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Review Article

Design starch: stochastic modeling of starch granule biogenesis

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Starch is the most widespread and abundant storage carbohydrate in plants and the main source of carbohydrate in the human diet. Owing to its remarkable properties and commercial applications, starch is still of growing interest. Its unique granular structure made of intercalated layers of amylopectin and amylose has been unraveled thanks to recent progress in microscopic imaging, but the origin of such periodicity is still under debate. Both amylose and amylopectin are made of linear chains of α-1,4-bound glucose residues, with branch points formed by α-1,6 linkages. The net difference in the distribution of chain lengths and the branching pattern of amylose (mainly linear), compared with amylopectin (racemose structure), leads to different physico-chemical properties. Amylose is an amorphous and soluble polysaccharide, whereas amylopectin is insoluble and exhibits a highly organized structure of densely packed double helices formed between neighboring linear chains. Contrarily to starch degradation that has been investigated since the early 20th century, starch production is still poorly understood. Most enzymes involved in starch growth (elongation, branching, debranching, and partial hydrolysis) are now identified. However, their specific action, their interplay (cooperative or competitive), and their kinetic properties are still largely unknown. After reviewing recent results on starch structure and starch growth and degradation enzymatic activity, we discuss recent results and current challenges for growing polysaccharides on granular surface. Finally, we highlight the importance of novel stochastic models to support the analysis of recent and complex experimental results, and to address how macroscopic properties emerge from enzymatic activity and structural rearrangements.

Introduction

Starch is the most widespread and abundant storage carbohydrate in plants and the main source of carbohydrate in the human diet. Moreover, it is increasingly used for biotechnological applications and serves as a resource for various nonfood products, including detergent and paper. Starch is also a very interesting subject of fundamental scientific research, because of its structural complexity. Granules are highly organized and densely packed structures, composed of glucose residues, which can reach sizes of up to 100 μm in diameter [1].

Polysaccharides (mostly cellulose, starch, and glycogen) constitute the most abundant polymer type in nature. Produced in both prokaryotic and eukaryotic cells, they vary in composition and linkage. Both starch and glycogen are complex polysaccharides composed of linear chains (α-1,4-bound) and branching points (α-1,6-bound). However, in starch, the formation and clustering of interchain double helices of defined lengths give rise to a semi-crystalline structure. The higher degree of organization of starch leads to fundamentally different physico-chemical properties: gylcogen is soluble in water while starch is insoluble [2]. Based on the distribution of chain length and branching pattern, glycogen is assumed to either have a fractal structure [3] or a random dendritic architecture [4].

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The enzymatic pathways for the production, degradation, and recycling of polysaccharides have been investigated for several decades. The recent progress in biochemical characterization and molecular genetics allows the identification and classification of starch producing and degrading enzymes. However, their precise mechanisms of action, their substrate specificity, and kinetics are still under debate. Soluble polysaccharides are characterized by two distinct ends: reducing and nonreducing. The combinatorial complexity of a pool of soluble glucans present inside a cell is determined by the set of enzymes, their substrate specificity, and kinetics, which can be described by typical rate laws. On the other hand, the unique semi-crystalline structure of starch granules results in an unusual liquid–solid interface where enzymatic reactions take place. As a consequence, starch growth and degradation cannot be described as processes in solution; therefore, new analytical and numerical tools are required.

We will begin this review by recapitulating the main features of starch structure and starch enzymatic activities, followed by a discussion on the experimental challenge for growing glucans on granule-like surfaces. Finally, reviewing various approaches to describe the biochemistry of insoluble polysaccharides with mathematical models, we will stress the importance to develop novel stochastic modeling methods.

Starch structure

Starch granules are organized at various levels (Figure 1) and their characteristics (volume fraction, rigidity, and fractal dimension) are essential for understanding their macroscopic properties such as rheological behavior and morphological-structural characteristics [5]. The structure of starch is particularly well described in refs [6–8].

Most granules are organized in concentric rings that are visible after treatment with acid or degrading enzymes using several microscopy techniques: light microscopy, atomic force microscopy, or scanning and transmission electron microscopy (SEM and TEM) [9–11]. These 'growth rings' are crystalline layers intercalated with amorphous lamellae. The origin of this periodicity remains unclear and under debate, but it has been proposed that it could be related to circadian rhythms and results from complex combinations of biological and physical processes [10].

Each crystalline growth ring (in contrast with the intercalated amorphous layer) exhibits a densely packed and organized amylopectin structure with internal 9 nm repeats [12], which result from a compact arrangement of double helices formed between α-1,4 chains. The formation of double helices is a physical stabilization of neighboring chains and is not catalyzed by enzymes. It occurs between chains of at least 9 glucose residues and is responsible for the crystalline structure of growth rings. As per some considerations, the double helices behave as biopolymer liquid crystals [13] and get arranged into more or less compact arrangements (A-type or B-type).

The amorphous layers of starch are composed of amylose, which constitutes 20–25% of the total weight of starch, depending on the species. Amylose is a linear molecule made of hundreds to thousands of glucose residues. Unlike amylose, both amylopectin (constituting up to 75–80% of starch weight) and glycogen are branched. The net difference between the latter two branched glucose polymers emerges from the distribution of the branch points (~10% and homogeneously distributed in glycogen, ~5% and heterogeneously distributed in amylopectin) and the typical degree of polymerization of the external linear chains (6–8 in glycogen and 12–16 in amylopectin depending on the species [14]).

The racemose, or tree-like structure of amylopectin, allows the distinction of three main categories of chains: A chains (external chains) do not carry any other chains, the C chain (considered as the 'root') is the only chain that has a reducing end [15], and B chains (grouping all other chains) carry other chains and can extend over several clusters of densely packed double helices [7].

Starch growth and degradation enzymes

After initiation of granule formation, starch synthesis involves three main enzymatic activities: elongation, branching, and debranching [16,17]. Starch synthases (SSs, elongating enzymes) are glycosyltransferases that create a new α-1,4 glucosidic bond via the transfer of the glucosyl moiety of ADP-Glc to the nonreducing end of an existing glucan chain. Branching enzymes cut part of a linear chain, cleaving an α-1,4 linkage, and create a new branching point (α-1,6 linkage) by either inter- or intramolecular transfer. In this process, a new nonreducing end is created, which can be further elongated. The third enzyme category contributing to starch growth is debranching enzymes. They are responsible for hydrolysis of α-1,6 linkages. The released chains are recycled by entering the pool of available glucose residues for elongation by SSs. Debranching can take place only on external branches that are not stabilized by forming double helices. Hence, it is hypothesized that

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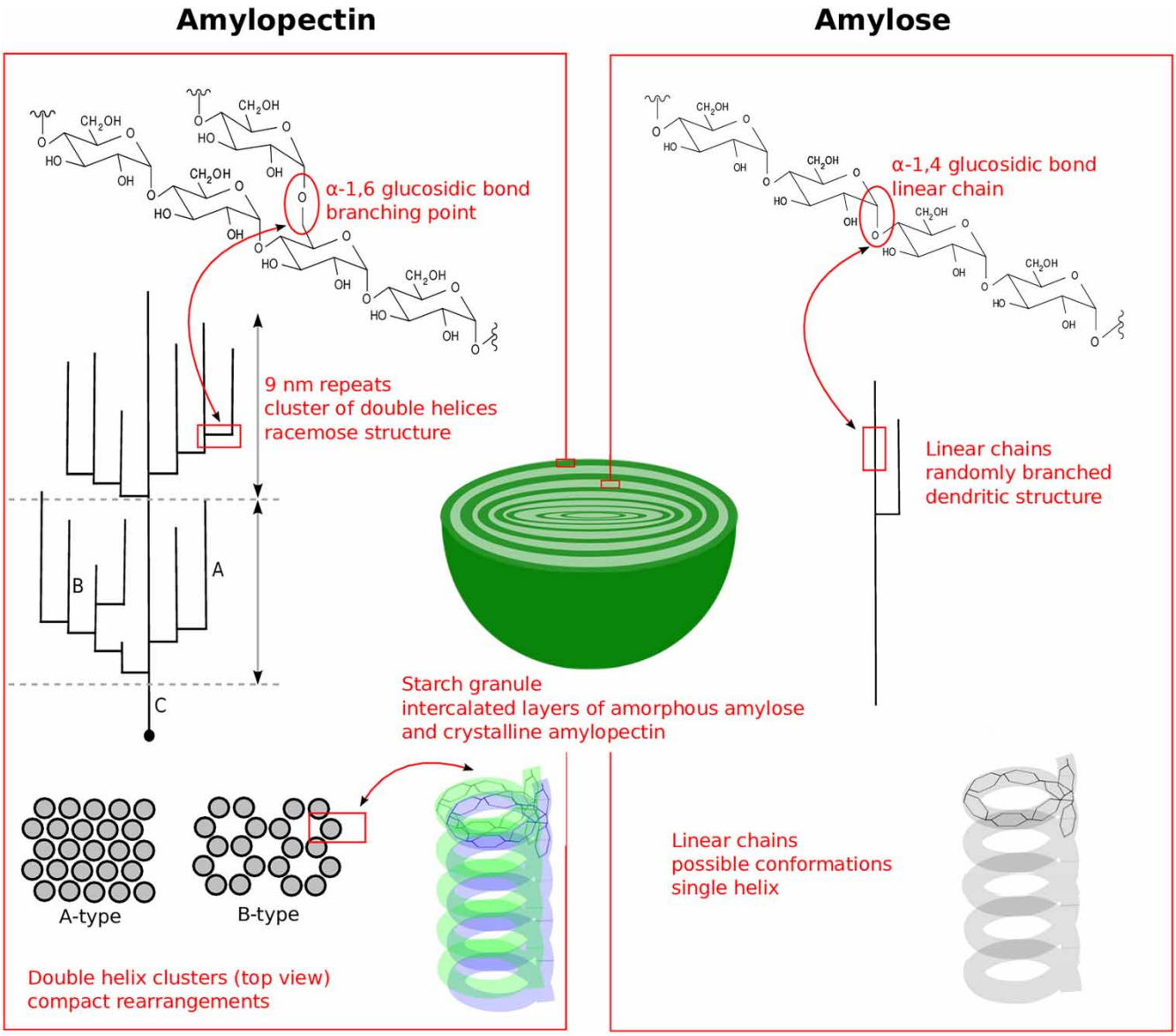

Figure 1. Structure and components of starch granules: amylopectin and amylose.

Starch granules are composed of concentric and intercalated "growth" rings of amylose and amylopectin. Amylose is mostly linear (α-1,4 bound glucose residues) whereas amylopectin has a tree-like structure (poylsaccharide chains are linked by α-1,6 bonds). Unbranched α-1,4 chains exhibit helical structures, which are stabilized by the formation of double helices. In amylopectin, double helices can form clusters of different arrangements that give rise to the semi-crystalline properties of the granule.

debranching is important to favor the tailoring of regular double helix clusters constituting the insoluble lamellar structures of starch.

It is still under debate to which degree starch synthesis and degradation occur simultaneously, and how these two antagonistic processes interfere. Experimental findings support the notion that phosphorylation takes place during starch biosynthesis [18], suggesting that some degrading activity could regulate starch synthesis.

Amylases are the first enzymes that have been discovered and isolated (by Anselme Payen in 1833), and their action has been extensively investigated ever since. Alpha-amylases are calcium metalloenzymes that react on random positions along α-1,4 chains and release either maltotriose and maltose from amylose, or maltose, glucose, and limit dextrin from amylopcetin. Contrarily, β-amylase reacts on the nonreducing ends of linear chains and catalyzes the hydrolysis of the second glucosydic bond, releasing maltose.

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Since 1904, it is known that potato starch contains small amounts of monoesterified phosphate groups [19]. The total amount in potato [0.2–0.4% (w/w)] is higher than in many other species [20]; however, regardless of its origin, starch is almost always phosphorylated to some degree [18,21]. Phosphorylation initiates degradation of starch by modification of amylopectin glucosidic residues. The collaborative action of dikinases (the glucan, water dikinase GWD, and phosphoglucan, water dikinase PWD) recruits two ATP molecules as phosphate donors and transfers one phosphate to water and the second to highly ordered insoluble α-chain glucans, hence, disordering and destabilizing amylopectin [22].

The characterization of the activity of individual starch enzymes was initiated in the early 20th century. This research experienced rapid progress in the last few decades, in particular for barley [23], *Arabidopsis thaliana* (synthases [7] and metabolism [24]), potato, and rice. However, most of our current knowledge is based on altering the expression of genes coding for starch enzymes. Clearly, when studying multiple mutants, the results are very difficult to interpret. Hence, the interplay between enzymes (collaborative action, or competition, formation of higher multi-modular enzymatic complexes) and the kinetics of the enzymatic activity is still poorly understood. The peculiar geometry of starch granules is an additional degree of complexity for understanding both growth and degradation processes. New *in vitro* (bio)chemical bottom-up strategies have been proposed to investigate polysaccharide growth on surfaces (summarized in ref. [25]) beyond the usual enzymatic experiments in solution.

In vitro biosynthesis of glycomaterials on surfaces

Several techniques for the detection of glycosylation on surfaces have recently been further developed such as mass spectrometry [26], radiolabeling [27], lectin affinity [28], or fluorescent affinity [29]. However, these methods are nonreal-time experiments and cannot reveal much about the kinetics of the reactions. In 2007, real-time analysis [30] and subsequent investigation of the kinetics [31] for the enzymatic extension of amylopectin have been performed using quartz crystal microbalance technology. More recently, microarray tools became new alternative strategies for easy immobilization of plant oligosaccharides, although the complex substructures of the glycans diminish resolution [32]. Thanks to surface plasmon resonance (SPR), it has been possible to track reactions on surfaces, but, only for nonenzymatic lectin binding onto glycans [33], or for unrealistic reaction conditions when enzymes (alternansucrase) are immobilized (on a carboxymethyl dextran chip) and the substrate (sucrose) is in solution [34].

The real-time tracking of glucan extension is required for investigating the kinetics of enzymes. Hence, microscopy (SPR and TEM) is now used to observe new generations of starch-like material, in particular glucans grown and immobilized on surfaces, such as sensor chips and nanoparticles. In ref. [35], the authors focus on *A. thaliana* phosphorylase AtPHS2, they track chain elongation on glucan-coated surfaces, and characterize the substructure formation of the grown α-chains into helical arrangements by iodine staining. Comparing reactivity for different surface densities of glucan chains, the authors found that low densities induce a rather similar behavior as in solution, whereas densely coated surfaces exhibit chain rearrangements and starch-like tightly packed double helices.

The miniaturization of these experiments is a great way to reduce signal-to-noise ratio, but the development of novel materials such as sensor surfaces is still a challenge. In that sense, gold nanoparticles are first choice for both recognition and transduction; among their remarkable properties, one can quote the ease to functionalize their surface and their amazing optoelectronic characteristics. More precisely, plasmon coupling between gold nanoparticles (based on their distance), as well as solvent, ligand, and temperature, may induce a shift in the SPR spectra and could potentially lead to use gold nanoparticles as colorimetric sensors derived from the conventional SPR method [36].

Starch modeling

From the 1960s to the 1990s, a myriad of models based on Michaelis–Menten kinetics have been proposed for the degradation of solubilized polysaccharide substrates, including cellulose, glycogen, and starch. They are particularly well reviewed in refs [37,38]. In parallel, other authors rather focus on the mechanisms of action of enzymes and aim at determining their active site of cleavage [39] in order to predict the pool of subsequently released glucans [40–44]. In 2009, Bansal et al. [45] tabulated and classified these results into four categories: empirical, Michaelis–Menten-based, adsorption in cellulose hydrolysis and soluble cello-oligosaccharides models. Dona et al. [46] reviewed in great detail *in vivo* and *in vitro* kinetic models for the digestion of starch. More recently, additional models based on Michaelis–Menten kinetics were proposed for the hydrolysis of

cellulose and chitin by a processive enzyme [47] and for the polymerization of a pool of hyaluronan polymers by a nonprocessive enzyme [48]. Michaelis–Menten-based models have been successfully employed to describe metabolic pathways in aqueous environments with relatively homogeneous spatial distributions of metabolites and enzymes. They rely on knowledge of the enzymatic parameters, such as catalytic rates and Michaelis constants. For complex polymer systems, a Michaelis–Menten-like mechanism is usually a rather crude assumption. Moreover, experimentally determined enzymatic parameters are difficult to interpret consistently, because of the complex substrate structure (see also below). However, Michaelis–Menten-based models can be very useful to discriminate between conflicting hypotheses regarding the underlying enzymatic mechanism, for example processive versus nonprocessive.

Additionally, Kartal et al. [49] investigated further analytical approaches and set the framework for applying statistical thermodynamics to carbohydrate-active enzymes. They showed that entropy is key for understanding the polydispersity of a pool of glucans in the presence of disproportionating enzyme. In this framework, different degrees of polymerization are associated with different energy states, reflecting the total bond energy between the monomers. This allows deriving a formal analogy to statistical thermodynamics, and the equilibrium distribution can be determined by maximizing the entropy of the system. Equilibrium distributions are described by a characteristic exponent, which is determined by the specific enzymatic mechanism and the initial experimental conditions. This exponent can be seen as a generalization of the equilibrium constant for polydisperse solutions. Chain length distributions and equilibrium entropy measured in *in vitro* experiments for various carbohydrate-active enzymes [disproportionating enzyme 1 (DPE1), DPE2, and phosphorylase] have provided excellent confirmation of the theoretical predictions. This approach can be extended to other enzymes, or systems of enzymes, acting on polydisperse substrates.

The modeling approaches described above are valuable to describe homogeneous systems of soluble substrates. However, starch granules exhibit a high degree of internal organization and are insoluble, which entails that the reaction space is confined. The structural properties of the granules are fundamental for starch growth and degradation, and branches can no longer be considered as isolated polymers. The accessibility of branches (constrained by the local environment) clearly affects the diffusion of enzymes, which can even be trapped inside starch granules [50,51]. Furthermore, the formation of double helices and their clustering directly affects the stability of the polysaccharide chains, drastically affecting the affinity of enzymes for this newly organized substrate. To rationalize enzymatic activity on starch is still a major challenge, and the complexity of the three dimensional structure of the granule has not yet been taken into account. In that perspective, we first review studies of polysaccharide degradation when the substrate is considered as a surface.

In 1981, Fan et al. [52] set up experiments for the hydrolysis of solid cellulose. They aimed at considering the substrate properties such as its structure and its surface area to estimate the adsorption of enzymes. They empirically established the relationship between hydrolysis, crystallinity, and surface area, showing that the rate of hydrolysis decreases faster with the crystallinity index than it increases with the specific surface area. Also for cellulose as substrate, Levine et al. [53] assumed simplistic random sequential adsorption and developed a detailed mechanistic model, taking into account the properties of the accessible surface for the enzyme adsorption. Playing with the size of starch granules and their biotic origin (rice, wheat, maize, cassava, potato, and sweet potato), Katano and co-workers provided some insights to the kinetics of glucoamylase-catalyzed hydrolysis [54]. They showed that the catalytic constant of the adsorbed enzyme increases with the density of the crystalline structure. These experimental findings were rather successfully compared with a three-step mechanism extended from standard Michealis–Menten. However, the X-ray characterization of the structure of starch granules was not systematically consistent with the measurements of the catalytic constant of the adsorbed enzyme, leading the authors to conclude that the structure of the amorphous regions and the impurity of the granule could play a role [55].

In addition to the experiments and heuristic models mentioned, some theoreticians also addressed the question of enzymatic reactions on surfaces. In 2013, Kartal and Ebenhöh [56] proposed a generic rate law to describe surface-active enzymes. Admitting the lack of consensus and the lack of canonical kinetic description in that field, they proposed a generic model and provided useful guidelines for experiments. For example, the theoretical results demonstrate that experiments performed in analogy to soluble substrates cannot be unambiguously interpreted if mass is used as the only characteristic of the substrate. In fact, in contrast with systems in solution, apparent V_{max} and K_M values depend also on the specific surface area and the enzyme concentration. Remarkably, the model is independent of the adsorption process assumed and could be incorporated into the mathematical analysis of more complex pathways.

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Numerical methods like Monte Carlo simulations allow additional exploration of molecular mechanisms of enzymatic reactions. Marchal et al. [57] exploited subsite maps from literature to model α-amylase action on starch amylopectin and predict the pool of polysaccharides generated. Subsites are uncorrelated positions surrounding the catalytic site of enzymes. They can bind to one glucose unit depending on their respective affinity for the substrate. Along the same baseline, Nakatani [58] provided substantial contributions. For β-amylase (as a multiple attack mechanism), 4-α-glucanotransferase (disproportionating enzyme) [59], and hyaluronidase (which catalyzes hydrolysis, transglycosylation, and condensation) [60], he simulated the enzymatic activities and systematically compared them with experimental data. These theoretical predictions, compared with experimental *in vitro* data, shed some light on the mechanism of action of isolated enzymes. However, none of these models on isolated single enzymes can capture the essential interplay of the various enzymes at work for starch growth. Remarkably, glucanotransferases were also modeled with a Gillespie algorithm to explain how a maltooligosaccharide buffer ensures constant provision of glucose phosphate [61]. In this case, the model was compared with *in vivo* results (for the conversion of starch to sucrose in *A. thaliana* leaves by night), suggesting that heterogeneity in polysaccharides could play a role in buffering the flux of carbon released from starch.

Numerical simulations are not only relevant for understanding detailed molecular mechanisms but also for larger-scale modeling of starch amylopectin. After building a computer matrix of potato amylopectin, and simulating its degradation under the action of α-amylase, Marchal et al. [62] analyzed its structure in terms of β-hydrolysis and A-chains to B-chains ratio. These output parameters were in good agreement with available data from the literature, but one should note that the model is not very sensitive to the input variables characterizing length and width of individual clusters of chains. In addition, the formation of new branches in amylopectin is implemented in such a way that positions on the substrate branch are systematically tested for their ability to support a new branch beginning from the first monomer. This leads to the attachment of the daughter branch always to the closest possible position from the substrate branch origin. This method artificially induces some order in the resulting tree-like structure and is somehow equivalent to consider the enzymatic activity as being fully processive rather than stochastic. Hence, the relevance of the algorithm to reflect biological facts can be questioned.

Conclusion

Starch biochemistry has been a very active field of research for more than a century. Owing to the remarkable properties and commercial applications of starch, it is still of growing interest. Biochemical and biological knowledge in terms of starch production, structure, and degradation has been reviewed. Starch degradation has early been intensively investigated and clearly benefited from the studies of the degradation of other polysaccharides, like cellulose and glycogen. Thanks to the fast progress of microscopic imaging, the complex structure of starch is better known. In addition, most enzymes involved in starch growth and degradation are now identified. However, their specific action, their interplay (cooperative or competitive), and their kinetic properties are still poorly characterized. In addition, it is not yet understood how the complete enzymatic activity (elongation, branching, debranching, and partial hydrolysis) and the physical and spontaneous formation of double helices of α-chains leads to the large-scale tightly packed structure with its specific macroscopic properties. In that perspective, modeling can be an essential approach. As reviewed here, polysaccharides are mostly either modeled as soluble systems, or the mechanistic details of the action of the digestive enzymes are numerically simulated. Hence, the precise mechanisms leading to the production of insoluble starch granules made of finely tailored α-chains are currently a major focus in polysaccharide research.

New avenues could be explored like bottom-up strategies that integrate biochemical and biological knowledge to build *in silico* large racemose starch-like objects. The numerical simulation of starch granule production or degradation should embody the complexity of the enzymatic activities (diversity of enzymes and substrate specificity) and the thermodynamic stabilization of the structure (formation of double helices). As outlined above, in the crowded and heterogeneous environment of starch granules, enzymatic activity cannot simply be modeled with classical chemical or enzymatic rate laws that were derived for spatially homogeneous systems. One major computing challenge here is to efficiently deal with the complex tree-like structure of the substrate and with the specificity and interplay of growth and degradation enzymes. In that perspective, new numerical tools are required and could highly benefit from numerical approaches that have been established in the field of stochastic physics. The Gillespie algorithm was initially developed from Monte Carlo approaches for the modeling of simple spatially homogeneous chemical systems. However, there is no reason why the Gillespie algorithm should not be applicable to inhomogeneous systems including substrates as complex as starch granules. The

underlying idea of the Gillespie approach is the association of all possible reactions with propensities reflecting their probabilities to take place in a given time interval. Therefore, with a careful definition of the possible reactions and their respective propensities, which depend on the particular substrate configuration, it is possible to apply a Gillespie-like methodology to heterogeneous and dynamically changing environments.

The statistical analysis of the structure of the simulated starch, for instance the distribution of branch lengths and branching patterns, would provide a theoretical tool to interpret complex experimental results, such as the starch structure of multiple mutants, in a more quantitative and rigorous fashion. Such modeling and statistical analysis would also shed more light on the kinetics of individual enzymes, assayed *in vitro*. In addition, the *in silico* simulation of starch production and degradation allows to better understand the problem how the specific macroscopic properties of starch granules (e.g. clusters of double helices, crystallinity, and solubility) emerge from underlying microscopic biochemical (substrate specificity, mode of action, and interplay of enzymes) and biophysical (thermodynamic stabilization) processes.

Abbreviations

DPE, disproportionating enzyme; SEM, scanning electron microscopy; SPR, surface plasmon resonance; SSs, starch synthases; TEM, transmission electron microscopy.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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