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Mini review

Starch granules: structure and biosynthesis

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Abstract

The emphasis of this review is on starch structure and its biosynthesis. Improvements in understanding have been brought about during the last decade through the development of new physicochemical and biological techniques, leading to real scientific progress. All this literature needs to be kept inside the general literature about biopolymers, despite some confusing results or discrepancies arising from the biological variability of starch. However, a coherent picture of starch over all the different structural levels can be presented, in order to obtain some generalizations about its structure. In this review we will focus first on our present understanding of the structures of amylose and amylopectin and their organization within the granule, and we will then give insights on the biosynthetic mechanisms explaining the biogenesis of starch in plants.

Keywords: Biosynthesis; Chemical structure; Starch

1. Chemical structure: molecular and macromolecular constituents

In most common types of cereal endosperm starches, the relative weight percentages of amylose and amylopectin range between 72 and 82% amylopectin, and 18 and 33% amylose (Table 1). However, some mutant genotypes of maize (*Zea mays*), barley (*Hordeum vulgare*), and rice (*Oryza sativa*) contain as much as 70%, amylose whereas other genotypes, called waxy, contain less than 1% (maize, barley, rice, sorghum). Recently, a waxy wheat (*Triticum aestivum*) starch has been obtained by classical breeding [1].

1.1. Amylose

Amylose is defined as a linear molecule of (1→4) linked α-D-glucopyranosyl units, but it is today well established that some molecules are slightly branched by (1→6)-α-linkages.

The oldest criteria for linearity consisted in the susceptibility of the molecule to complete hydrolysis by β-amylase. This enzyme splits the (1→4) bonds from the non-reducing end of a chain releasing β-maltosyl units, but cannot cleave the (1→6) bonds. When degraded by pure β-amylase, linear macromolecules are completely converted into maltose, whereas branched chains give also one β-limit dextrin consisting of the remaining inner core polysaccharide structure with its outer chains recessed [2].

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Table 1
Number-average MW ($\bar{M}_n$), weight-average MW ($\bar{M}_w$) of potato and wheat amyloses

Starch	$\bar{M}_n \times 10^{-5}$	$\bar{M}_w \times 10^{-5}$	References
Potato	10.3	—	[2]
	—	7.0	[5]
	1.3	9.0	[6]
	4.6	8.7	[7]
Wheat	2.1	—	[2]
	—	5.1	[5]
	0.6	3.9	[6]
	2.8	5.8	[7]

Takeda et al. [3] developed an extensive study of amylose fine structure, based upon the successive use of β-amylase and isomylase. No effective methods for the separation of linear and branched amyloses are known, so all results concerning amylose branching have been obtained by assuming the existence of two quite distinct populations, one strictly linear and the second one characterized by a 40% β-amylolysis limit [3]. This low value suggests that the branch linkages are frequently located rather near the reducing terminal end and/or they have multiple branched side chains. β-Limit dextrins of branched amyloses also show properties (iodine binding capacity, molecular weight) close to those of the corresponding original amyloses, while remaining completely different from those of amylopectin. This observation confirms the relevance of the distinction between amylose and amylopectin. Indeed, no structural continuum is observed between these two types of α-glucans.

The branched molecule amounts range from 25 to 55% on a molecular basis [3,4]. Using fractionated amyloses, this level of branching was shown to increase continuously as a function of the molecular weight [2]. The average number of secondary chains attached through occasional $(1 \rightarrow 6)$ branch points to the branched molecules are in the wide range 4–18. Hizukuri et al. [4] found an average of two to eight branch points per molecule, the side chains from four to over 100 glucosyl units in length. By further fractionation of β-limit dextrins, they concluded that branched amylose has some tiny clusters of short chains rather than a pure comb structure. The major drawback of the aforementioned approach is to give averaged information.

Another useful approach to this problem of amylose branching involves measuring the volume occupied by the polymer in solution. This volume may be represented by the radius of gyration, $\bar{R}_G$, which is dependent upon the molecular weight (MW). The molecular weight dependence of $\bar{R}_G$ can be described by the power law, $R_i \sim M_i^n$, where the exponent n is a kind of Mark–Houwink coefficient, and M_i and R_i are, respectively, the molecular weight and the radius of the component i of a series of particles of the same architecture but different molecular weights.

The coupling of HPSEC (high-performance size-exclusion chromatography) with a detector based upon light scattering or viscosimetry leads to $R_i \sim M_i^n$ relations over the whole range of fractionated molecules M_i. The purpose of this approach is to determine up to which point amylose chains in solution behave differently from strictly linear chains. Amylose fractions extracted by aqueous leaching of corn starch using 5°C steps over a temperature range of 65–95°C [5] were studied by the aforementioned techniques. Molecular weight dependences of macromolecular sizes were consistent with the behaviour of expanded chains in good solvent. Indeed, the hydrodynamic coefficient n was estimated within the 0.6–0.7 range for the $R_G \sim M^n$ relation, suggesting therefore an extended linear random coil in a large range of MW (3×10^5–9×10^6 g/mol).

We therefore conclude that the presence of branches does not alter significantly the solution behaviour of amylose chains, which remains identical to that of strictly linear chains.

Measurements of both molecular weight distributions and average molecular weights have been performed on a large number of starch samples of different botanical origins. In contrast to proteins which are genetically coded, polysaccharides occur with a molecular weight distribution, usually represented by the average molecular weights: number-average MW ($\bar{M}_n$), weight-average MW ($\bar{M}_w$), or z-average MW ($\bar{M}_z$). Important discrepancies are observed in the literature due to: (i) the biological origin of amylose, leading to uncontrolled variations in the biosynthesis mechanisms; and (ii) molecular degradation occurring during amylose fractionation [6]. The different values (Table 2) quoted in the literature for potato and wheat amyloses illustrate these variations. Intrinsic viscosity remains a basic technique to calculate the viscosity-average MW (M_v), using the Mark–Houwink coefficients measured on different solvents [2].

Table 2
Radius of gyration, $\bar{R}_G$, and weight-average MW ($\bar{M}_W$) of maize and wheat amylopectins

Source	$\bar{R}_G$ (nm)	$\bar{M}_W \times 10^{-6}$	References
Maize	105	10	[2]
	60	19.5	[13]
	229	22	[15]
	260	333	[34]
		10	[35]
	205	10	[36]
	13	60	[37]
	205	75–100	[38]
Potato	160	195	[2]
	144	47	[37]
		44	[38]
	195	14–36	[39]

Polydispersity depends on the botanical origin but also on extraction procedures: high temperatures of leaching give values up to 10 [8]. The molecular sizes of the branched molecules are 1.5–3.0-fold higher than those of linear molecules. No significant differences are observed between average molecular sizes of cereal amyloses on the one hand, and those of tuber and rhizome starches on the other hand.

Nowadays, amylose solutions can be easily characterized by size-exclusion chromatography coupled on-line to multi-angle laser light scattering (SEC–MALLS). However, the hydrodynamic differences between amylose (R_G 7–22 nm; [8]) and amylopectin (R_G 21–75 nm; [9]) are not in favour of a complete resolution between both components, and the use of combined detectors should provide direct access to the complete molecular weight distribution of starch rather than each component separately.

Different solvents were proposed in the literature: DMSO, aqueous alkaline solution, acidic conditions or DMAc/LiCl. Some authors reported quantitative problems due to incomplete recovery after SEC [10]. However, this problem should not be misinterpreted by reducing the difficulties to solvent quality. The system solvent–size exclusion column–temperature has to be considered as a whole. Its overall efficiency will be assessed considering both chromatographic recovery and linear calibration, demonstrating an exclusion effect. Efficient and reproducible fractionation of amylose and whole starch have been reported by Yu and Rollings [11,12], Fishman and Hoagland [13], Roger and Colonna [5,14] and Bello-Perez et al. [15]. Using a specific optimization algorithm, experimental molecular weight distributions (MWD) obtained by SEC–MALLS offers the possibility of fitting them to mathematical MWD such as normal distribution, 'most probable' (MP) distribution, and 'log–normal' (LN) distribution. Good agreements were obtained using either a sum of overlapping Gaussian curves [13] or a 'most probable' model [5].

It is interesting to calculate the number of amylose chains present inside a single starch granule. Assuming an amylose content of 25% and a granule density of 1.5, with an average relative molecular weight of 500000, for 20-μm-diameter granules the number could be 1.8×10^9.

Flexibility of polymeric chains is considered on the basis of the dimensionless quantity C_∞ (the characteristic ratio), generally defined as the ratio between the dimensions of real and freely jointed chains. An equivalent characteristic is the persistence length a, defined as the average projection of an infinitely long chain on the initial tangent of the chain. Amylose chains in solution ($a = 1.71$ nm) are more flexible [5] than those of modified cellulose (cellulose diacetate $a = 4.8–7.2$ nm; carboxymethyl cellulose $a = 8.0–12.0$ nm), but stiffer than those of pullulan ($a = 1.2–1.9$ nm). Therefore, all of these polysaccharides belong to the class of loosely jointed polysaccharides, in contrast to stiff polysaccharides [16] such as xanthan ($a = 310 \pm 40$ nm) and scleroglucan ($a = 180 \pm 30$ nm). This conformational feature explains why amylose has a low intrinsic viscosity value compared to other polysaccharides.

Another specific feature of interest concerning amylose is its capacity to bind iodine. The existence of I_{3-} and I_{5-} was checked using Raman spectral measurements and UV/Vis, coupled with theoretical analyses. Yu et al. [17] demonstrated that the four dominant polyiodide chains which coexist are longer species such as I_9^{3-}, I_{11}^{3-}, I_{13}^{3-} and I_{15}^{3-}. An interesting result is the demonstration of the absence of participation of I_2 in the polyiodide chain.

1.2. Amylopectin

Amylopectin is the highly branched component of starch: it is formed through chains of α-D-glucopyranosyl residues linked together mainly by $(1 \rightarrow 4)$ linkages but with 5–6% of $(1 \rightarrow 6)$ bonds at the branch points.

Amylopectin is a branched polysaccharide composed of hundreds of short $(1\rightarrow4)$-α-glucan chains, which are interlinked by $(1\rightarrow6)$-α-linkages. The multiplicity in branching is a common feature of both amylopectin and glycogen. The basic organization of the chains is described in terms of the A, B and C chains as defined by Peat et al. [18]. Thus, the outer chains (A) are glycosidically linked at their potential reducing group through C_6 of a glucose residue to an inner chain (B); such chains are in turn defined as chains bearing other chains as branches. The single C chain per molecule likewise carries other chains as branches but contains the sole reducing terminal residue. The ratio of A-chains to B-chains, which is also referred to as the degree of multiple branching, is an important parameter. Using careful enzymatic experiments [19], the A:B ratio has been shown to be 0.8–2.2 on a molar basis and 0.4–1.0 on a weight basis. Nevertheless, a proportion of B-chains must carry more than one A-chain. Manners [20] reported A:B ratios ranging between 1.1–1.5, with A:B potato amylopectin at 1.2, whereas Hizukuri [21] assigns a 0.8 A:B ratio to potato amylopectin. This discrepancy appears to be due both to accumulated errors while harvesting experimental data and to the very nature of the methods used. The general rule is that amylopectins have rather more A-chains than B-chains, with ratios ranging from 1.0:0 to 1.5:1. These values are consistent with the cluster and Meyer's structures, but not with those of Haworth and Staudinger, as described in the Manners review [20].

Very significant information is gained from chain distributions profiles. The debranching enzymes, isoamylase and pullulanase, specifically hydrolyze the branch linkages and produce the short linear chains. SEC [21,22] and high-performance anion-exchange chromatography with pulsed amperometric detection [22–24] are the two basic techniques used to estimate the chain length distributions. Amylopectin is built with three types of chains: short chains, S, consisting of both outer (A) or inner (B) chains with a mean polymerization degree (DP) ranging between 14 and 18, inner (B) long chains, L, of DP 45–55, and a few B-chains of DP above 60. Hizukuri [21] even detected a third peak as a weak shoulder on the L population for wheat, tapioca and tulip amylopectins. Differences related to the botanical species were mainly concerned with the L/S ratio expressed on a molar basis. This ratio was estimated at 5 for amylopectins from B-crystalline type (potato) starch and at 8–10 for normal cereal amylopectins from A-crystalline type (normal genotypes) granules.

Polymodal chain distributions have been reported by Hizukuri et al. [22,23] and Koizumi et al. [25]. On 11 amylopectins from different species, chromatograms exhibited periodic waves of DP 12, except edible canna and yam amylopectins, where the period might be DP 15. Therefore, the chains were fractionated into four different fractions with DP ranging in the intervals 6–12, 13–24, 25–36 and more than 37. Amylopectins with high and low amounts of the DP 6–12 fraction respectively, show A- and B-type X-ray diffractions of starch granules respectively. Hence, Hizukuri et al. [4] suggested that these S-chains with DP between 6 and 12 determined the starch crystalline allomorph. The DP 6–12 fraction should play an important role in the determination of starch crystallite polymorphs. Nevertheless, Ong et al. [6] considered that there was limited evidence to assess this influence of S-chains on the allomorphism since important variabilities of these ratios are observed inside the same botanical species. The involvement of the amylopectin model remains, however, based upon correlation. The relative amounts of each of the polymodal chain populations is genetically coded through the selectivity of the starch biosynthetic enzymes. In addition, physical effects due to short chain crystallization during synthesis also have to be taken into account.

Without doubt, the most important feature of this branched molecule is that S-chains are found in discrete clusters. Nowadays, the cluster structure concept originally proposed by Robin et al. [26] and by French [27] has found wide acceptance. Several lines of evidence supporting this structure have accumulated (Fig. 1). The localization of the α-$(1\rightarrow6)$ linkages has been defined only by using a sequential enzymatic method. This analysis has been performed both on native amylopectins together with their corresponding β-limit dextrins [28–30], lintners [26,31] and Naegeli [32] amylodextrins from granular starches. These chains are not linked together randomly, as in the Meyer and Bernfeld model but, rather, are organized in a cluster structure. The two aforementioned L-chain populations represent the backbone supporting the S-chains-bearing clusters. It was for the first time refined into a statistical

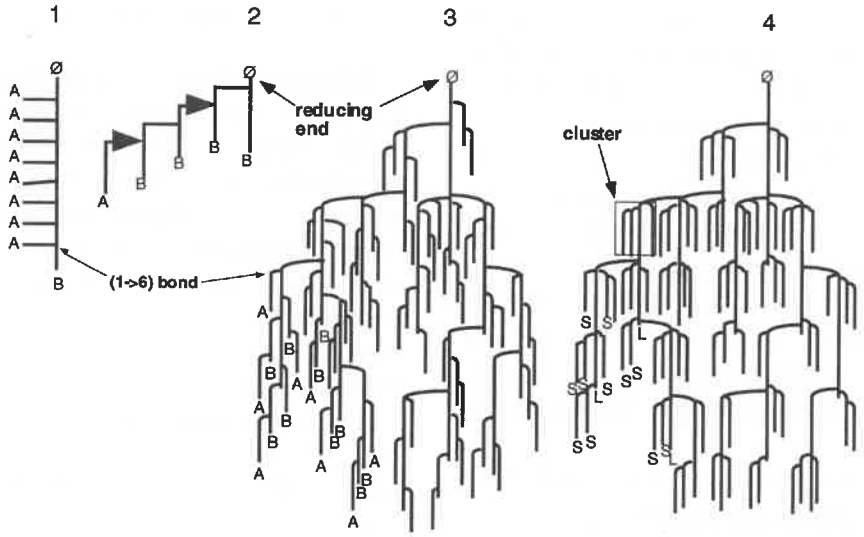

Fig. 1. Diagrams of the molecular structure of amylopectin as proposed by (1) Haworth, (2) Staudinger, (3) Meyer and (4) Meyer redrawn as a cluster-type architecture.

model by Burchard and Thurn [35]. Branching points are arranged in clusters which are not distributed randomly throughout the macromolecule. An average chain length of 20–23 units interconnects two clusters. The success of the cluster-type model proposed by Robin et al. [26,31] and French [27] is explained by its ability to account for the higher viscosity of amylopectin when compared to glycogen, for the involvement of amylopectin chain in crystallinity for the degradation pattern displayed by α-amylases. A-chains can be considered as the limiting factor defining crystallite thickness. Assuming classical models for A- and B-crystallites, involvement of S-chains in crystallite thickness leads to an helicoidal length of about 5.7 nm, with 16 glucosyl units, each one giving a repeating distance of 0.35 nm per glucose. The lateral assembly of clusters could explain the formation of crystallites. However, the exact global conformation of amylopectin inside a granule remains unknown.

Another enzymatic approach to the problem of amylopectin structure is obtained through α-amylolysis. Zhu and Bertoft [33] observed a non-random hydrolysis of potato amylopectin with the formation of intermediate products presenting relative molecular weights above 30000. These dextrins resulted from the preferential hydrolysis of intercluster linkages. However, dextrins contained molecules with several clusters, therefore complicating their interpretation.

Alternative experimental strategies, such as those based on the combined use of static and dynamic light scattering [34,35], rely directly on analyses performed on the native molecule without any molecular weight reduction. Different models for amylopectin were examined through comparison of calculated and experimentally determined particle scattering functions from combined static and dynamic light-scattering studies. The data prove that amylopectin is an heterogeneously branched polymer, in agreement with the proposals of French [27] and Robin et al. [26,31]. For maize amylopectin, these authors concluded that each L-chain has 1.4 clusters made of 3.22 S-chains on average, whereas Robin et al. [26] assumed exactly two clusters per L-chain. The distance between two clusters on the same B-chain is 22 glucosyl units on average. This modelling based upon the cascade branching theory should be renewed in the light of the last refinements of Hizukuri and his colleagues [21–23].

This branched character based upon short chains explains two main features of amylopectin. Thus, 100 g of amylopectin bind less than 1 mg of iodine, giving a λ_{max} around 540–550 nm. The low iodine binding capacity is based upon the formation of an arrangement of four iodine atoms more or less arranged linearly within the cavity of the helix structure of 11 glucosyl units [36]. This fits nicely with the abundant S-chain populations. The β-amylolysis susceptibility of all amylopectin is low (54–58%), whatever its botanical origin or purification procedure [2].

Amylopectin has one of the largest relative molecular weights (10^7–10^9), but mostly in excess

of 10^8 (Table 2). However, there is a lack of knowledge about the molecular weight distribution, as all commercial chromatographic phases are unable to fractionate this polymer on a molecular weight basis. For degraded amylopectins [11,12], the quantitative branching characterization via size-exclusion chromatography and online low-angle light scattering detection was in good agreement with theoretical predictions obtained by branching modelling. Due to its branched character, amylopectins have low intrinsic viscosity (120–190 ml/g) despite huge molecular weights.

As for amylose, it is interesting to calculate how many amylopectin chains are present inside a single starch granule. Assuming an amylopectin content of 75%, with a weight-average relative molecular weight of 50000000, for a 20-μm diameter granule and a density of 1.5, the number could be 5.4×10^7.

Atypical amylopectins have been observed from high-amylose genotypes from maize [40] and pea [41], where they represent up to 20% of total starch. Called intermediate material because of their medium iodine binding capacities, they can be distinguished from normal amylopectins by (i) longer L-chains with 5–15 glucosyl units more, and by (ii) low-molecular-weight amylopectin with a lower S/L ratio.

Despite intensive investigations, the fine structure of the wild-type amylopectin clusters and the relative proportions of the side chains still need to be determined. Here the main limitation is the inability of all actual analytical systems to fraction amylopectin according to size. Transmission electron microscopy (TEM) [42] of starch molecules deposited from dilute solution and rotary shadowed with platinum enables us to probe the heterogeneity within the amylopectin family. Waxy maize amylopectin could be described as about 28% circular space filling patches containing branched clusters and 72% asymmetric linear containing branched. This structural problem still continues to elude scientists, as the molecular architecture of this polymer is of importance to explain its physicochemical properties. A detailed knowledge of the internal molecular structure would help to describe mechanisms of crystallization, biosynthesis and organization of amylopectin within starch granules.

1.3. Minor components

Minor components associated with starches correspond to three categories of materials: (i) particulate material, composed mainly of cell-wall fragments; (ii) surface components, removable by extraction procedures; and (iii) internal components. Lipids represent the most important fraction associated with the starch granules. High contents are generally observed for cereal starches: 0.8–1.2 and 0.6–0.8% for wheat and normal maize, respectively.

The main constituents of surface components are proteins, enzymes, amino acids and nucleic acids. Some components can be extracted without granule disruption: approximately 10% of proteins and 10–15% of lipids. A number of proteins [43] associated with wheat starch granules isolated by aqueous extraction from grain or flour have received the most attention. Some appear to be integral components of the granule structure, whereas others appear to be associated with the granule. One of the starch granule proteins, friabilin, was studied in detail because of its association with changes in wheat grain endosperm texture, from soft wheat to hard wheat [44]. It consists of several different polypeptides, in which puroindoline polypeptides are mostly represented. The reason for the differential binding of these proteins to the surfaces of hard and soft wheat starch granules remains to be found [45]. Association of friabilin with starch could be considered as artefactual. Being located at the starch granules peripheries, the puroindoline-β-polypeptides would presumably become accessible for adsorption to the granule surface immediately after flour wetting. Implication of lipid binding in the mechanism by which friabilin polypeptides could be related either on a direct bridge of friabilin on lipid zones or a protein conformational change of friabilin.

Triglycerides represent a major fraction of surface lipids of maize and wheat. Glycolipids and phospholipids would correspond to amyloplast membrane remains [46]. The location of the lipids at the surface of starch granule is still unknown.

In contrast, internal components are composed mainly of lipids. Proteins, including granule bound starch synthase, are minor. Extraction procedures have been optimized by Morrison and coworkers [47–50] on the basis of water-saturated butan-1-ol at 95–100°C. The presence of internal lipids is a characteristic of cereal starches (Table 3).

In contrast to tubers and legume starches, cereal starches are characterized by the presence of monoacyl lipids (free fatty acids (FFA) and lysophospholipids (LPL)) in amounts positively correlated to amylose content [47]. Wheat, barley, rye, and other triticale starches contain almost exclusively LPL, whereas other cereals contain mainly free fatty acids together with a minority of lysophospholipids (Table 3). Lysophosphatidylcholine is the major lipid found for both wheat and maize, with palmitic and linolenic acids. For barley starches, the fatty acid composition of the lipids becomes progressively more unsaturated as lipid content increases, but this pattern is less consistent in starches from maize [48]. Most waxy starches have negligible lipids. In the family of maize starches, by comparing different mutants, lipid content appears most directly correlated with long-chain linear α-1,4-glucan (i.e. the backbone of amylose) revealed by enzymatic debranching. In starch from wheat and barley harvested at various stages of grain development, both amylose and LPL contents increase with maturity. The picture that is thus emerging is that of a large A-type starch granule displaying a gradient (from the hilum to the periphery) of increasing amylose and LPL [49].

Table 3
Free fatty acids and lysophospholipids present in purified cereal starches

Source	Free fatty acid content range	Lysophospholipid content range
Barley		
Waxy	0.03–0.04	0.12–0.75
Normal	0.03–0.05	0.47–1.14
High amylose	0.05–0.09	0.86–1.36
Maize		
Waxy	0.01–0.05	0.01–0.03
Normal	0.30–0.53	0.16–0.35
High amylose	0.38–0.67	0.26–0.61
Rice		
Normal	0.22–0.50	0.41–0.86
Wheat		
Normal	0	0.78–1.19
Waxy	0	0.07–0.17

Values represent % total dry wt.; data quoted in Morrison and coworkers [49,50].

The presence of LPL and FFA is still a surprising finding from a biological point of view. Monoacyl lipids are usually associated with lipolysis, and lysophospholipids are potentially harmful because they can cause cell membrane lysis. Hydrolysis of amyloplast lipids should have led to the presence of monoacylgalactosylglycerides.

Numerous studies have observed a correlation between these monoacyl lipids and the functional properties of barley [50], oat [51] and wheat [52] starches. Monoacyl lipids will induce the formation of amylose–lipid complexes during gelatinization. They will restrict swelling, dispersion of the starch granules and solubilization of amylose, thus generating opaque pastes with reduced viscosity and increased pasting temperatures.

Mineral fractions are negligible in cereal starches in contrast to tuber starches. Phosphorus is the most important one, which can easily tracked using nuclear magnetic resonance (^{31}P-NMR) [53]. Cereal starches contain phosphorus that is mainly in the form of phospholipids. Root and tuber starches are unique in that they contain phosphate monoesters, with an exceptionally high level in potato (0.089%). Phosphate monoesters of all these starches were located mainly on the primary C-6 alcohol function and less on the secondary C-3 function of the glucosyl unit.

2. From the chain to the crystal

Starch is biosynthesized as semi-crystalline granules with varying polymorphic types and degrees of crystallinity. As powder diffraction patterns of native starch are difficult to interpret because of both poor crystallinity and molecular structure complexity, many structural models of starch were established from recrystallized amylose. The different conformations of amylose and amylopectin which can be present at the crystalline or amorphous state are described.

2.1. A- or B-crystals and double helices

A- and B-type fibrillar or lamellar crystals can be prepared by deacetylation of amylose triacetate fibers [54,55] or precipitation from dilute solutions of low polymerization degree amylose [56]. The recent models for A- and B-type structures are based on parallel double-stranded helices, right-handed [54,55] or left-handed [57,58] and packed

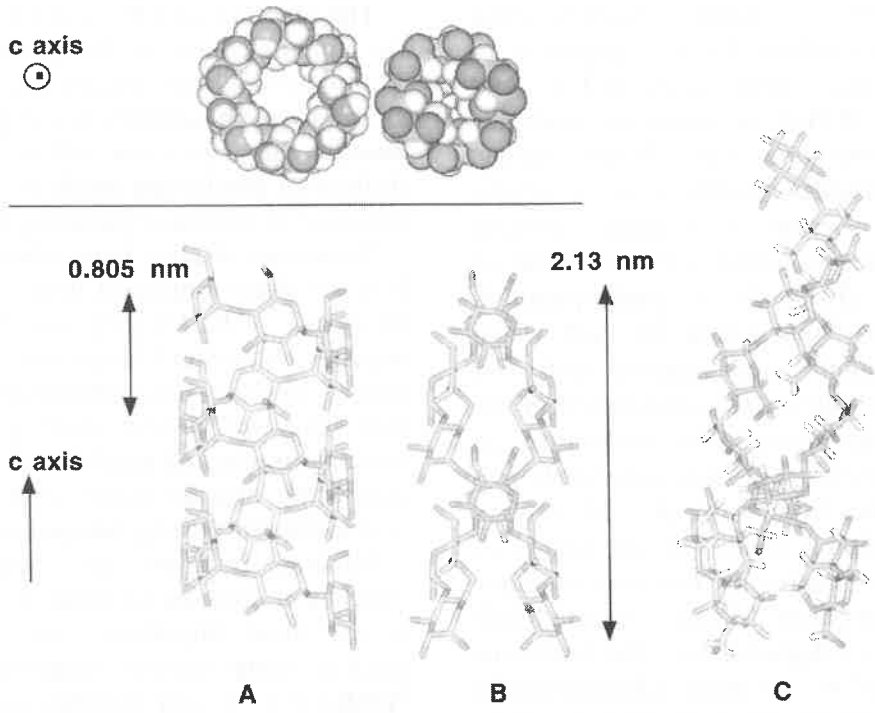

Fig. 2. Helical conformations present in starch and starch components: (A) 6-fold single helix (pitch 0.805 nm); (B) 6-fold left-handed double helix (pitch 2.13 nm); (C) double helix between two short chains in amylopectin. When looking at solid rendering views at the top of the figure, it is clear that the single helix presents a central cavity, contrary to the double helix.

antiparallel [54,55] or parallel [57,58] in the unit cell. The left-handed form (Fig. 2B) is energetically preferred to the right-handed form [57], but Muller et al. [59] showed, using computer simulations of X-ray scattering curves of amylose gels, that they do not scatter in a significantly different way. Schulz et al. [60] from a crystallographic study of a polyiodide–maltohexaose complex, proposed an amylose antiparallel double helix with a central cavity which can adopt, upon removing the complexing molecules, a conformation very close to the parallel stranded one (six residues/turn and a pitch height of about 2.1 nm). The antiparallel packing is strongly debated since it seems incompatible with the cluster model of amylopectin and the biosynthetic pathway. The most recent models for A and B amylose structures are based upon 6-fold left-handed double helices with a pitch height of 2.08–2.38 nm [57,58]. In the A-structure [57,58], these double helices are packed with the space group B2 in a monoclinic unit cell ($a = 2.124$ nm, $b = 1.172$ nm, $c = 1.069$ nm, $\gamma = 123.5°$) with eight water molecules per unit cell (Fig. 3A). In the B-type structure [58], double helices are packed with the space group $P6_1$ in a hexagonal unit cell ($a = b = 1.85$ nm, $c = 1.04$ nm) with 36 water molecules per unit cell (Fig. 3B). The symmetry of the double helices differs in A and B structures, since the repeated unit is a maltotriosyl unit in the A form and a maltosyl unit in the B form [58]. Independent evidence for the individuality of each glucosyl residue in maltosyl and maltotriosyl units comes from solid-state ^{13}C-NMR. The C1 peak in A-form spectra is a triplet while it is a doublet in spectra of the B form [61,62].

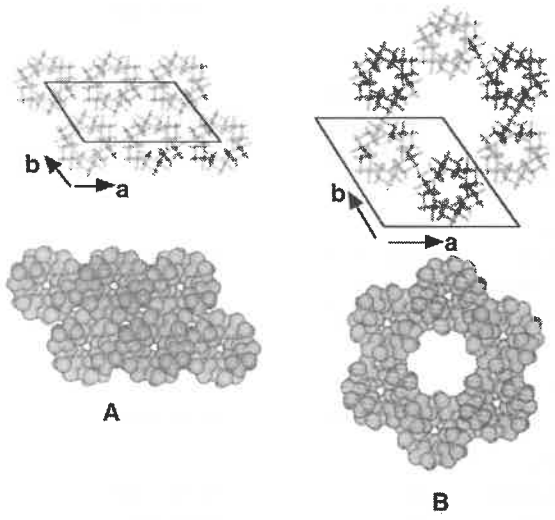

Fig. 3. Crystalline packing of double helices in A-type (A) and B-type (B) amylose. Projection of the structure onto the (a, b) plane.

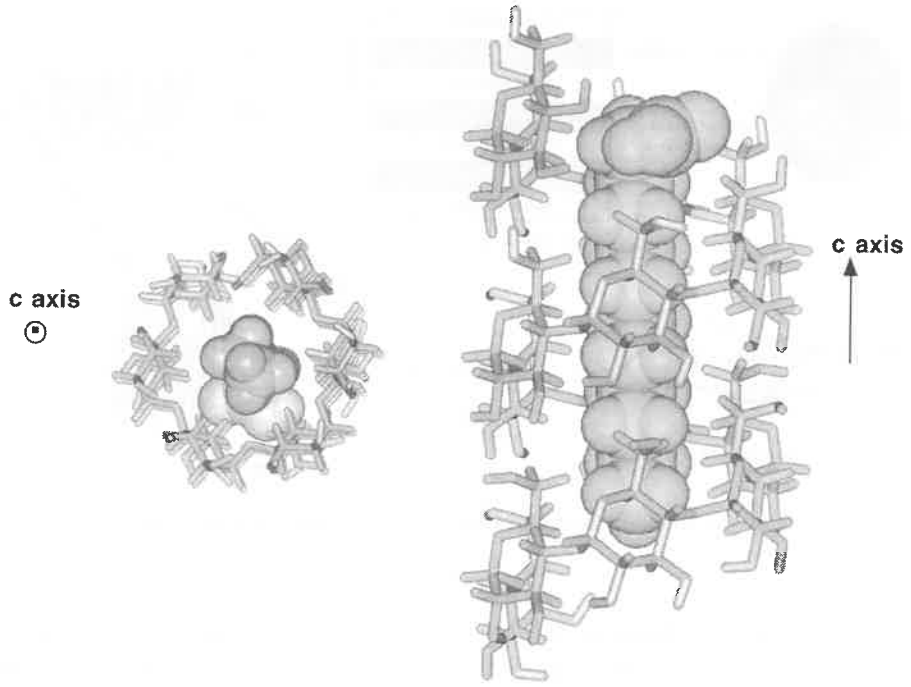

Fig. 4. Molecular modelling representation of amylose–fatty acid complexes showing the inclusion of the aliphatic part (C12) of the fatty acid inside the hydrophobic cavity of the amylose single helix.

2.2. The amylopectin conformation

Amylopectin is usually assumed to support the framework of the crystalline regions in the starch granule. Two independent studies [63,64] showed that the branch point does not induce extensive defects in the double helical structure (Fig. 2C). Only dihedral angles Φ and Ψ of residues adjacent to the (1→6) linkage were modified slightly with regard to their values in the double helix. The calculated conformation differs from that determined by crystallography for the trisaccharide panose (αGlc(1→6)αGlc(1→4)αGlc), which has a primary structure equivalent to that of the branch point of amylopectin but contains a specific hydrogen bond. The conformation of chain segments between branching points is not well known up to now.

2.3. Amylose as single helices

V-amylose is a generic term for amyloses obtained as single helices co-crystallized with compounds such as iodine, DMSO, alcohols or fatty acids. Although such compounds are required for formation of the V-type structure, they are not systematically included in the amylose helix. The characteristic Vh structure obtained with linear alcohols and fatty acids has been most extensively studied [65–67]. The chain conformation consists in a left-handed six residues per turn helix (Fig. 2A) with a pitch height of 0.792–0.805 nm (i.e. a rise per monomer of between 0.132 and 0.136 nm). In the case of amylose–lipid complexes, it is assumed that the aliphatic part of the lipid is included inside the amylose helix (Fig. 4), while the polar group lies outside, being too large to be included [68–70]. In the Vh-type, the most common form obtained by complexation of amylose with lipids, the single helices are packed in an orthorhombic unit cell ($a = 1.37$ nm, $b = 2.37$ nm, $c = 0.805$ nm) with the space group $P2_12_12_1$ and 16 water molecules within the unit cell. The amylose–lipid complexes can be crystalline or amorphous depending on the temperature at which they form [67,71]. The two forms cannot be evidenced by differential scanning calorimetry (DSC) since they yield very similar melting/decomplexing enthalpy and temperature values. The latter form consists in individual complexed single helices which are not involved in a crystalline packing.

The double helical conformation is still questionable since many authors reported some Vh to B transition upon rehydration [72,73]. It is unlikely that any drastic conformational change such as defolding/refolding can be provoked by such

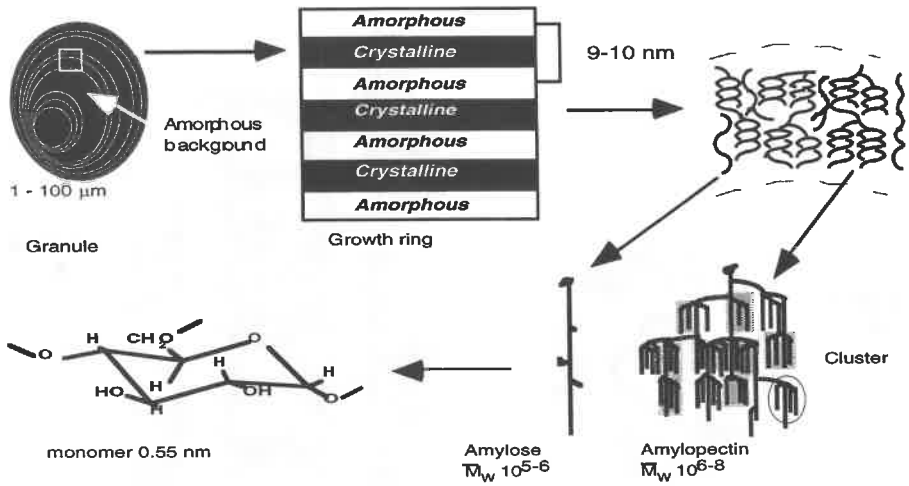

Fig. 5. Schematic representation of the different structural levels of the starch granule and the involvement of amylose and amylopectin.

mild treatment as rehydration, and Saito et al. [72] proposed that the helical conformations ascribed to both Vh- and B-types are not very different.

2.4. The conformation of amylose or amylopectin in the dry amorphous state

Very few reports are available concerning the conformation of amorphous amylose and amylopectin. Trommsdorf and Tomka [74] calculated a detailed model for dry amorphous starch (amylose) with different densities (including 1.5 g/cm^3) and compared the differential radial distribution functions evaluated from X-ray scattering measurements and from their model structures. Their model allowed a molecular view of the specific interactions in amorphous starch which is strongly networked by hydrogen bonds but no experimental evidence was reported by the authors.

3. From the crystal to the starch granule

The starch granule organization is very complicated and depends strongly on the botanical origin. Despite several decades of investigation on the crystalline ultrastructure of starch, many questions remain unresolved such as the respective contribution of amylose and amylopectin to crystallinity, the distribution of ordered and unordered areas in the granule, the size distribution of crystalline areas or the organization of mixed A- and B-type granules. We present here the most commonly accepted data about the different levels of organization within the starch granule. A schematic representation of the granule architecture is given in Fig. 5.

3.1. Size and shape of starch granules

In plants, starch accumulates in many different photosynthetic or non-photosynthetic tissues. One of the most abundant and universally distributed forms of the storage polysaccharide concerns transient starch, which is accumulated during the day in plant leaf cells and broken down at night to achieve a more or less constant supply of sucrose to the non-photosynthetic tissues. However, very little information is available concerning either the structure or the morphology of such granules.

Major attention has been devoted to the long-term form of storage present in endosperm of cereals, parenchyms of tubers, cotyledons of legume seeds. These differences are noteworthy as starch biosynthesis will occur in different genetic ploidy backgrounds: 3*n* for wheat endosperm, 2*n* for tubers and cotyledons (assuming a diploid plant in each case which is not accounted for by hexaploid wheat or tetraploid potato). Sizes vary from 1 μm up to 100 μm. The most interesting feature remains the granule shape (Table 4). This demonstrates that the biosynthetic pathway can shape the granule in accordance with the physical constraints encountered during cellular starch deposition due to the presence of other subcellular compounds such as the shape assumed by the amyloplast membrane.

Table 4
Morphological features of starch granules and amylose content in major plant sources

Source	Amylose content (% total starch)	Size	Average (μm)	Shape
Barley normal (wild)	21–24	A-granule	20	
		B-granule	2–3	
Wheat normal (wild)	25–29	A-granule	30	Discs
		B-granule	2–3	Perfect spheres
Wheat waxy	1.2–2.0	One type		
Maize normal (wild)	25–28	One type	30	Polyhedral and rounded
Maize waxy	0.5	One type	15	
Maize high amylose	60–73	One type	5–25	Highly elongated irregular filament
Oat		A-granule	15	Compound
		B-granule	2–3	Oval
Potato normal (wild)	18–21	One type	40	Large oval
Potato amylose free	1	One type	40	Large oval
Pea *RR* (wild)	33–36	One type	30	Oval
Pea *rr*	66–72	One type	50	Compound
Pea *rbrb*	23–32	One type	20	Round
Pea *rr rbrb*	49	One type		Compound

Some sources contain compound granules, corresponding to the fusing of different granules developing simultaneously within a single amyloplast. Other special forms occurs in high amylose types where non-symmetrical shapes are observed, more especially with high amylose content (above 50%). This emphasizes further the importance of amylopectin as an order parameter acting during starch granule morphogenesis.

Some cereals including oat, wheat and barley present large (A-granule) and small (B-granule) granules, whose biosynthesis occurs at two different stages of development. Contradictory results have been published on the respective amylose and lipids contents of A- and B-granules. The basic problem is to explain how these different populations are deposited in the plant tissue. No satisfactory explanation has yet emerged from biosynthesis studies with respect to the origin of this dual population.

3.2. Surface of starch granules

Starches of corn, sorghum, millet, large granules of wheat, rye and barley observed by scanning electron microscopy (SEM) were found to have pores [75]. However, it appears that some granules contain many pores, others a few, and some none on the surface observed. Hall and Sayre [76], making similar observations, identified the presence of artefacts produced during granule purification. Gallant and Guilbot [77] also identified chemical or physicochemical events which could occur during the preparation and observation of samples. Moreover, all these experiments do not take into account the propensity of the granule to swell when water is absorbed, inducing higher porosity inside the amorphous matrix by inducing higher molecular mobility (plasticizing role of water). In fact, starch granules, as it has been demonstrated for gels [78], can be considered as porous material exhibiting both external and internal surface area. The external surface area is determined by the shape and size of the particle which can be determined using microscopy or light scattering. The internal surface area is defined by the capillary structure of the hydrated particle and the size of the penetrating reagent.

Surfaces of wheat and potato starch granules, including surface morphology and minor component composition, have been observed using atomic force microscopy (AFM) [79]. The author observed small protusions (e.g. 10–50 nm) on wheat granules and larger ones (200–500 nm) on potato starch granules. Because of the novelty of this approach, both a wider panel of starches and

milder granule purification conditions are awaited to confirm these results.

3.3. Macromolecules orientation

When the starch granules are observed under polarized light, a characteristic dark cross (centred at the hilum) is seen which has led to the granules being considered as distorted spherocrystals [80]. The sign of birefringence is positive with respect to the spherocrystal radius ($n_e - n_0 = 0.015$) which theoretically indicates that the average orientation of polymer chains is radial. The intensity of birefringence strongly depends on the shape and on the orientation of the granules with regard to the light beam. Therefore, for non-spherical granules, it is more accurate to say that the orientation is perpendicular to the growth rings and to the surface of the granule [80]. This was also confirmed by solid-state light scattering of starch granules [81]. More recently, Buléon et al. [82] and Waigh et al. [83] using microfocus X-ray diffraction with a 2-μm beam, showed that the orientation of polymer chains in potato starch was very strong on the edges of the granule and perpendicular to the surface of the granule. A lower level of orientation was found in the more internal regions and at the hilum, but the interpretation was more complicated since in the centre of the granule, the beam probably averages over helices pointing forward, backward and sideways. No specific orientation at a 2-μm scale was found for wheat starch granules, either on the edges or in the centre [82], which means that the radial orientation in such granules is weak and limited to very small domains. Similar results were obtained on potato and wheat starch by Chanzy et al. [84] and Helbert and Chanzy [85], respectively, using electron diffraction (1 μm^2 area) on thin sections of partly hydrolyzed starch. However, in both studies, the diffractograms were not as well resolved because of the great amount of inelastic scattering inherent to this technique.

3.4. The alternating ultrastructure of starch

Research, to date, has been concentrated on crystalline areas because of the paucity of analytical means for detailed studies of the amorphous zones. Detailed knowledge regarding the structure, the organization and the arrangement of the granule may be obtained studying remnant residues after acid or enzymatic hydrolysis (Fig. 6). Coupled to the introduction of improved analytical and microscopic techniques, the different levels of the starch granule organization have begun to be visualized.

The distribution of crystalline and amorphous areas of starch has been approached through acid hydrolysis. Under these conditions, granules weaken, crack and show a lamellar organization [86]. The layered concentric shell structure of starch granules has been observed by SEM or transmission electron microscope (TEM) after acid hydrolysis [87]. The more resistant lamellae are believed to represent the crystalline part of the granule. Amorphous areas which are supposed to be more susceptible to acid hydrolysis would be hydrolyzed first. Starch granules are usually believed to consist of alternating 120–400 nm thick amorphous and semi-crystalline layers [80,88]. The organization of semi-crystalline shells has been widely studied by electron microscopy of thin sections of granules with silver staining [89,90] or fragments of granules [88,91]. It was also assessed on entire granules using small-angle X-ray [92,93] or neutron scattering [94]. Considerable evidence now supports the finding that the crystalline shells consist of a regular succession of alternating amorphous and crystalline lamellae as reviewed by French [80]. The sum of the sizes of one amorphous and one crystalline lamella ranges from 9 to 10 nm (Fig. 5). Nevertheless, detailed knowledge regarding the structure, organization and arrangement of lamellae is still limited. No precise determination of the lateral (tangential) dimensions of the elementary crystallites inside the crystalline lamellae has been obtained up to now. From the observation of the 1.6-nm reflection in potato starch X-ray diffractograms, Hizukuri and Nikuni [95] propose a crystallite size of 14.7 nm. This reflection arises from the 100 plane and therefore yields some information about the number of times the inter-helical distance is contained in the crystallites. Sterling [96] from observations performed on partly gelatinized potato starch granules showed the presence of radially oriented fibrillar elements of 10–20 μm in length and 0.3–1 μm in diameter. Oostergetel and van Bruggen [97], from electron diffraction data and silver staining of potato starch fragments, concluded that the semi-crystalline domains form a network of left-handed superhelices (diameter 18 nm, pitch 10 nm), which could be a well-ordered skeleton for

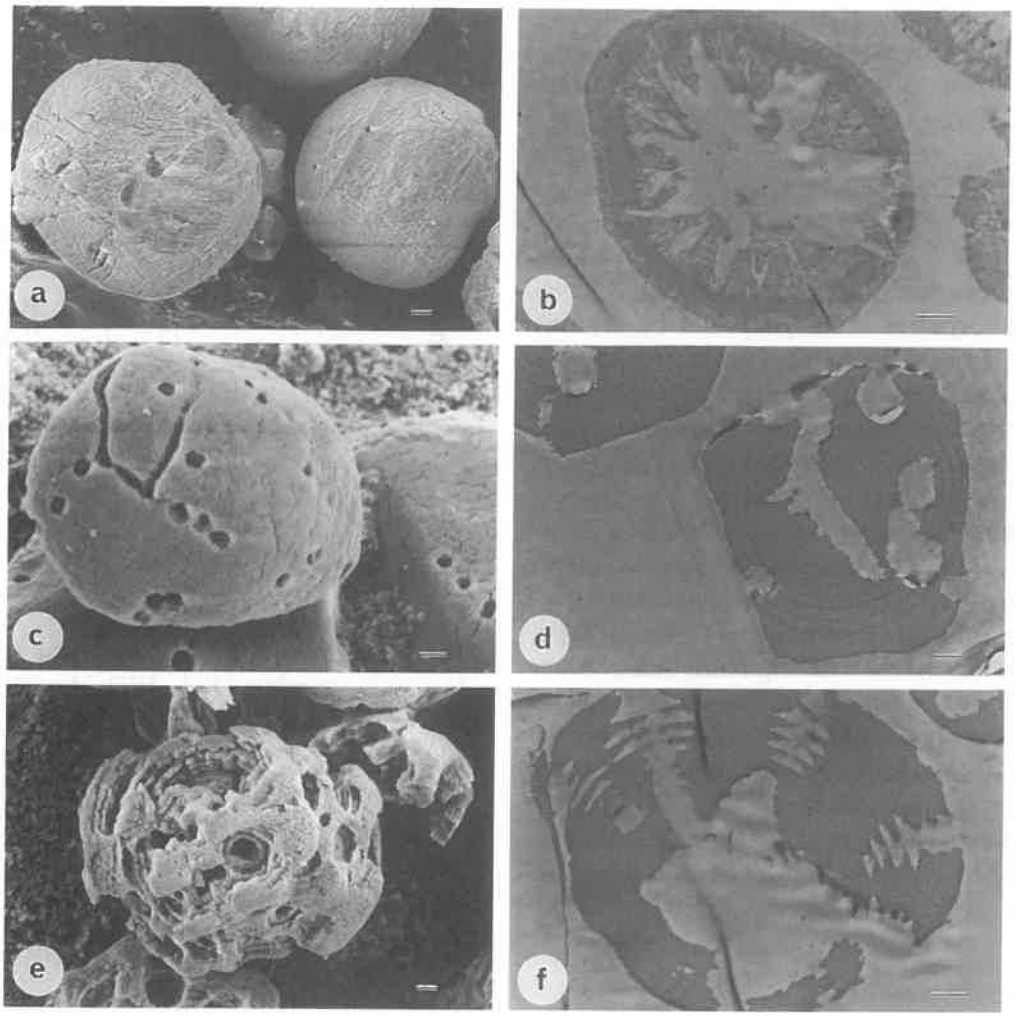

Fig. 6. Electron micrographs of maize starch granules after hydrolysis by *Aspergillus fumigatus* (K-27) alpha-amylase: (a, c, e) SEM, and (b, d, f) TEM. Micrographs (a) and (b) show high-amylose maize with an extent of hydrolysis of 50%; (c) and (d) show normal maize with an extent of degradation of 15%; (e) and (f) show waxy maize with an extent of degradation of 22%. Bars correspond to 1 μm [100].

the starch granule (Fig. 7). Lastly, on the basis of electron and atomic force microscopy observations, Gallant et al. [98] and Baldwin [79] proposed that lamellae are organized in spherical blocklets which range in diameter from 20 to 500 nm, depending on the botanical origin of starch and their location within the granule. These conclusions are based on observations made on thin silver stained sections of hydrolyzed granules and on protusions observed by AFM at the surface of potato and wheat starch granules. Thomson et al. [99] also observed by AFM the surface of wheat starch granules, and saw features between 50 and 450 nm in diameter. Such features compare well with the surface protusions seen by Baldwin [79]. Unfortunately, Thomson et al. believed them to be a contaminant, possibly protein; thus, further confirmations are needed for to validate the blocklet structure concept.

The crystalline material, as defined by its volume fraction, morphology, distribution and crystalline type, is considered to be an important factor in defining the rate and extent of enzymatic hydrolysis. Investigation of starch amylolysis is a way to refine our understanding of starch granule structure. Indeed the crystalline material behaviour during hydrolysis shows susceptibility differences, reflecting some structural features. Some authors claim that during α-amylolysis the less crystalline parts of the starch granules are more easily digested than the crystalline parts. Although enzymatic hydrolysis of starch can sometimes lead to a layered and concentric shell structure, as well as for acid hydrolysis, no work has reported an

increase in crystallinity after amylolysis of native starch granule. Moreover, previous experiments [100] have shown that the botanical type determines the composition of the resistant structures recovered. These structures consist either of highly degraded and pitted granules (even for a hydrolysis extent as low as 15–25%) or fragmented external shells (Fig. 6), suggesting that the structures do not strictly correspond to the alternative crystalline and amorphous shells proposed by Jenkins et al. [101]. Results could be interpreted on the basis of the fact that arrangement of crystallites inside the granule is responsible for the enzymatic susceptibility, whatever the crystallite type. It is difficult to design experiments which provide meaningful analysis of the intricate relation existing between different structural levels and direct amylolytic susceptibilities. Starches which are very resistant towards amylolysis are of the B type, such as potato or cereal with high amylose content starches. One of the factors limiting their hydrolysis could be the nature of the granule surface with respect both to crystallinity [82] and to the presence of adsorbed non-starch material [102]. Both would impede enzyme action. The new techniques, e.g. synchrotron radiation or AFM, would allow these factors to be investigated.

3.5. The crystalline nature of starch

Native starch granules exhibit two main types of X-ray diffraction diagrams (Fig. 8), the A type for cereal starches and the B type for tuber and amylose-rich starches [103–105]. Another C-type diffraction diagram, which has been shown to be a mixture of A- and B-type diagrams [71], is characteristic of most legume starches [30], and also from cereals grown in specific conditions of temperature and hydration [106]. The crystalline V-form characteristic of amylose complexed with fatty acids and monoglycerides, which appears upon gelatinization of starch, is rarely detected in native starches [107], although it has been proved, using solid-state NMR, that amorphous amylose–lipid complexes are present in native maize, rice and oat starches [70].

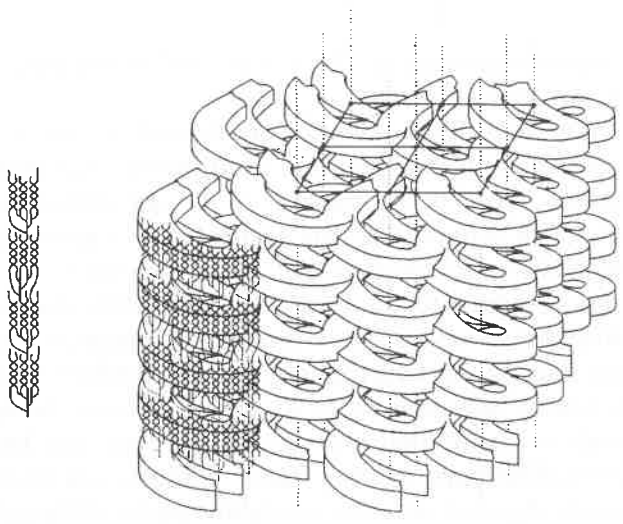

Fig. 7. Schematic model for the arrangement of amylopectin in potato starch in a 'super-helical' organization (from Oostergetel and van Bruggen [97]).

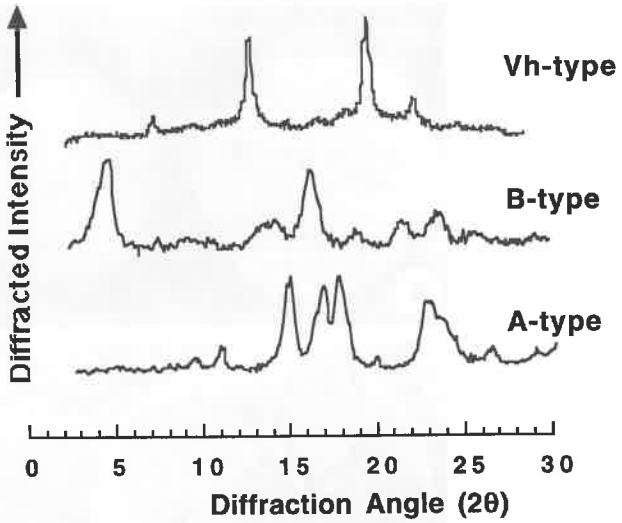

Fig. 8. X-ray diffraction diagrams of A-, B- and Vh-type starch.

The structural models established from A and B amylose crystals were transposed to crystalline regions of starch, since the main reflections contained in the powder diffraction diagrams of native starch were present in the diffractograms of these crystals. More recently, Buléon et al. [82] have recorded some fibre-like diffraction patterns within single potato starch granules using microfocus (2 μm) synchrotron analysis. These diagrams have proved to be very similar to those obtained from the B-type amylose fibrillar crystals obtained by Wu and Sarko [54], which shows that the experimental data used for solving the B-type structure of amylose can be directly transposed to B-type starch.

As mentioned above, amylopectin is usually assumed to support the framework of the crystalline regions in the starch granule. Hence, the short chains with polymerization degrees ranging be-

tween 15 and 18 probably have a double helical conformation (Fig. 5) compatible with the models described above. Moreover, the same short chains were used, after extraction from native starch by mild acid hydrolysis, for the preparation of the A and B lamellar crystals mentioned above. In any case, the lamellae are believed to represent the crystalline (side-chain clusters) and the amorphous regions (branching regions) of the amylopectin molecule according to the model of Robin et al. [26]. A major uncertainty remains concerning the relative content of amorphous lamellae in the different models proposed. The density of the starch granule is very high (about 1.5 depending on the water content [108]) and slightly greater than that of lamellar crystals of A-amylose [57]. In the 9-nm repeat (crystalline + amorphous lamellae), the crystalline region represents 5–6 nm, assuming the double-helical model of Imberty and Perez [63] and a polymerization degree of 15 for amylopectin short chains. It is unlikely that only the branch points of amylopectin are present within the amorphous lamellae. In that case a density of 1.5 could not easily be reached.

The conformation of free amylose in the native starch granule is not known. It could also be partly involved in double helices with amylopectin short chains in the crystalline regions [109]. Kasensuwan and Jane [110], using NMR on an insoluble fraction of cross-linked starch, showed that amylose molecules are randomly interspersed among amylopectin and must be located in close proximity with amylopectin for them to be cross-linked. They concluded it is plausible that some large molecules participate in double helices with amylopectin, especially since amylose can only be completely extracted at temperatures above 90°C. Moreover, amylose chains could be involved in amylose–lipid complexes. V-crystallinity is rarely detected in native starches and most native cereal starches containing fatty acid and/or monoglycerides yield no diffraction diagram characteristic of the Vh structure. Gernat et al. [107] determined some amount ranging from 15 to 25% of the total crystalline phase for amylose-rich starches from maize and wrinkled pea. High-amylose starches are known to contain greater amounts of lipids; the extraction, purification and drying of starch processes may easily induce amylose–lipid complexes upon heating. Nevertheless, experimental evidence was given by Morrison et al. [70] that such complexes may be present in native starches in the amorphous state. In that case amylose chains are involved in separate (isolated single helices) inclusion compounds or arrangements of too limited an extent to show the Vh-type diffraction diagram. Such structures can reorganize in a detectable crystalline packing (propagation) in the presence of heat and moisture [71]. Morrison [111] determined amounts of lipid-complexed amylose ranging from 15–20% of total amylose for maize and wheat to 37–59% for waxy barley.

The crystalline nature of starch depends probably on both genetic control and climatic conditions during the plant growth. The chain length involved in the crystalline phase and the branching pattern in amylopectin molecules influence the crystallinity and the crystalline type [4]. However, temperature and hydration conditions during the plant growth may also induce some important changes in the A:B composition [106]. As for preparation of amylose crystals [56], the A type is obtained preferentially in dry and warm conditions as opposed to the B type (wet and cold conditions). In any case, it is still a real challenge for anyone working on starch biosynthesis and structure to elucidate the mechanism of starch crystallization and granule formation.

3.6. Starch and degree of crystallinity

A variety of techniques has been used to determine the absolute crystallinity of native starch [80,109] but the values obtained are very dependent on the technique used (Table 5). NMR and infrared spectroscopy yield something close to helicity while the easily degradable fractions drawn from acid or enzymatic hydrolysis curves may include a part of crystalline regions. Lastly, in methods based on moisture regain, porosity and surface of the granules have a strong influence. The major effort has been devoted to the use of X-ray diffraction. The determination of crystallinity of native starch is tricky because of the small crystal size and the role of hydration. Two main types of techniques are commonly used based on internal and external comparison, respectively. In the first ones, the areas corresponding to the respective contributions of amorphous and crystalline scattering are evaluated, as it was first described by Hermans and Weidinger [112]. In the second type of technique, experimental diagrams are compared to 100% crystallinity and completely amorphous diagrams [113]. Such a technique may

Table 5
Degree of crystallinity (%) of different starches determined by acid hydrolysis, X-ray diffraction and solid-state NMR [26,115,116]

Starch	Acid hydrolysis	X-ray diffraction	^{13}C-NMR
A-Type			
Normal maize	18.1–27.0	38–43	42–43
Waxy maize	19.7–28	38–48	48–53
High amylose maize	18.1	25	38
Wheat	20.0–27.4	36–39	39
Rice		38–39	49
B-Type			
Potato	18.1–24.0	25–40	40–50
Cassava	24.0	24	44

be very useful to determine relative crystallinities and compare some starches in the native state or during processes, but may only yield absolute crystallinity if the amorphous and crystalline standards have a true 0 and 100% crystallinity. The methods based on internal reference are used for determination of absolute crystallinity but there are such discrepancies in the published values that it is difficult to rely on such absolute crystallinity values. The values vary from 15 to 45% depending not only on the origin and the hydration of starch but also on the technique used [104,108,109]. In any case, the crystallinity has to be determined at a well defined hydration since it has been proved to depend strongly on it. For example, the crystallinity of B-type starches increases from 8 to 33% H_2O [108,114]. Gidley and colleagues [115,116], by comparison of solid-state NMR and X-ray diffraction data, observed that the double helix content in native starches (which is the detected order by NMR) is considerably higher than the crystallinity determined from X-ray diffraction. It was therefore concluded that molecular ordered regions containing double helices not involved in crystalline arrays were present within the granule due to either amylose or amylopectin.

Acid hydrolysis supposed to yield the crystalline resistant part of the granule is used to determine amorphous and crystalline parts. When the percentage of carbohydrate solubilized by acid is plotted versus time, the curve showed two different steps (Fig. 9). The first high rate step is attributed to the amorphous part of the granules while the second step tends towards a plateau [26]. Acid hydrolysis of starch granules might be deconvoluted into two first-order kinetics distinguishing the hydrolysis behaviour of two main structural organizations. Plotting $100/(100-X)$ (where X represents the hydrolysis extent in percent) versus time, makes these two phases conspicuous. The second part of the curve may be extrapolated at time zero, allowing the part of the granule susceptible to acid hydrolysis to be estimated, which is believed to correspond to the amorphous part. However, this degradable fraction seems to be more linked to amylose content than to crystallinity of the granule. Increasing amylose content appears to decrease the starch granule degradation by acid [117]. High amylose content mutants of maize or pea starches show a hydrolysis rate 2-fold less important than the normal or high amylopectin content equivalent starches. Remnant residues after acid hydrolysis of high-amylose starches show a polymerization degree higher than those obtained for other starches. While preferential acid hydrolysis fits with current observations and thinking regarding the possible organization of amorphous and crystalline zones, the validity of the acid hydrolysis method to determine content and organization of the crystalline part should be examined carefully. However, this approach is useful to isolate more crystalline residues and therefore to assess the organization of the starch granule crystalline regions. For instance, Oostergetel and van Bruggen [97], on the basis of observations using lintnerized starches, have been able to propose a three-dimensional reconstruction of the residual crystallites.

4. From precursors to macromolecules: an overview of starch biosynthesis

The presence of only one type of sugar residue and two distinct chemical linkages in starch granules have mislead biologists to believe that biosyn-

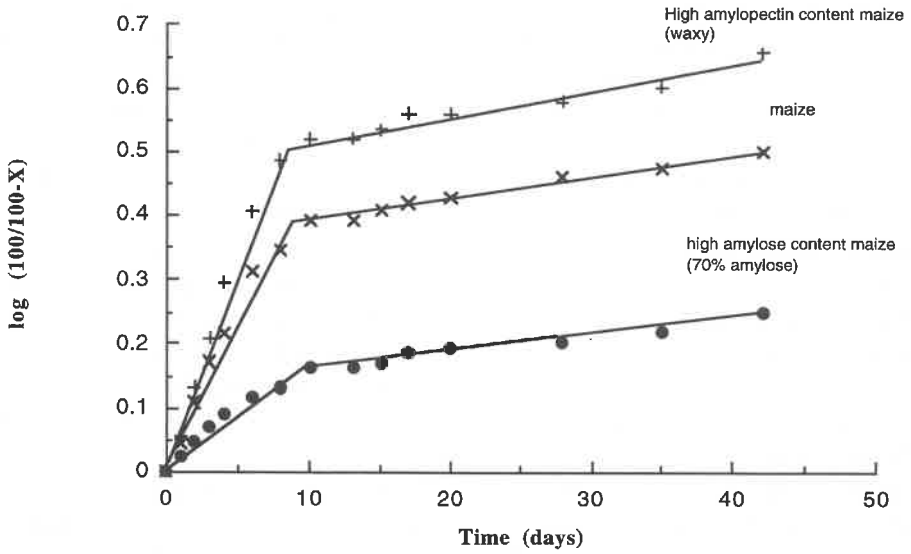

Fig. 9. Hydrolysis of waxy, normal and high-amylose-content maize starches in 2.2 N HCl at 35°C. Percentage of hydrolyzed starch (X) vs. time of hydolysis.

thesis of amylose and amylopectin consisted of a logical economical and exceedingly simple pathway. Such pre-conceived ideas were largely responsible for a long lasting lack of interest in starch biosynthesis. Similarly, such apparent and misleading simplicity discouraged many structural biologists from studying the structure of these polysaccharides, thus favouring investigations dealing with proteins, nucleic acids and complex oligo- or polysaccharides. This situation has been slowly changing and the picture of starch emerging today is that of a highly organized structure requiring sequential action of many different biosynthetic steps for its biogenesis.

4.1. The synthesis of the glucosyl–nucleotide substrate

An overview of starch biosynthesis is given in Fig. 10. ADP–glucose synthesis through ADP–glucose pyrophosphorylase occurs in all starch-accumulating plastids examined to date. Ghosh and Preiss [118] established that plant ADP–glucose pyrophosphorylases were regulated through allosteric activation by 3-PGA and inhibition by orthophosphate. Levi and Preiss [119] subsequently found that cyanobacterial glycogen synthesis through AGPase (ADP–glucose pyrophosphorylase) was regulated in a similar fashion. The in vivo concentrations reported for the effectors and substrates of AGPase fully support the hypothesis that the 3-PGA/P_i ratio regulates the rate of starch biosynthesis in plant leaves (reviewed by Preiss [120]). In storage tissues, AGPases with similar regulatory properties were purified from potato tubers, cereal endosperms and pea embryos (reviewed by Preiss [121]). In addition to these enzymes, evidence for the existence in endosperm of cereals of a cytosolic form of AGPase is accumulating. In barley, both the purified and recombinant starchy endosperm enzymes display a lowered sensitivity to 3-PGA activation [122]. Since the other enzymes of the starch pathway are located in the plastid, this observation implies the existence of an import mechanism. The adenylate translocator encoded by the maize *BT1* gene could fulfil this function [123]. Indeed the phenotype due to the maize *bt1* mutations

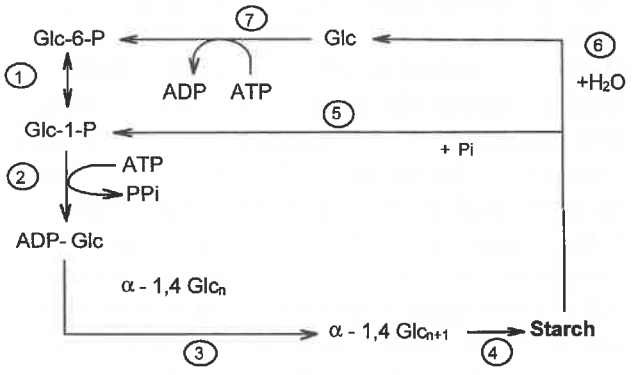

Fig. 10. General scheme for starch biosynthesis: (1) phosphoglucomutase; (2) ADP–glucose pyrophosphorylase; (3) granule-bound and soluble starch synthases; (4) branching enzymes; (5) starch phosphorylase; (6) amylases, debranching enzymes, maltases; (7) hexokinase.

consists of a dramatic decrease in starch amount accompanied by a substantial increase in ADP–glucose concentration [124], both of which are to be expected in the absence of the putative translocator. It must be stressed that these results do not question either the importance of AGPase as a rate-limiting step or that of allosteric regulations of the enzyme. The existence of the cytosolic enzyme can be envisioned as a late refinement of the biosynthetic pathway particularly suited to the production of storage starch from sucrose in cereals.

Ever since the initial purification of the spinach leaf enzyme by Ribereau-Gayon and Preiss [125], it appeared that all plant enzymes purified to date, including those from unicellular green algae, are heterotetramers ($\alpha_2\beta_2$) containing two so-called large and small subunits of related primary structures and similar molecular mass within the 50–55 kDa range [121]. Cyanobacteria were proved to contain homotetramers [126]. Expression of the corresponding cDNAs in *Escherichia coli* demonstrates that the small subunits are essential for catalysis while the large subunits modulate the response to the allosteric effectors [127,128]. This was also confirmed by mutant analysis in higher plants and green algae [129–131].

The in vivo evidence supporting the function of AGPase in starch biosynthesis can be traced back to the pioneering work of Tsai and Nelson [132] who found a selective defect in ADP–glucose pyrophosphorylase in the *sh2* (shrunken 2) mutants of maize that displayed a 70% reduction in starch content. Mutations of AGPase have since been described in many other plant species including *bt2* [133] and *sh2* in maize, *adg1* [134] and *adg2* [135] in Arabidopsis, *rb* [136] in peas, and *sta1* [129,131] and *sta6* in *Chlamydomonas reinhardtii*. Moreover, antisense RNA directed against the small subunit of the potato tuber AGPase successfully inhibited starch synthesis in tubers [137]. Conversely, plastid-directed expression of mutant bacterial AGPase with unusually high specific activity successfully increased starch content in transgenic potatoes [138]. In maize, revertants of *sh2* mutants were isolated with increased AGPase activity [139]. These revertants also lead to starch overproduction. To summarize the impressive amount of data generated through these mutant analyses, we will state that both up- and down-regulating AGPase lead to proportional increases and decreases in the rates of starch biosynthesis. These experiments thus establish AGPase as the major control point for starch biosynthesis in plants. The work performed in Arabidopsis and *C. reinhardtii* also demonstrate that the absence of the small subunit encoded by *ADG2* and *STA6* leads to a collapse of starch biosynthesis, while disappearance of the large subunit encoded by the *ADG1* and *STA1* genes yields an incompletely defective phenotype. Absence of the large subunit of the *Chlamydomonas* heterotetramer yields an enzyme selectively unresponsive to 3-PGA activation, confirming the function established for the large subunit in the regulation of AGPase activity [129,131].

In cyanobacteria, glucose-1-P generated through phosphoglucomutase from glucose-6-P is an essential step of many different biochemical pathways. In plants, distinct phosphoglucomutases encoded by different genes are found both in the cytosol and in the plastid. In addition, hexose phosphate transport across the plastid membranes has been evidenced in pea embryos [140], *C. reinhardtii* (Uwe Klein, personal communication, 1997) and wheat [141,142]. It presently appears that the generation of glucose-1-P for starch biosynthesis from plastidic phosphoglucomutase is the sole function of this enzyme. This made possible the isolation of mutants defective for the plastidic form of the enzyme. The severity of the phenotype largely depended on the nature of the hexose-phosphates translocated between the cytosol and the plastid. An extremely severe phenotype (a 96–99% drop in starch content) was reported for the *rug3* mutants of pea [143], the *pgmP* [144] mutant of Arabidopsis and the phosphoglucomutase mutant of *Nicotiana sylvestris* [145]. On the other hand, a disappearance of plastidic phosphoglucomutase is correlated to a less severe phenotype in *C. reinhardtii* [131] (an 85–95% decrease in starch amounts). It must be pointed out that glucose-1-P translocation was reported for *C. reinhardtii* chloroplasts, while the selective import of glucose-6-P was documented in peas. Plastidic phosphoglucomutase would also be logically required for metabolism of glucose-1-P generated by phosphorylase during starch breakdown. However, evidence for this dual function is still lacking.

Mutants defective in the supply of the glucosyl nucleotide through defects in ADP–glucose pyrophosphorylase or phosphoglucomutase all display, as expected, a substantial decrease in the rates of starch biosynthesis and therefore in yield.

Little attention was paid to the structure of the residual starch in these mutants. Van den Koornhuyse et al. [131] showed that in *C. reinhardtii*, mutants defective either for phosphoglucomutase or for the large subunit of ADP–glucose pyrohosphorylase accumulate polysaccharides whose structure were identical to those of transitory starch. Transitory starch is accumulated during the day by leaf cell chloroplasts and broken down during the night. The structure of transitory starch differs from that of the storage polysaccharide by a decrease in amylose content and a modification in amylopectin structure [146]. In microalgae such as *C. reinhardtii*, transitory starch-like polysaccharides are synthesized during the log phase [147], while storage starch synthesis requires a block in cell divisions such as that achieved under nitrogen starvation. The mutants defective for the large subunit of AGPase in *C. reinhardtii* accumulate under nitrogen starvation a polysaccharide virtually identical to that synthesized by log-phase wild-type cultures [131,147]. The balance of enzyme activities known to be involved in starch biosynthesis was not influenced by the drop in substrate supply. It was therefore proposed that the profound modifications witnessed in the structure were due to the presence of elongation enzymes characterized by different affinities for their common ADP–glucose substrate. It is known for instance that GBSSI (granule-bound starch synthase I), the enzyme responsible for amylose biosynthesis, displays of all starch synthases the lowest affinity for the nucleotide–sugar [121]. It thus appears logical to detect a net relative decrease in amylose biosynthesis in these mutants. Van den Koornhuyse et al. [131] thus concluded that substrate supply could directly control polysaccharide structure. This would directly tie starch structure to carbon metabolism and optimize it either for photosynthesis (short-term carbon storage) or for long-term carbon storage. It would be, in this respect, of great interest to assay the crystallinity of storage and transient starches, and to measure the rate of starch degradation in each case in vitro using the same set of catabolic enzymes.

4.2. Elongation of the α-1,4-linked glucans

It was apparent ever since the pioneering work of Leloir and colleagues [148,149] that glucose was transferred from ADP–glucose to the non-reducing end of a growing α-1,4-linked glucan, thus generating an extra glucose with the simultaneous release of ADP. The enzyme was identified by Leloir et al. as associated with starch granules [148] and was subsequently called granule-bound starch synthase. GBSS is also able to use non-physiological concentrations of UDP–glucose as a substrate with lesser efficiency. Other enzymes were later identified in the soluble extracts. At least one of the soluble enzymes is found as a minor granule-associated protein [150] while GBSSI often constitutes over 90% of the total proteins bound to starch. All these enzymes are thought to be active components of the starch synthesizing machinery. It is not yet entirely clear as to the exact number of soluble isoforms required to make starch. Two such forms are detected in *C. reinhardtii* [151,152] and pea embryos [153], while three were found in potato tubers [154] and maize endosperm [155]. Since the relative contributions of the different soluble starch synthases may vary widely between organs and species, it is quite possible that the third soluble enzyme escaped detection in green algae. It is also possible that the number of soluble isoforms increased during evolution of vascular plants. The picture is further complicated by a report describing primer-dependent glucan synthases outside the chloroplast [156]. The soluble starch synthases can be distinguished from GBSSI and the glycogen synthases by the presence of N-terminal extensions. While these extensions are unlikely to be active in catalysis they may be involved in the building of multi-enzyme complexes together with the appropriate branching enzymes.

It has been known, since the discoveries by Nelson and Rines [157] regarding the enzymatic deficiency in the waxy mutant of maize, that GBSSI is responsible for the biosynthesis of the amylose fraction. Mutations leading to defects for GBSSI have been isolated in an ever increasing number of species, including *wx* (waxy) maize [158], *wx* rice [159], *wx* barley [160], *wx* wheat [161], *amf* (amylose-free) potato [162], *lam* (low amylose) pea [163], *wx* amaranth [164] and *sta2 C. reinhardtii* [165]. All mutants accumulate during storage normal amounts of starch granules containing amylopectin with wild-type crystalline organization. These important results establish that amylose is not required for the biogenesis of normal granules. A number of studies focusing on the synthesis of amylose in vitro establish that GBSSI

incorporates glucose both in amylopectin and amylose according to the conditions used in these experiments. Leloir originally noted a stimulation of GBSSI by high concentrations of maltooligosaccharides [148] and found incorporation of radioactive glucose into both starch fractions. In a very recent study, Denyer et al. [166] showed that, in the absence of these oligosaccharides, the labelled product synthesized in vitro by GBSSI was confined to the amylopectin fraction. However in the presence of very high maltooligosaccharide concentrations, GBSSI massively incorporated glucose into amylose-like glucans. The effect could be mimicked by concentrated crude potato tuber or pea embryo homogenates, suggesting that maltooligosaccharides could be at work in vivo. Denyer et al. [166] thus speculate that maltooligosaccharides could trigger amylose synthesis in storage starch when their concentration becomes high enough to be physiologically active. It is thus tempting to suppose that the oligosaccharides are used as primers for the synthesis of amylose within starch granules. In vivo evidence supporting the involvement of GBSSI in amylopectin synthesis was produced by Maddelein et al. [167]. In this study, genetic interaction experiments clearly showed that defects in GBSSI strongly reduced amylopectin synthesis in particular mutant backgrounds.

The first evidence for a function of the soluble starch synthases in the biosynthesis of amylopectin can be traced back to 1980 when Preiss and Boyer [168] showed that the maize 'dull' mutants displayed a strong reduction in the activity of one particular soluble starch synthase. However, both SSII and BEIIa (branching enzyme) clearly decreased in the maize *du* mutants and it was not until 1998 that Gao et al. [169] demonstrated that *DU* was the structural gene for a maize-soluble starch synthase of 186 kDa related to the SSIII of potato tubers. The phenotype of dull mutants consists of a small reduction in starch amount, the appearance of a more highly branched amylopectin and the accumulation of 15% of starch as intermediate material. According to the genetic background, maize dull mutants can also be considered as accumulating high-amylose starch, thus suggesting a selective defect in amylopectin biosynthesis. Two analogous mutants have been reported in *C. reinhardtii* (*sta3*) [151] and in peas (*rug5*) [143,170]. The *C. reinhardtii* mutants displayed a marked decrease in starch content, a high-amylose phenotype and a substantial modification of the amylopectin structure. The latter consisted of relative decrease in intermediate-size chains and an increase of very small glucans with a maximum of six glucose residues in the chain-length distribution. The crystallinity of the starch collapsed and switched from the A to the B type. The addition of a defect in the GBSSI structural gene leads in these mutants to the production of minute amounts of a very highly branched granular polysaccharide with C-type crystalline organization [152]. All these defects could be traced back to the disappearance of a 115-kDa soluble starch synthase activity. While this is the sole defect witnessed in the mutants, it remains to be shown that the *STA3* locus encodes the missing starch synthase. Similar mutants were isolated in peas which also displayed a reduction in starch content, an increase in amylose percentage and a decrease of intermediate-length glucans within the residual amylopectin. All these modifications are due to the disappearance of one particular soluble starch synthase. In this case, however, the mutant gene is known to encode SSII which is also found associated to the starch granule (GBSSII) in peas and potatoes. Finally, antisense constructs directed against the minor SSIII of potato tubers yielded T-shaped cracks at the surface of potato starch granules and increased levels of phosphate [171]. However, the structure of the polysaccharides were nowhere near as affected as those caused by *sta3*, *rug5* or *du* mutations. Another likely candidate for a soluble starch synthase defect is the maize *su2* (sugary) mutant which displays many of the phenotypic attributes of the pea *rug5* and *C. reinhardtii sta3* mutants [172]. Both the nature of the enzymological defect and the function encoded by *SU2* still need to be established. All these experiments prove that the soluble starch synthases are major components of the amylopectin synthesis machinery. It is too early, however, to speculate on the respective functions of the different starch synthase isoforms. In order to get a clearer picture, we definitively need additional information on the in vitro specificities of the recombinant enzymes. Equally important are investigations performed on the enzymes extracted from the plant tissues. Indeed these enzymes are likely to exist as active complexes together with specific branching enzymes, as was suggested by the finding of a BEIIa defect in the dull mutant of maize. Moreover, we still need selection of addi-

tional mutants and genetic interaction studies before the selective functions of each starch synthase in the complex pattern of amylopectin synthesis become clearer.

4.3. Introducing the α-1,6 branch

A branching enzyme, formerly called the Q-enzyme, was described in potato tubers as far back as 1945 [173]. The potato enzyme (BEI) was subsequently shown to be able to catalyze inter-chain transfer, together with possible intra-chain transfers [174]. In all plants analyzed in sufficient detail, there appears to be at least one isoform of branching enzyme belonging to two distinct families of enzyme (reviewed by Martin and Smith [175]). Enzymes of the A family (BEIIa and BEIIb from maize, BEI from peas, BEIII from rice) prefer amylopectin as an in vitro substrate and transfer short chains, while enzymes of the B family (potato Q-enzyme, maize BEI, pea BEII, rice BEI) prefer longer amylose-like chains and transfer longer chains [176,177]. It has also been recently shown that branching enzymes differ with respect to the minimal size of the glucan that can be used as a substrate for branching [178]. In vitro experiments performed with recombinant *E. coli* glycogen and maize starch branching enzymes suggest that this minimal size ranges from DP 12 for both glycogen branching enzyme and A-family starch branching enzyme to DP 16 for starch branching enzyme of the B family [178]. Moreover, GBE (glycogen branching enzyme) transfers chains ranging between DP 5 and DP 16 while the size of the chains transferred by the plant branching enzyme ranges from DP 6 to over DP 30. Another difference between branching enzymes of the A and B families can be found in the ability displayed by branching enzymes of the A family to transfer very short chains from DP 3 to DP 9 with a clear maximum at DP 6 and DP 7. Another distinction comes with the response of these enzymes to orthophosphate [179]: branching enzymes of the B family are activated 5-fold by orthophosphate while enzymes of the A family show a 1.5-fold maximal activation. It presently appears that both types of enzymes also differ with respect to expression patterns during starch accumulation. In cereal endosperm [179] and pea embryos [180], constitutive expression of branching enzymes of the A family was found while enzymes of the B family were systematically expressed later. Clear-cut involvement of branching enzymes of the A family in amylopectin synthesis has been demonstrated in pea [181], rice [182] and maize [183]. In all cases, the mutants displayed a similar phenotype: a reduction in starch correlated to a large increase both in amylose and in a fraction that was called intermediate material (a name which is seemingly applied to polysaccharides of very different structure [41]). In maize this is accompanied by a switch from the A to the B type of diffraction pattern and by a net loss in crystallinity.

Despite the resemblance in chain-length distributions shown between amylopectin and the in vitro products generated by branching enzymes on amylose-like glucans, branching enzymes do not mediate starch biosynthesis when expressed in *E. coli* [184]. In fact, recombinant bacteria lacking their own glycogen branching enzyme synthesize glycogen-like polymers and not starch in the presence of either or both maize endosperm branching enzymes. However, these glycogen-like polymers also displayed chain-length distributions which are in agreement with their in vitro specificities. We may conclude here by stating that the branching enzymes, while important, are not sufficient to lead to starch biosynthesis. Other structural determinants have thus to be sought. These may be found in the interaction existing between the branching enzymes and the starch synthases of diverse specificities. Another intriguing possibility is that additional functions may be required to order the polysaccharides during synthesis.

4.4. Pre-amylopectin processing

The *sugary-1* mutants of maize have been known for decades to accumulate, in addition to starch, a novel form of water-soluble polysaccharide (WSP) [185]. The structure of this WSP can be compared to that which characterizes animal glycogen and was consequently named phytoglycogen. As early as 1958, Erlander [186] proposed that glycogen could be considered as a precursor of starch biosynthesis. His initial suggestion was that amylopectin would be produced by debranching this precursor and that amylose would be subsequently generated through debranching of amylopectin. Ever since this proposal, several researchers have been actively searching for a debranching enzyme deficiency in *su1* maize. Surprisingly, the first differences re-

ported between wild-type and *su1* maize consisted in the appearance of an unusual form of branching enzyme in the mutants [187–189]. This activity, tentatively called phytoglycogen branching enzyme, was thought responsible for the formation of a polysaccharide with an abnormal branching ratio: phytoglycogen. The presence of phytoglycogen in the mutants would thus be explained by the presence of an abnormal form of branching enzyme and would not necessarily reflect a block in the amylopectin synthesis pathway. This hypothesis is still elegantly sustained nowadays by Manners [190]. It is, however, an established fact that of all the endosperm mutants of maize, *ae* (amylose-extender) is the only mutation which prevents phytoglycogen synthesis [191]. Since it is has been demonstrated that *AE* encodes BEIIa (a standard branching enzyme of the A family), we must therefore assume BEIIa to be phytoglycogen branching enzyme. Since *AE* and *SU1* are known to be distinct genes, *SU1* cannot thus be the structural gene for phytoglycogen branching enzyme. A first clue with respect to the nature of *su1* came with the observation made by Pan and Nelson [192] that *su1* maize lacks one form of debranching enzyme. This activity was shown to be able to cleave pullulan: a homopolymer of maltotriosyl residues linked by α-1,6 branches at the non-reducing ends. A definitive proof concerning the nature of the *SU1* gene product was established by James et al. [193] who found that *su1* encoded a particular debranching enzyme. However, the sequence displayed high degrees of homology with bacterial isoamylases. Isoamylase-type debranching enzymes are reported to cleave the branches of glycogen and amylopectin but not of pullulan. Moreover, the recombinant *SU1* gene product was demonstrated to lack pullulanase activity (Alan Myers, personal communication, 1997). Similar results were obtained in rice, where again the presence of phytoglycogen could be correlated to the decrease of pullulanase activity [194]. In this case no reports concerning the presence of phytoglycogen branching enzyme were made. In addition, the sugary deficiency of rice did not encode the rice pullulanase that was cloned [194,195]. In *C. reinhardtii*, mutants accumulating less than 1% of the wild-type amount of granular starch were shown to synthesize up to 5% of water-soluble polysaccharide in the form of phytoglycogen. The phenotype was correlated to the disappearance of an 88-kD debranching enzyme activity [196]. It has been shown recently that the native purified enzyme displayed an isoamylase type of specificity and was unable to cleave pullulan. The severity of the phenotype displayed by the *C. reinhardtii sta7* mutants proves that debranching of an intermediate of amylopectin synthesis is mandatory in order to get significant starch synthesis in plants. On the basis of these results and also of those concerning the minimal requirements of the branching enzymes, a hypothetical scheme for amylopectin synthesis has been proposed by several researchers [197].

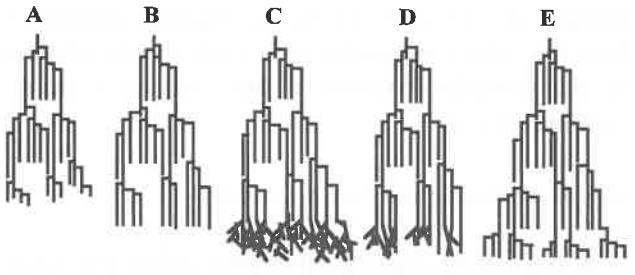

Fig. 11. A two-dimensional model for starch biogenesis occurring at the surface of the granule. Elongation starts from a trimmed amorphous lamella depicted in (A), and proceeds through (B) until the critical size needed to accomodate the branching enzymes is reached. Then, because of the presence of high branching enzyme specific activities, random branching will occur (C). Debranching activities will simultaneously trim the loosely branched glucans down (D). This will prevent phytoglycogen synthesis and spare the tightly spaced branches, thereby generating the next amorphous lamella (E) [196].

The model proposed in Fig. 11 for plant amylopectin synthesis explains how the asymmetrical distribution of branches in amylopectin could be generated.

Briefly, a previously synthesized amorphous lamella containing tightly spaced branches is being elongated by a specific soluble starch synthase. No branching occurs at this first step. We explained this [197] by assuming that branching is prevented because the branching enzymes need a minimal length for the glucan to adapt in their catalytic site. As soon as this length is attained, unordered branching and elongation occur simultaneously. At the same time, debranching enzymes will trim down the structure into the next amorphous lamella. This model was named the discontinuous synthesis model for amylopectin synthesis. It explains the amylopectin cluster constant size throughout the plant kingdom [101], and relates the size of the crystalline lamella and therefore of the amylopectin cluster to the minimal requirements of the branching enzymes.

Quite interestingly, it was subsequently found that maize BEI and BEII require, respectively, a minimum number of 16 and 12 glucose residues for branching to occur [178]. A glucan of 12 residues is of the very same size as that measured for the crystalline lamella of maize amylopectin. It is comforting to realise that mutants of maize defective for BEII and thus retaining only BEI show an increase of the size of this lamella from that which characterizes a DP 12 glucan to that of a DP 16 glucan [198].

4.5. The beginning of starch: priming the starch granule

Starch granules are known to display a core known as the hilum with a relatively disorganized structure. It is generally thought that the number of granules and therefore their size distribution is limited through low-frequency starch granule priming. It is tempting to speculate that polysaccharide synthesis priming might be the major biochemical event associated with hilum formation. Because glycogen and amylopectin belong to the same family of storage polysaccharides, it is suspected that synthesis of these molecules might be primed by a common mechanism. The presence of proteins with autoglucosylating abilities physically associated with mammalian glycogen granules and glycogen synthases has been documented for years (for review see Alonso et al. [199]). A mechanism for glycogen granule priming has been suggested to involve UDP–glucose-mediated autoglucosylation of these glycogenins through *O*-glycosylation of specific tyrosine residues followed by elongation catalyzed by the glycogen synthases. In yeast, cloning of glycogenin-analogue structural genes was achieved using the double-hybrid technique [200]. This technique allows for detection of any gene product physically interacting with a protein of interest, in this case yeast glycogen synthase. This led to the identification of a protein phosphatase and a glycogenin-like protein. Two yeast glycogenins were thus detected and both genes were disrupted [200]. Inhibition of any of the two *GLG* genes did not yield a clear phenotype, while blocking both severely impaired glycogen synthesis. This result is of considerable importance for our understanding of polysaccharide synthesis and establishes the biological relevance of glycogenin proteins. It provides a piece of evidence that was needed by those supporting glycogenin's function in polysaccharide priming. However, it must be stressed that because the whole *Saccharomyces cerevisiae* genome has been sequenced, we are presently certain that the yeast mutant strains carrying the double gene disruptions do not synthesize any other protein with significant homology to mammalian or fungal glycogenins. Yet these mutants still synthesise 1–5% of the wild-type amount of glycogen, which throws into the question the absolute requirement of these proteins for polysaccharide priming. Indeed, expressing a high-specific-activity mutant yeast glycogen synthase on a multi-copy vector fully restores glycogen synthesis in the mutants (Peter Roach, personal communication, 1997). This result might suggest either that the mutant glycogen synthase might satisfy itself with otherwise inefficient biological primers or that the synthase by itself can prime the reaction at a low frequency. Because of the comparatively small number of polysaccharide granules, low-frequency priming might be sufficient to warrant full starch priming. Both bacterial glycogen synthase [201] and soluble starch synthases purified from maize endosperm [202] and spinach leaf [203] are capable of priming glucan synthesis in the presence of 0.5 M citrate. This result has been confirmed by the purification of recombinant maize soluble starch synthase [204]. It is therefore quite possible that both starch or bacterial glycogen granule priming do not require the autoglucosylating glycogenin-like proteins identified so far. Indeed, it might very well be that such proteins [205–207] which favour UDP–glucose over ADP–glucose for autoglycosylation are required for priming the synthesis of other polysaccharides. This is further suggested by the nature of the glucosyl–protein linkage, which was shown to be β-glucosylarginine [208] instead of α-glucosyltyrosine, and by the fact that no homology could be found between glycogenin and the so-called amylogenin cDNA sequences [208]. It is thus possible that animal or fungal glycogenins might have evolved later in order to assist both the shaping and high-frequency priming required for eukaryotic glycogen synthesis.

5. Towards tailoring novel starch polymers in plants

Three different strategies are available to modify starch biosynthesis in plants in order to obtain

Table 6
Phenotypes of mutants of *C. reinhardtii* defective for loci involved in starch biosynthesis

Locus	Phenotype	Iodine stain	Starch[a] (%)	WSP[a] (%)	Defective enzyme[b]	Maize[c]	Pea[c]
STA1	Low starch	Yellow	5	0	AGPase (LS)	*sh2*	*rb*
STA2	No amylose	Red	100	0	GBSS	*wx*	*lam*
STA3	High amylose	Green	25	0	SSS II	*du*	*rug5*
STA4	High amylose	Green	40	0	Unknown		
STA5	Low starch	Yellow	10	0	Pgm	—	*rug3*
STA6	Low starch	Yellow	0.1	0	AGPase (SS)	*bt2*	
STA7	Low starch	Yellow	0.1	5	Isoamylase	*su1*	
STA8	High amylose	Green	20	5	Unknown		

[a] Starch and water-soluble polysaccharide amounts accumulated by mutant strains expressed as percentages of the amount of starch accumulated by wild-type reference strains. These relative amounts were assayed from nitrogen-starved cultures.
[b] AGPase (SS) and AGPase (LS) stand for ADP–glucose pyrophosphorylase small and large subunits, respectively.
[c] The names of analogous loci are listed for maize and peas, respectively. For instance —, *rug3* means that no analogue was reported for maize, while the *rug3* locus of pea is the analogue of *sta5*; *su1*, — means that *su1* is the maize analogue of *sta7* while no analogue was reported in peas.

starch-like polymers with novel functional properties:

- Knocking out biosynthetic enzymes through selection of mutations;
- Pulling down biosynthetic enzyme expression by mutations or by directing antisense RNA against them;
- Expressing heterologous genes related or unrelated to the biosynthetic pathway.

5.1. Knocking out biosynthetic enzymes through selection of mutations

Mutations in genes encoding biosynthetic enzymes often prevent completely the synthesis of active protein, thereby blocking the biochemical step controlled by the missing enzyme. The severity of the phenotypes witnessed in vascular plants can vary because of slight differences due either to functional redundancy of some of the multiple enzyme forms or minor differences in their plastidial or extraplastidial cellular localization. It is comforting to notice, however, that the same basic effects are found in all plants examined to date. The plant systems most intensively studied are maize, pea, rice, Arabidopsis and the unicellular green alga *C. reinhardtii*. Table 6 includes all genetic loci identified by mutation in *C. reinhardtii*. The phenotypic effects in pea and maize are analogous. The only locus of importance that has not been identified in algae consists of the equivalent of the maize *ae* (amylose-extender) or pea *r* mutations inactivating a branching enzyme of the A family (BE A) and leading to the production of high-amylose starch [181,183].

Selection of such mutants and their use in breeding programmes enables the starch-processing industry to choose from a variety of starch polymers with no or very little amylose (carrying mutations in the GBSSI structural genes) or, on the contrary, with very high amylose (carrying mutations either in the BE A or SS (soluble starch synthase) structural genes). Blocking the supply of ADP–glucose or the pre-amylopectin processing pathway leads to significant yield loss and accumulation of sucrose in seeds or tubers of vascular plants, offering a set of sweet-flavoured storage organs for the food industry. In some circumstances, blocking ADP–glucose supply can lower the amylose content, while preventing normal processing of pre-amylopectin can also lead to high-amylose starch. Breeding these characteristics into polyploid crops, however, has been hampered by the presence of multiple functional genes and the recessivity of the mutant blocks. These technical bottlenecks are now being obviated through the use of transgenesis and the antisense RNA strategy.

5.2. Reducing expression of biosynthetic enzymes by directing antisense RNA against them

Introducing part of a gene coding for a starch biosynthetic enzyme transcribed in the opposite direction will lead to the production of antisense

RNA and therefore a partial block in the expression of the sense RNA from the corresponding plant gene. Antisense RNA mediated blocks are dominant and can thus be successfully applied to hexaploid wheat or tetraploid potato breeding. Moreover, expression of genes corresponding to proteins possibly involved in biosynthesis but for which mutations have not been selected can also be reduced through the use of this technique. This allowed very recently the production of both high-amylose and amyloseless potato starch. In addition, the biochemical function of the R1 protein in amylopectin phosphorylation was recently discovered through this strategy [209]. However, the block occasioned by the antisense RNA is often incomplete. Consequently, the benefits to be expected will be limited by the amount of residual activity which in many cases can be sufficient to overcome the antisense block. On the other hand, incomplete blocks may also be desired in a few instances. With the exception of novel functions such as that mediated by R1, the benefits to be expected are the same as those offered by the mutant approach as exemplified in Table 6.

5.3. Expressing heterologous genes related or unrelated to the biosynthetic pathway

From the information available in this review, it seems obvious that the biosynthetic pathway can be manipulated to produce novel polymers through heterologous gene expression. For instance, expression in potato tuber amyloplasts of a gene encoding a deregulated *E. coli* AGPase with high specific activity triggers a net increase in starch biosynthesis and content [138]. Expressing bacterial debranching enzymes or bacterial glycogen branching enzymes [210] and glycogen synthases [211] will interfere with the corresponding starch pathway in an often unpredictable way. An increase in amylopectin branching has thus been achieved with glycogen branching enzymes in potato tubers [210]. Moreover, increasing cereal starch phosphorylation through heterologous overexpression of the potato R1 cDNA can no longer be considered a far-fetched dream. Last but not least, expressing genes unrelated to the starch or glycogen pathways could lead to the introduction of novel linkages or of other chemical groups including other carbohydrates. This entirely new polysaccharide design is currently being undertaken.

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