Hamburg, Germany).

**X-ray diffraction.** X-ray diffraction analyses were performed with MD\textsubscript{cryst} and native starch granules in a fully hydrated state using a NanoStar (Hejazi \textit{et al.} 2008). Crystallite size was quantified according to Scherrer equation.

**Thin-layer chromatography.** Neutral and phosphorylated glucans were separated by thin-layer chromatography using PEI cellulose plates according to Hejazi \textit{et al.} (2008). Following chromatography, radioactivity was quantified using a phosphor imager (Hejazi \textit{et al.} 2008).

**Protein quantification.** Buffer-soluble proteins were quantified using the microversion of the BIO-RAD protein assay kit according to Bradford (1976).

**HPAEC-PAD analysis.** Neutral and phosphorylated maltodextrins were analyzed using a HPAEC-PAD device (DX-600 system, Dionex, Idstein, Germany) and a CarboPac PA 100 or PA 1 column. Prior to sample application, the column was equilibrated for 10 min with a mixture of 5 mM sodium acetate and 100 mM NaOH. Following sample application maltodextrins were eluted by using a linear sodium acetate gradient (1 mL min\(^{-1}\); 5 to 500 mM) dissolved in 100 mM NaOH. Eluate fractions (0.5 mL each) were collected and each fraction was mixed with 3 mL scintillation cocktail. Radioactivity was monitored using a liquid scintillation counter (for details see Hejazi \textit{et al.} 2008).

**Coulter Counter.** Size distribution of the crystalline maltodextrins was determined using a Coulter Counter Multisizer 3 using a 100 µm aperture tube (range 2-60 µm; Beckman, Krefeld, Germany). For measurements, 150 µg of crystallized maltodextrins were resuspended in 75 mL Coulter ISOTON II Diluent (Beckman).

**Mass spectrometry.** Neutral and phosphorylated maltodextrins were separated by chromatography on graphitized carbon black column and mass spectrometry was performed using a Bruker Reflex MALDI-TOF (Bruker Daltonik, Bremen, Germany) in the positive-ion mode (Hejazi \textit{et al.} 2008).

**Light scattering experiments.** The relative changes in the light scattering intensities of suspensions of both the A- and B-type allomorph were monitored at an angle of 90° using the fluorescence spectrometer Fluorolog-3 (Horiba Jobin Yvon GmbH, Germany). The wavelengths of both the excitation and emission monochromators were set at 532 nm. Measurements were performed using a 1 cm fluorescence cell (Hellma, Müllheim, Germany) placed into a temperature-controlled sample holder equipped with peltier elements. To ensure
homogeneity, the suspension was continuously stirred. The temperature of the suspension increased with a rate of 5°C per min. To avoid multiple scattering, the crystalline maltodextrins particle suspensions were diluted with water to give an extinction at 532 nm not exceeding 0.7.

**Scanning electron microscopy.** Following three steps of washing and centrifugation (as above) the pelleted crystalline MD were resuspended in water and were lyophilized. Specimen were coated with gold and analyzed by scanning electron microscopy using a (QuantaTM, Philips, Hamburg, Germany).

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**References:**


Fig. 1. Physical and physicochemical properties of the A- and B-type allomorph of MD\textsubscript{cryst}.

A. MD patterns of MD\textsubscript{cryst} of the B-type (procedure I) and the A-type (procedure II) allomorph as revealed by HPAEC-PAD. Aliquots of both particle suspensions were solubilized by heat treatment and equal amounts (30 µg glucosyl residues each) were applied to a PA100 column. In the eluate carbohydrates were monitored by pulsed amperometric detection. Signal intensities (relative units but equal sensitivity) are plotted against the elution time. DP6 and DP15: MD having a DP of 6 and 15, respectively.

B. Size distribution of the A- and B-type allomorph of MD\textsubscript{cryst} as revealed by Coulter Counter. For both particle preparations, size distribution was monitored before (solid line) and after incubation for 10 min (30°C) in the absence of GWD but in the presence of the reaction buffer under continuous agitation (broken line).

C. X-ray diffraction patterns of crystalline maltodextrins obtained by procedure I (B-Type) and procedure II (A-Type) as determined by using a NanoStar instrument. For comparison, native starch granules from kernels of wild type \textit{Zea mays} L. (Maize) or from tubers of \textit{Solanum tuberosum} L. (Potato) were analyzed under identical conditions.

D and E. Polarization light microscopic evaluation of A-type (D) and B-type (E) MD\textsubscript{cryst}. magnification: 600x

F. Temperature-dependent light scattering of A- or B-type MD\textsubscript{cryst}. Light scattering at 532 nm (relative units) is plotted against the temperature.

Fig. 2. The action of recombinant stGWD on A-type or B-type crystalline maltodextrins. The specific enzyme activity was determined by the GWD standard assay using 10 min incubation. Reaction was terminated by heating and aliquots of the entire reaction mixture were analyzed by TLC. Total $^{33}$P incorporation into the maltodextrins was quantified by phosphor imaging. The specific activity of stGWD is plotted against the amount of crystalline maltodextrins applied to the respective assay mixture. Open symbols: A-type allomorph; closed symbols: B-type allomorph.

Fig. 3. stGWD-dependent $^{33}$P incorporation into soluble or insoluble MD derived from the A-type (A) or the B-type (B) allomorph of MD\textsubscript{cryst}. The GWD standard assay was used except the incubation time was extended to 60 min. At intervals, the soluble and insoluble
matodextrins were separated by centrifugation for 10 min at RT. The pellets were washed twice with water and dissolved in water. Following solubilization (5 min at 95°C), aliquots of both fractions were analyzed by TLC and phosphor imaging. Closed symbols: $^{33}$P content of the pelleted (phospho)glucans; open symbols: $^{33}$P content of the soluble (phospho)glucans.

**Fig. 4.** Scanning electron micrographs of both the A- and the B-type allomorph of MD$_{cryst}$ without and following the stGWD-mediated phosphorylation. A and B: A-type allomorph MD$_{cryst}$. C and D: B-type allomorph. E and F: A-type allomorph following 3 h phosphorylation by stGWD. G and F: B-type allomorph following 3 h phosphorylation by stGWD. In bar is equivalent to 20 µm (A, C, E, G) or rather 10 µm (B, D, F, H).

**Fig. 5.** The action of recombinant atPWD on prephosphorylated A- and B-type crystalline maltodextrins. A-type (A) or B-type (B) allomorphs of crystalline maltodextrins (4 mg each) were prephosphorylated with recombinant stGWD (250 ng each) using unlabeled ATP. At intervals the prephosphorylation was terminated by SDS. The pelletable prephosphorylated maltodextrins were then incubated with recombinant stPWD (250 ng each) and $[\beta^{33}$P]ATP. Following 10 min of incubation the reaction mixture was separated into a pellet and a supernatant by centrifugation (10 min at RT). The $^{33}$P content of both the soluble and insoluble fractions was determined by thin-layer chromatography and phosphor imaging.
Table 1: Structural features of crystalline A- and B-type maltodextrins and of native starch granules as revealed by X-ray diffraction analysis.

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<tr>
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<th>Degree of Crystallinity</th>
<th>Crystallite Size [nm]</th>
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<tr>
<td>Potato Tuber</td>
<td>0.78</td>
<td>9.8</td>
</tr>
<tr>
<td>Maize Kernel</td>
<td>0.52</td>
<td>8.8</td>
</tr>
<tr>
<td>A-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td>1</td>
<td>15.2</td>
</tr>
<tr>
<td>B-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td>1</td>
<td>18.5</td>
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Table 2: GWD-mediated phosphorylation and solubilization of crystalline A- and B-type maltodextrins. The patterns of mono-, di- and triply phosphorylated α-glucans in the soluble and insoluble state. Four mg of A- or B-type maltodextrins were incubated with GWD for 1 h at 30°C under continuous agitation. The samples were separated into soluble and insoluble fractions by centrifugation for 10 min at RT. The pellets were resuspended in water using the equal volume than that of the respective supernatant. Following solubilization for 10 min at 95°C samples were passed through a 10 kDa membrane filter and the resulting filtrate was loaded onto HPAEC column. Eluting compounds were detected by pulsed amperometry. 0.5 mL fractions were collected, mixed with 3 mL scintillation cocktail, and the radioactivity was monitored using a liquid scintillation counter. Radioactivity of the various phosphoglucons is given as % of the total $^{33}$P content. Total glucosyl residues solubilized were subjected to acid hydrolysis and were then quantified using the photometric hexokinase/glucose 6-phosphate dehydrogenase assay. n. d., not detected.

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<th></th>
<th>Phosphorylated MD [%]</th>
<th>Glucosyl Residues Released [µg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>singly</td>
<td>doubly</td>
</tr>
<tr>
<td>A-type MD$_{\text{cryst}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>65.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Pellet</td>
<td>21.3</td>
<td>0.33</td>
</tr>
<tr>
<td>B-type MD$_{\text{cryst}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>53.6</td>
<td>12.7</td>
</tr>
<tr>
<td>Pellet</td>
<td>29.2</td>
<td>0.9</td>
</tr>
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</table>
Table 3: Prolonged GWD action results in a massive solubilization of the crystalline maltodextrins. MD<sub>cryst</sub> was incubated for 12 h with recombinant GWD. At intervals the released maltodextrins were separated by centrifugation, hydrolyzed and the glucose content were determined enzymatically. The data represent the mean of four replicas and the SD.

<table>
<thead>
<tr>
<th>Incubation time [h]</th>
<th>Glucosyl Residues Released [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-type</td>
</tr>
<tr>
<td>1</td>
<td>6.2 ± 3.1</td>
</tr>
<tr>
<td>2</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>13.8 ± 3.3</td>
</tr>
<tr>
<td>5</td>
<td>15.3 ± 2.4</td>
</tr>
<tr>
<td>6</td>
<td>16.6 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>21.9 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>28.6 ± 1.4</td>
</tr>
<tr>
<td>9</td>
<td>31 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>38.9 ± 1.1</td>
</tr>
<tr>
<td>11</td>
<td>43.3 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>45 ± 1.1</td>
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</table>
Table 4: The inhibition of the recombinant stGWD by two soluble maltodextrin preparations. As a control, equal concentrations of a commercial dextran from *Leuconostoc mesenteroides* were applied. In a final volume of 50 µl, soluble MD was preincubated with 250 ng of recombinant GWD for 15 min at RT. The reaction was started by adding 2 mg of the B-type maltodextrin. Following incubation for 10 min at 30 °C under continuous agitation the reaction mixtures were stopped by heating for 5 min at 95°C. Aliquots (2 µL each) were loaded onto TLC plates and the \(^{33}\)P incorporation into maltodextrin was quantified by phosphor imaging. MD\(_{\text{sol}}\), B-type maltodextrin had been solved by heat treatment. MD\(_{\text{ICN}}\), soluble maltodextrin was purchased from ICN. The data represent the mean of three replicas and the SD.

<table>
<thead>
<tr>
<th>MD [mg]</th>
<th>GWD Activity [%]</th>
<th>GWD Activity [%]</th>
<th>GWD Activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MD(_{\text{sol}})</td>
<td>MD(_{\text{ICN}})</td>
<td>Dextran</td>
</tr>
<tr>
<td>0</td>
<td>100 +/- 3.0</td>
<td>100 +/- 2.0</td>
<td>100 +/- 5.0</td>
</tr>
<tr>
<td>0.5</td>
<td>67.4 +/- 0.6</td>
<td>61.9 +/- 0.8</td>
<td>100 +/- 3.6</td>
</tr>
<tr>
<td>1.0</td>
<td>41.9 +/- 1.5</td>
<td>42.8 +/- 0.9</td>
<td>100 +/- 2.2</td>
</tr>
<tr>
<td>1.5</td>
<td>33.7 +/- 1.0</td>
<td>33.4 +/- 1.0</td>
<td>85 +/- 2.6</td>
</tr>
<tr>
<td>2.0</td>
<td>23.3 +/- 0.8</td>
<td>7.7 +/- 0.8</td>
<td>42 +/- 0.5</td>
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</table>
Table 5: PWD-mediated phosphorylation of crystalline A- and B-type maltodextrins. The patterns of mono-, di- and triply phosphorylated α-glucans in the soluble and in the insoluble state. Four mg of prephosphorylated A- or B-type maltodextrin were incubated with 250 ng PWD for 10 min at 30°C under continuous agitation. Reaction was stopped by adding EDTA. The soluble and insoluble maltodextrins were separated by centrifugation (10 min at RT) and the pellets were resuspended with the same volume than that of the supernatant. Following heat treatment for 10 min at 95°C, samples were passed through 10 kDa filter and the resulting filtrate was analyzed by HPAEC. 0.5 mL fractions were collected, mixed with 3 mL scintillation cocktail and the radioactivity was measured using scintillation counter.

<table>
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<tbody>
<tr>
<td></td>
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<td>doubly</td>
</tr>
<tr>
<td>A-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>5.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>39</td>
<td>17.6</td>
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<tr>
<td>B-type MD&lt;sub&gt;cryst&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td>Supernatant</td>
<td>15.8</td>
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<tr>
<td>Pellet</td>
<td>8.4</td>
<td>4.7</td>
</tr>
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</table>
Fig. 1. Physical and physicochemical properties of the A- and B-type allomorph of MD$_{cyst}$.

A. MD patterns of MD$_{cyst}$ of the B-type (procedure I) and the A-type (procedure II) allomorph as revealed by HPAEC-PAD. Aliquots of both particle suspensions were solubilized by heat treatment and equal amounts (30 µg glucosyl residues each) were applied to a PA100 column. In the eluate carbohydrates were monitored by pulsed amperometric detection. Signal intensities (relative units but equal sensitivity) are plotted against the elution time. DP6 and DP15: MD having a DP of 6 and 15, respectively.

B. Size distribution of the A- and B-type allomorph of MD$_{cyst}$ as revealed by Coulter Counter. For both particle preparations, size distribution was monitored before (solid line) and after incubation for 10 min (30°C) in the absence of GWD but in the presence of the reaction buffer under continuous agitation (broken line).

C. X-ray diffraction patterns of crystalline maltodextrins obtained by procedure I (B-Type) and procedure II (A-Type) as determined by using a NanoStar instrument. For comparison, native starch granules from kernels of wild type Zea mays L. (Maize) or from tubers of Solanum tuberosum L. (Potato) were analyzed under identical conditions.

D and E. Polarization light microscopic evaluation of A-type (D) and B-type (E) MD$_{cyst}$ magnification: 600x

F. Temperature-dependent light scattering of A- or B-type MD$_{cyst}$. Light scattering at 532 nm (relative units) is plotted against the temperature.
Fig. 2. The action of recombinant stGWD on A-type or B-type crystalline maltodextrins. The specific enzyme activity was determined by the GWD standard assay using 10 min incubation. Reaction was terminated by heating and aliquots of the entire reaction mixture were analyzed by TLC. Total $^{32}$P incorporation into the maltodextrins was quantified by phosphor imaging. The specific activity of stGWD is plotted against the amount of crystalline maltodextrins applied to the respective assay mixture. Open symbols: A-type allomorph; closed symbols: B-type allomorph.
Fig. 3. stGWD-dependent $^{33}$P incorporation into soluble or insoluble MD derived from the A-type (A) or the B-type (B) allomorph of MD$_{cryt}$. The GWD standard assay was used except the incubation time was extended to 60 min. At intervals, the soluble and insoluble matodextrins were separated by centrifugation for 10 min at RT. The pellets were washed twice with water and dissolved in water. Following solubilization (5 min at 95°C), aliquots of both fractions were analyzed by TLC and phosphor imaging. Closed symbols: $^{33}$P content of the pelleted (phospho)glucans; open symbols: $^{33}$P content of the soluble (phospho)glucans.
Fig. 5. The action of recombinant atPWD on prephosphorylated A- and B-type crystalline maltodextrins. A-type (A) or B-type (B) allomorphs of crystalline maltodextrins (4 mg each) were prephosphorylated with recombinant stGWD (250 ng each) using unlabeled ATP. At intervals the prephosphorylation was terminated by SDS. The pelletable prephosphorylated maltodextrins were then incubated with recombinant stPWD (250 ng each) and [-3P]ATP. Following 10 min of incubation the reaction mixture was separated into a pellet and a supernatant by centrifugation (10 min at RT). The 33P content of both the soluble and insoluble fractions was determined by thin-layer chromatography and phosphor imaging.