Yasunori Nakamura *Editor*

Starch

Metabolism and Structure

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Preface

Rapid progress of research in recent years on the biosynthesis, degradation, and structure of starch produced in photosynthetic and non-photosynthetic tissues from a variety of plant species and in algae, including land plants and green algae (Chloroplastida), red algae (Rhodophyceae), and cyanobacteria, lead us to understand the whole scope of the specific metabolic systems in plants. At the same time we must revise our earlier concept of starch-related metabolism by dealing with the newly revealed dynamism of regulation of starch biosynthesis.

Genome analysis indicates that higher plants have evolved two different starch biosynthetic processes in photosynthetic and non-photosynthetic tissues by differentiation of key enzymes for starch biosynthesis, i.e., starch synthase, starch branching enzyme, and starch debranching enzyme, into multiple isozymes having specific catalytic properties. Concomitantly, plants have refined the fine structure of amylopectin, the major component of starch, so that it can be densely packed into the semi-crystalline granules having distinct size and morphology depending on plant species and tissues.

Numerous past investigations vigorously and comprehensively performed worldwide by biochemical, genetic, and molecular approaches especially since the early 1990s have established a concrete basis for contributions of individual isozymes to the fine structure of amylopectin and amylose and for their expression patterns altered by various tissues and their developmental stages. However, despite these invaluable fundamental results, at present we cannot help feeling that for a better understanding of the dynamic aspects of regulatory mechanisms for starch biosynthesis and its degradation, novel concepts should be established regarding the functional interactions between enzymes and between the enzyme(s) and glucans/dextrins. Also, we should formulate new concepts regarding the multiple functions of individual enzymes, responding to changing physiological and environmental conditions.

Detailed comparative analysis of the chain-length distribution of amylopectin from various plant sources has provided new insights into the basic skeleton of the

distinct amylopectin fine structure. Although amylopectin has long been believed to be composed of the unit structure referred to as the cluster, which includes the amorphous lamellae and the crystalline lamellae with a total size of about 9 nm universally found among the plant kingdom, Bertoft and his colleagues recently proposed an alternative model of the amylopectin structure referred to as the "backbone model" (see Chap. 1). The features of the backbone model are, first, that the construction and direction of chain-elongation (and possibly the biogenesis as well) of the cluster-linking chains (B2- and B3-chains) are distinct from the cluster chains (A- and B1-chains), while in the traditional "cluster model" the natures of both chains are not necessarily distinguished with the exception of their chain-lengths. Second, the backbone model has proposed a novel notion that the building blocks are actually the structural units constituting the individual cluster, whereas no such structural units are assumed in the cluster model. However, due to methodological limitations, the actual, exact fine structure of amylopectin remains unproven.

The fact that plants can synthesize starch granules efficiently and at a high rate and accumulate them with a specific gravity of about 1.6 (Rundle et al. 1994. J Am Chem Soc 66:130–134) at the huge amounts inside plastids inclines us to conceive that the synthesis of starch granules makes it possible for plants to survive on land. To approach the secret and miracle of their capacities, we must always pay attention to the relationship between each biochemical event during starch metabolism and the structure of glucans intervening in the enzyme actions and contributions. In this book, novel findings of the fine structures of amylopectin (Chap. 1) and amylose (Chap. 2) and the crystalline structure of starch granules (Chap. 3) are introduced and their significance is concisely explained.

We can expect that during the coming decade basic and applied studies will focus on the mechanism for dynamic features of enzyme–enzyme and enzyme–glucan interactions although these attempts will provide us with quite new findings, and each interaction must be significantly involved in the regulation of starch biosynthesis and degradation. In this book, each chapter describes the present status of our understanding of the related topics and mostly includes future perspectives. Many chapters also explain the advantages and limitations of the modern methodologies used. Thus, we believe that the book is of great use for young scientists and students who are engaged in or majoring in the starch science fields including basic science, biotechnology, and applied science such as applications for food and bioplastics to learn the whole scope of starch metabolism and its structure.

Finally, I, as an editor of the book, express my deep and sincere gratitude to all the chapter authors for their great contributions by sharing the aim of publishing a good, updated reference book for all scientists who are interested in starch science. I especially thank Professor Martin Steup for discussing the content of the book and reviewing many manuscripts.

Akita, Japan Yasunori Nakamura

Contents

Part I
Structure

Chapter 1
Fine Structure of Amylopectin

Eric Bertoft

Abstract Starch granules consist of two major polyglucans, namely, branched amylopectin and essentially linear amylose. In all nonmutant starches, amylopectin is the major component and is responsible for the internal structure of starch granules, which is the native, semicrystalline form of starch. The granules, irrespective of the plant source, consist of granular rings of alternating amorphous and semicrystalline polymers. On a smaller scale, blocklets as well as crystalline and amorphous lamellae have been identified. Amylopectin is generally accepted as the contributor to the lamellar structure, but the nature of blocklets is only beginning to be resolved. Amylopectin consists of numerous chains of glucosyl units that are divided into short and long chains. These chains are organized as clusters that have been isolated by using endo-acting enzymes, and the fine structure of the clusters have been investigated. The clusters consist of still smaller, tightly branched units known as building blocks. The organization of the clusters and building blocks in the macromolecular structure of amylopectin is to date uncertain, and two schools exist at present suggesting that amylopectin either has a treelike branched cluster structure or a building block backbone structure. The structural features of amylopectin and the two models presently in debate are discussed in this chapter.

Keywords Amylopectin structure • Cluster structure • Building blocks • Starch granules • Amylopectin models

Amylopectin is generally the major component of starch and constitutes 65–85 % of the matter in the starch granules (Table 1.1) (Fredriksson et al. 1998; Gérard et al. 2001; Hoover 2001). However, in some mutant plants, the amylopectin content is much higher – it can even reach 100 % – and the sample is then known as "waxy starch." Some mutant plants possess high-amylose starches with low amylopectin content. In these starches,the morphology of the granules is often

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Table 1.1 Crystalline structure and amylose content of some starch granules

Starch source	References	Allomorph	Relative crys-tallinity (%)	Lamellar repeat distance (nm)	Crystalline lamella thickness (nm)	Amylose content (%)
Barley	[a]	A	23.3			28.3
	[b]			8.9		
	[c]				4.9	
Wheat	[d]	A	24.0			33.2
	[e]	A	32	9.9		37.0
	[b]			9.0	4.3	
Waxy maize	[a]	A	31.4			0
	[c]				4.2	
	[f]			8.8		
	[e]	A	47	10.3		1.0
Rice	[a]	A	29.1			19.5
	[g]				4.1–4.6	12.8–24.2
	[h]	A	37.1			13.2
	[b]			8.7		
Sweet potato	[h]	A	34.4			19.8
Tapioca	[a]	A	29.9			17.6
	[h]	A	35.8			17.9
	[e]	A	32	9.8		29.8
	[b]			9.1		
Potato	[a]	B	26.4			17.9
	[h]	B	29.8			18.0
	[e]	B	43	9.0		14.0
	[i]	B		8.8	5.5	

[a]Bertoft et al. (2008)
[b]Jenkins et al. (1993)
[c]Genkina et al. (2007)
[d]Kalinga et al. (2013)
[e]Vermeylen et al. (2004)
[f]Jenkins and Donald (1995)
[g]Koroteeva et al. (2007b)
[h]Srichuwong et al. (2005b)
[i]Kozlov et al. (2007a)

defect with a lot of elongated granules (Banks and Greenwood 1973; Tester et al. 2004; Kubo et al. 2010; Jane et al. 1994; Glaring et al. 2006). The granules in waxy plants, however, are indistinguishable from those of normal amylose-containing granules (Fuwa et al. 1978; Jane et al. 1994; Song and Jane 2000). This shows that amylopectin is the principal component that contributes to the structure of starch granules.

1.1 Internal Architecture of the Starch Granule

Although starch granules from diverse plants have a large range of different sizes and shapes (Jane et al. 1994), their inner architecture appears remarkably similar. The ubiquitous structures within starch granules are granular rings, blocklets, and lamellae, which can be observed with different techniques, because they represent structural levels ranging from micrometers down to the nanometer scale.

1.1.1 Granular Rings

Granular rings are also known as "growth rings." The rings have been known as long as the starch granules – they were described already in the early 18th century by Leeuwenhoek, who developed a more practical version of the light microscope (Seetharaman and Bertoft 2012a). They appear as alternating light and dark rings with an approximate thickness of a few hundred to several hundred nanometers. The rings tend to be thicker in the interior part of the granule and become thinner toward the periphery (Pilling and Smith 2003; Wikman et al. 2014). By a limited treatment of cracked granules with amylase, the rings become more clear and appear thinner and more numerous by scanning electron microscopy (Fulton et al. 2002).

Although the granular rings are well known, the reason for their existence and their exact nature is still unclear. It was suggested already in the 19th century that the rings are laid down periodically as a result of the day and night (diurnal) cycle (Fritzsche 1834; Meyer 1895). Indeed, starch granules from barley and wheat grown in constant light were shown to lack rings (Sande-Bakhuizen 1926; Buttrose 1960, 1962). However, potato starch granules retained rings when grown under similar conditions, which suggest that yet unknown mechanisms regulate the formation of the rings (Buttrose 1962; Pilling and Smith 2003). The fact that treatment in diluted acid results in higher relative crystallinity in the remaining starch granules (Sterling 1960; Muhr et al. 1984; Vermeylen et al. 2004; Utrilla-Coello et al. 2014) suggests that amorphous areas are eroded by the acid, which in turn suggests that the granules consist of alternating amorphous and crystalline rings. Because amylose is considered to exist mostly in the amorphous state in the granules, it has been generally assumed that the amorphous rings mostly consist of amylose (Atkin et al. 1999). However, the fact that granular rings exist in both amylose-containing and waxy starches shows that amylopectin also participates in the amorphous rings.

The crystalline rings are often called semicrystalline because, as we shall see, they are not completely crystalline. Amylopectin is thought to be the major component in these rings, but amylose is probably also a part of them (Koroteeva et al. 2007a; Kozlov et al. 2007b). It should be noted, however, that the semicrystalline rings were named as crystalline by Gallant et al. (1997) and the amorphous rings were named "semi-amorphous," although the reason for these labels remained

unclear. In the work of Tang et al. (2006), the semicrystalline rings were considered "hard shells" and the amorphous were called "soft shells." Thus, the view of the nature of the granular rings remains a matter of debate in the literature.

1.1.2 Blocklet Structure

Small, birefringent units inside starch granules around one micrometer in size were observed in the 1930s and called blocklets or "Blöckchen" by the German scientists Hanson and Katz (1934a, b). They treated starch granules in diluted acid before the granules were allowed to swell in a calcium nitrate solution and described blocklets as ordered in both radial and tangential directions when observed in an ordinary light microscope. Later, these structures seem to have been forgotten, but they were redescribed by Gallant et al. in 1997. Blocklets are now observed by atomic force microscopy (AFM) of native or acid-treated granules, and, therefore, it cannot be taken for granted that these blocklets are the same as the "Blöckchen" described by Hanson and Katz in 1934. Nevertheless, blocklets were observed as protrusions on the surface of starch granules. The sizes of these blocklets on potato starch granules range between 50 and 300 nm according to Baldwin et al. (1998) or are around 30 nm according to Szymonska et al. (2003). On granules from sweet potato, maize, rice, and wheat, the blocklets were reported to have similar sizes of approximately 20–40 nm (Ohtani et al. 2000). However, Dang and Copeland (2003) reported that blocklets in rice are 100 nm wide and 400 nm long and suggested that they span a whole growth ring. Blocklets were also observed on the surface of granules isolated from wheat at different stages of the development of the kernel endosperm. It was found that blocklets on the surface of granules from early developmental stages are larger and have more fuzzy contours than at later stages and at maturity (Waduge et al. 2013).

Gallant et al. (1997) suggested a general model for the blocklet structure of starch granules. According to these authors, blocklets fill up the whole interior of the granule. Large blocklets build up the semicrystalline rings (or "crystalline" rings according to the terminology by Gallant et al.), whereas smaller blocklets build up the amorphous ("semi-amorphous") rings. Chauhan and Seetharaman (2013) studied blocklets in acid-treated (and subsequently dried) potato starch granules and confirmed the model by Gallant et al. Baker et al. (2001) found that native maize granules have blocks 400–500 nm in size that span the whole growth ring. Similar structures were reported by Atkin et al. (1998). After acid treatment, smaller blocklets 10–30 nm in size were observed within the rings (Baker et al. 2001). Chauhan and Seetharaman (2013) reported that blocklets fused together when acid-treated granules were exposed to water vapor during the observation by AFM. This indicates that the blocklet is a flexible structure that is readily observed in the dry state and takes up water under humid conditions.

Tang et al. (2006) have also proposed a model for the blocklet structure in starch granules. In their view, there exist perfect and defect (less perfect) blocklets.

The former mainly build up "hard shells" (corresponding to semicrystalline rings), and the latter are found in "soft shells" (corresponding to amorphous rings).

As blocklets so far have not been isolated from starch granules, the exact composition of a blocklet is not known. Whether the composition of the blocklets, or the structure of their molecular components, is similar or different in the alternating granular rings is so far only a matter of speculation. Dang and Copeland (2003) assumed that the blocklets consist of amylopectin and calculated that the blocklet dimensions suggests that they contain about 280 clusters of amylopectin side chains. Ridout et al. (2003, 2006) also assumed that the blocklets largely consist of amylopectin and that they are embedded in an amorphous matrix that mainly consists of amylose. This matrix, they found, swells and gives rise to the amorphous granular rings, which appear bright and soft. The dark, semicrystalline rings arise because they contain defects that do not swell, which results in a discontinuation of the swelled areas.

1.1.3 Lamellar Structure

A lamellar repeat distance of approximately 10 nm in starch granules was first reported by Sterling in 1962 using small-angle X-ray diffractometry. This was ascribed to alternating crystalline and amorphous lamellae in the semicrystalline granular rings (Cameron and Donald 1992). Later, Jenkins et al. (1993) refined the calculations to be around 9 nm and reported that this is a ubiquitous repeat distance for practically all types of starch (Table 1.1). Moreover, Jenkins and Donald (1995) found that the distance is similar irrespective of the amylose content in the granules: In waxy starch granules, the amorphous lamellae tend to be thicker and the crystalline lamellae thinner, whereas in high-amylose granules, the situation is the opposite, but the general average repeat distance remains roughly the same.

The responsible molecular component for the lamellar organization is amylopectin. The crystalline lamella is built up by double helices of short, external chains of amylopectin with an average 11–15 glucose residues (Manners 1989; Bertoft et al. 2008). Theses double helices crystallize into either a so-called A-type allomorph (typical in cereal starches) or a B-type allomorph (common in root and tuber starches) (Imberty et al. 1991), details of which are described in Chap. 3. Essentially, the A-type crystal exhibits a monoclinic unit cell involving six double helices and only little water (Imberty et al. 1988; Popov et al. 2009), whereas the B-type crystal is hexagonal, also involving six double helices, but with a central cavity filled with water (Imberty and Pérez 1988). The strands of the double helix are left handed and consist of six glucose residues per turn and a pitch of 2.1 nm. The length of the double helices corresponds approximately to the experimentally estimated thickness of the crystalline lamellae, i.e., 4.0–6.5 nm, depending on the type of starch (Table 1.1) (Kiseleva et al. 2005; Genkina et al. 2007; Koroteeva et al. 2007a; Kozlov et al. 2007a).

The amorphous lamella is situated directly adjacent to the crystalline lamella, and several of these lamellae units form "stacks" in the semicrystalline ring (Cameron and Donald 1992). The average number of repeat units appears to depend on the sample, but is in the order of 15–25 (Daniels and Donald 2003). The amorphous lamella consists of the internal part of the amylopectin molecule with most, or at least plenty, of the branches in amylopectin (French 1972). This part of the amylopectin molecule cannot, therefore, crystallize, but its structure is believed to be of importance for the crystallization of the double helices (O'Sullivan and Pérez 1999; Vamadevan et al. 2013). In comparison to the detailed knowledge of the structure and organization of the double helices in the crystalline lamella, the structure of the amorphous lamella is only poorly known. The details, however, begin to emerge, as we will see later on.

The involvement of amylose in the lamellar structure is also not well known to date and is a matter of debate. It appears, however, that amylose is a part of the structure and possibly interferes with the crystalline arrangement of the double helices of amylopectin (Koroteeva et al. 2007a; Kozlov et al. 2007b). The amylose may be intertangled with the internal chains of amylopectin in the amorphous lamella, and it might also extend into the crystalline lamella, or even through several layers of lamellae. Yuryev and coworkers have suggested that the amylose involvement depends not only on the amount of amylose in the starch but also on the specific plant species (Koroteeva et al. 2007a; Kozlov et al. 2007a, b).

The relation between the lamellar organization and the blocklet structure of the granular rings is to date only a matter of speculation. Possibly, the lamellae build up the blocklets in the semicrystalline rings. It is interesting to notice that the lamellae are only observed in wetted starch samples, but not in dry granules. This was explained on the basis of a side-chain liquid-crystalline model of branched polymers (Waigh et al. 2000b), in which the amorphous chain segments of the amylopectin are connected to the double helices and act as spacer arms: In the dry state (the so-called nematic stage), the double helices are disorganized, whereas in the wet stage (the smectic stage), the spacer arms move the double helices and align them into the observed crystalline lamellar distance. Apparently, this coincides with the swelling of the blocklets and might contribute to the appearance of the lamellae (Bertoft and Seetharaman 2012). Tang et al. (2006) suggested that the lamellar structure is found in blocklets in both semicrystalline and amorphous rings, but the structure of the blocklets is more defect in the latter rings.

1.2 Amylopectin: The Major Starch Component

Amylopectin consists of numerous short chains of α-(1,4)-linked D-glucose residues. The chains are interlinked through their reducing end side by α-(1,6)-linkages. Together the chains form a very large macromolecule with an average molecular weight (M_w) in the order of 10^7–10^8 (Aberle et al. 1994; Millard et al. 1997; Buléon et al. 1998a), and thereby the size is about one or two order of

magnitude larger than the almost linear amylose molecules (Aberle et al. 1994; Buléon et al. 1998a). Exact M_w values are, however, difficult to achieve, because the molecules are prone to aggregate in solution and to breakdown due to harsh dissolving methods, resulting in too high or too low estimations, respectively. The number-average value (M_n) is considerably smaller than M_w (McIntyre et al. 2013). The polydispersity index (M_w/M_n) is therefore large (Erlander and French 1956; Stacy and Foster 1957).

Each macromolecule has as many nonreducing ends as there are chains, but only a single glucose residue that has a free reducing end group. Takeda et al. (2003) labeled this group with the fluorescent dye 2-aminopyridine. By size-exclusion chromatography (SEC) of labeled samples, these authors showed that the amylopectin component from a range of diverse starches in fact consists of three size fractions: The largest amylopectin molecules have a number-average degree of polymerization (DP_n) of 13,400–26,500 depending on the plant species. Intermediate-sized amylopectins have DP_n 4400–8400 and the smallest molecules have DP_n 700–2100. Large amylopectin molecules were the most abundant in all starches they studied.

As in the starch granule, there exist several structural levels within the amylopectin macromolecule, namely, the unit chains, larger clusters of the chains, and the ultimate small, branched units of chains known as building blocks. Each of these levels is discussed in more detail below.

1.2.1 Unit Chains of Amylopectin

The chains in amylopectin have been divided into a range of different types by different authors. Unfortunately, the meaning of the different divisions and nomenclatures is not always apparent and has resulted in a lot of confusion and misunderstanding in the literature throughout the later decennia. Indeed, a correct understanding of the terminology is essential for a meaningful interpretation of the structure of the macromolecule. The terminology of the chains is therefore outlined in detail below.

1.2.1.1 Major Chain Categories

Already in 1952, Peat et al. (1952b) suggested a basic – and very useful – nomenclature of chains in amylopectin, which focuses on the mode of participation in the molecular structure. They realized that, based on the then generally accepted model of the structure, which had been proposed by Meyer and Bernfeld (1940), some chains carry other chains, whereas some chains do not. The latter chains were called A-chains, whereas the former chains were named B-chains. In addition, they suggested that the sole chain that carries the free reducing end group, but otherwise is similar to the B-chains, should be called C-chain. This practical nomenclature

was soon accepted by the research community. Moreover, the model by Meyer and Bernfeld (1940) implied two other principle types of chain segments: external and internal. External chains are considered as segments that extend from the outermost branch to the nonreducing end of the chain, whereas all other segments are internal. It follows that A-chains are completely external and each B-chain (and the C-chain) has one external chain segment, whereas the rest of the chain is internal.

In order to make use of this nomenclature, it is necessary to have experimental tools that distinguish the chains from each other, so that they can be identified and quantified. The first tools in this direction were based on the specific action of starch-degrading (amylolytic) enzymes. The methods were developed simultaneously with the discovery of the enzymes in the 1940s and 1950s. Thus, the enzymes phosphorylase (from rabbit liver) (Hestrin 1949; Illingworth et al. 1952) and β-amylase (from sweet potato and soybean) (Hestrin 1949; Peat et al. 1952a) were found to be so-called exo-acting enzymes, i.e., they hydrolyze the chains in amylopectin (and in amylose) from the nonreducing end until they approach the most exterior branch point, which possesses a barrier that the enzymes cannot bypass. The resulting, resistant molecule is called a limit dextrin (LD) and contains the entire internal part of the original amylopectin macromolecule together with shorter external chain stubs that the enzymes leave in front of the outermost branch points (Fig. 1.1). The relative extent of hydrolysis of the amylopectin molecule by phosphorylase or β-amylase is known as the φ- and β-limit value, respectively (Hestrin 1949; Walker and Whelan 1960b). Phosphorylase and β-amylase have also been used in sequence, which results in a so-called φ,β-LD (Hestrin 1949; Lii and Lineback 1977). Either of the limit values can be used for the estimation of the average external chain length (ECL) (Bertoft 1989; Manners 1989) and is summarized for some different samples in Table 1.2. It should be noted that ECL is only an average value, and the actual size distribution of the external chains is to date still unknown. Nevertheless,

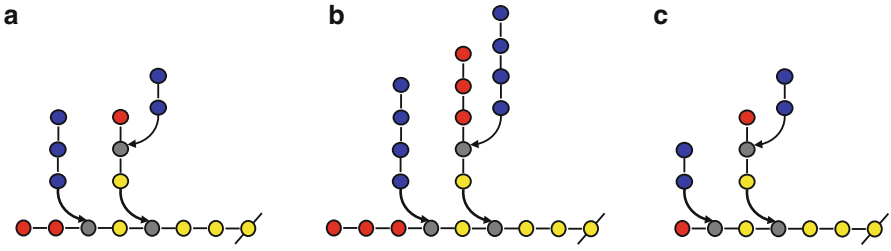

Fig. 1.1 Principal structure of limit dextrins: (**a**) β-LD, in which A-chains remain as two or three glucose residues and the external chain stubs of B-chains have one or two residues; (**b**) φ-LD, in which all A-chains have four residues and all external B-chain stubs have three residues; (**c**) φ,β-LD, in which all A-chains have two residues and all external B-chain stubs have one residue. *Blue circles* symbolize A-chains, *red circles* external B-chains, *gray circles* are glucose residues involved in a branch linkage (*arrows*), and *yellow circles* are residues in internal segments of B-chains. Note that the chain segment that carries the reducing end (/) is regarded as an internal segment

Table 1.2 Structural parameters of amylopectin from diverse plants[a]

Starch source	References	Structure type	CL	ECL	ICL	TICL	S:L	A:B
Barley	[b]	1	17.5	11.2	5.3	12.3	19.4	1.0
Wheat	[c]	1–2	17.7	12.3	4.4	12.7	16.2	1.4
	[d]		18				12.9	
Waxy maize	[b]	2	18.1	11.9	5.1	12.0	13.5	0.9
Rice	[b]	2	17.8	11.7	4.9	12.3	11.5	1.1
	[d]		18				10.8	
Sweet potato	[e]	2–3	19.6	12.8	5.8	14.0	10.1	1.1
	[d]		20				8.9	
Tapioca	[b]	3	18.8	12.4	5.3	14.6	11.0	1.3
Potato	[b]	4	23.1	14.1	8.0	19.9	6.3	1.2
	[d]		22				6.5	

[a]Structural type is based on the internal unit chain distribution of amylopectin; *CL* chain length, *ECL* external chain length, *ICL* internal chain length (between branches), *TICL* total internal chain length (the whole internal B-chain length), S:L ratio of short to long chains, A:B ratio of A- to B-chains
[b]Bertoft et al. (2008)
[c]Kalinga et al. (2013)
[d]Hanashiro et al. (2002)
[e]Zhu et al. (2011a)

ECL corresponds rather well to the reported thickness of the crystalline lamellae in starch granules (Table 1.1) (Kiseleva et al. 2005; Genkina et al. 2007; Koroteeva et al. 2007a; Kozlov et al. 2007a).

Internal chain segments are defined as the segments between the branches in amylopectin (Fig. 1.1). Just like for the external chains, there exists no method that can clarify the size distribution of these segments, but the average internal chain length (ICL) can be estimated when the average chain length (CL) and ECL is known (Bertoft 1989; Manners 1989). ICL is much shorter that ECL and some examples are shown in Table 1.2.

In addition to ECL and ICL, the limit dextrins can be used to estimate the relative number of A- and B-chains, if the LDs are debranched using specific enzymes that attack the α-(1,6)-linkages (isoamylase and pullulanase). Pioneering experiments with such enzymes were conducted in the 1950s (Hobson et al. 1951; Peat et al. 1952b), and the enzymes became commercially available a decade later. The method is based on the fact that the different exo-acting enzymes leave external chain stubs with specific lengths in the resistant dextrins. Phosphorylase, which removes glucose from the external chains (producing glucose 1-phosphate), hydrolyzes A-chains until four glucose residues remain, whereas the external segments of the B-chains become three residues long (Bertoft 1989). Therefore, chains with a degree of polymerization (DP) of 4 correspond to A-chains. The remaining B-chains in the φ-LD are all longer than DP 4, as illustrated in Fig. 1.1. β-Amylase produces maltose from the nonreducing ends, i.e., it removes two glucose residues simultaneously. If the enzyme is added to a φ-LD, it removes one maltose from each external chain stub

(whereby the number of maltose molecules produced corresponds to the number of chains in the molecule), and in the resulting φ,β-LD, all A-chains correspond to maltosyl chain stubs (Bertoft 1989). When β-amylase is used alone, without prior phosphorolysis, the A-chains remain as either maltosyl or maltotriosyl chain stubs depending whether the original external chain had an even or odd number of residues (Fig. 1.1). The external B-chain stub is DP 1 or 2 (Summer and French 1956). Because β-LDs are comparatively easy to prepare, they have been more frequently used in structural studies. However, as in the example shown in Fig. 1.1, some B-chains of the β-LD have DP 3, i.e., they are of the same length as half of the A-chains. This might interfere with the estimation of A-chains when using β-LDs, albeit in most cases the number of B-chains with DP 3 in β-LDs is very low. The number-based ratio of A:B-chains in some plants is listed in Table 1.2. In most plants, especially several with A-type allomorph crystallinity, the ratio is close to 1.0, albeit wheat appears to be an exception with a comparatively high ratio, whereas B-type crystalline starches tend to have somewhat higher ratio (Bertoft et al. 2008).

1.2.1.2 Unit Chain Distribution

The invention of gel-permeation chromatography made it possible to separate molecules by size and contributed to a much better understanding of the structure of amylopectin. The first size distribution of the chains in amylopectin was published by Lee et al. in 1968. Gunja-Smith et al. (1970) showed for the first time that amylopectin and glycogen have different structures, because glycogen has a unimodal size distribution of only short chains, whereas amylopectin has a bimodal distribution. Therefore, two additional groups of major chains are distinguished in amylopectin, namely, short (S) and long (L) chains (Fig. 1.2). S-chains constitute the major group and have size distributions from DP 6 up to approximately DP 36 in most samples and a peak DP around 11–15, which depends on the sample (Koizumi et al. 1991; Srichuwong et al. 2005b; Vermeylen et al. 2004; Bertoft et al. 2008). L-chains possess normally a peak, or sometimes only a shoulder, around DP 43–50 (Fredriksson et al. 1998; Hanashiro et al. 2002; Bertoft et al. 2008). The number of L-chains is much smaller than S-chains, and the ratio of S:L-chains is quite different in starches from different plants (Table 1.2). Typically, A-type crystalline starches – especially cereal starches – have a high ratio between 10:1 and 22:1, whereas B-type crystalline samples have ratios between 6:1 and 8:1 due to a comparatively high amount of L-chains (Biliaderis et al. 1981a; Hanashiro et al. 2002; Bertoft et al. 2008). This results in longer average chain lengths (CL) of the whole unit chain population, and it was shown that the B-type allomorph crystalline is accompanied by longer CL than the A-type (Hizukuri 1985).

Hizukuri (1986) found that amylopectin preparations from several samples possess polymodal size distributions of unit chains, i.e., the L-chains consist of at least two, maybe three, groups of chains. Moreover, the peak of S-chains possessed a shoulder approximately at DP 15–19, which suggested that S-chains also consist of at least two subgroups of chains. From the classical model by Meyer and Bernfeld

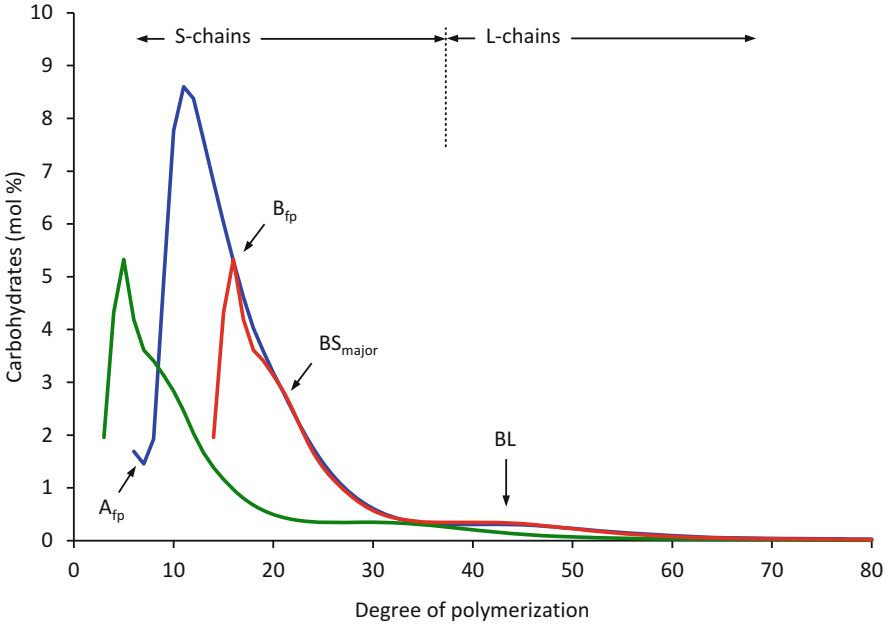

Fig. 1.2 Molar-based unit chain profile of amylopectin from finger millet (*blue line*) and its internal unit chain profile of the B-chains obtained from the φ,β-LD (*green line*). A reconstruction of the position of the internal chain profile (*red line*) was made by adding the average ECL to each of the B-chains of the φ,β-LD. Different chain categories are shown based on the unit chain profile of the amylopectin (A_{fp}-, S- and L-chains) and the reconstructed B-chain profile (Bfp-, BS_{major}, and BL-chains, of which BL-chains correspond to L-chains) (Courtesy of G. A. Annor, University of Guelph, Canada)

(1940), A-chains can generally be supposed to be shorter than B-chains. This led Hizukuri (1986) to suggest that the S-chains consist of A-chains with DP 6–15 and short B-chains, which he named B1-chains, with DP 15–36. The L-chains were all considered as B-chains and were subdivided into B2-chains (with a peak DP around 38–45), B3-chains (peak DP 62–74), and B4-chains (peak DP not clearly distinguished at DP > 80). He also suggested that the subgroups of long B-chains are involved in the interconnection of clusters of chains, details of which are discussed in Sect. 1.3.1. It is of importance to notice, however, that there is no experimental method to date that can measure the actual size distribution of A-chains. In fact, indications of the existence of long A-chains have been found in few samples, albeit in extremely small quantities (Bertoft 2004b; Bertoft et al. 2008).

Hanashiro et al. (1996) used high-performance anion-exchange chromatography (HPAEC), which gives a very high resolution of chains up to approximately DP 60. They found indications of a certain periodicity of DP 12 when they compared the unit chain distributions of amylopectin from several different plant species. They suggested that the shortest chains of fraction fa, which had DP 6–12, were the A-chains, whereas fractions fb_1 (DP 13–24), fb_2 (DP 25–36), and fb_3 (DP > 36)

corresponded to the B1-, B2-, and B3-chains, respectively. Thereby they suggested that the size ranges were shorter than earlier given by Hizukuri (1986) for the same subcategories of chains. This issue will be discussed in more detail later on. It should be noted already here, however, that the ratio of A:B-chains calculated based on this division becomes far lower than the actual A:B-chain ratio that can be measured based on debranching of limit dextrins, because fraction fa includes, in fact, only a fraction of all A-chains in amylopectin (Bertoft et al. 2008).

In addition to unit chains with DP up to the order of 100, some amylopectin samples have been shown to contain very long chains, also named "extra long" or "superlong" chains (Hanashiro et al. 2005; Inouchi et al. 2006). These chains have DP corresponding to several hundred glucose residues and are therefore of the same length as typical amylose chains. Indeed, these chains are apparently synthesized by the same enzyme as amylose, namely, granule-bound starch synthase I (GBSSI), and the chains do not exist in amylopectin from waxy samples (Aoki et al. 2006; Hanashiro et al. 2008). Very long chains have been found in comparatively large amount especially in *Indica* varieties of rice (Takeda et al. 1987), but they were detected in small amounts also in *Japonica* rice and in several other samples, e.g., cassava, sweet potato, potato, maize, and wheat (Charoenkul et al. 2006; Hanashiro et al. 2005; Noda et al. 2005; Shibanuma et al. 1994; Takeda et al. 1988; Laohaphatanaleart et al. 2009; Zhu et al. 2013). It appears that these very long chains mostly are a type of B-chains with longer or shorter external segments (Hanashiro et al. 2005; Laohaphatanaleart et al. 2009).

The size distribution of the C-chain was investigated by Hanashiro et al. (2002), who debranched amylopectin that had been labeled with 2-aminopyridine. The fluorescent C-chains were analyzed by SEC in amylopectin preparations from several different plant species, and it was shown that in most cases the C-chain possesses an almost unimodal size distribution from DP ~10 to DP ~ 100 and a peak DP around 38–43. In yam starch, however, the peak was at DP 49, and in high-amylose maize, it was as high as DP 80. Several samples also possessed a shoulder around DP 21–25. As there is only one C-chain in each amylopectin molecule, the broad size distribution shows that the size of the C-chain is very different in the individual molecules.

1.2.1.3 Internal Unit Chains and Structural Types of Amylopectin

The size distribution of the internal chains in amylopectin, which are part of the B-chains as discussed above, has been studied by debranching limit dextrins (Akai et al. 1971; Atwell et al. 1980; Biliaderis et al. 1981a; Klucinec and Thompson 2002; Lee et al. 1968; MacGregor and Morgan 1984; Mercier 1973; Robin 1981; Shi and Seib 1995). Yao et al. (2004) and Xia and Thompson (2006) debranched the β-LDs of normal and mutant maize amylopectin and found that the short B-chains possessed two size categories with peaks at DP 5 and DP 8 or 9. These chain categories were named B1a- and B1b-chains, respectively (Yao et al. 2004). Bertoft et al. (2008) analyzed the φ,β-LDs of amylopectin from a range of different plants

and found that these chain categories were, in fact, common for all samples. Because the size distribution profile of the shortest chains with DP 3–7 (and the peak at DP 5 or 6) appeared to be specific for a particular plant, they suggested the name "fingerprint" B-chains (B_{fp}-chains), whereas the major part of the short B-chains with DP 8–23 was called BS_{major}-chains. The long internal B-chains with DP > 23 (BL-chains) corresponded apparently to the same categories of B2- and B3-chains as found in the whole amylopectin (Fig. 1.2).

Bertoft et al. (2008) divided the starch samples into four different structural types based on the internal unit chain profile of their amylopectin component. Long chains in type 1 amylopectins are typically not clearly distinguished from the short BS-chains, i.e., a groove in the chromatogram between BS- and BL-chains does not exist because the size distribution of the short chains is broad and overlap largely with the long chains. Further, the number of BL-chains is low, which results in a high ratio of BS:BL-chains (approximately 7.3–9.4). Type 1 amylopectin was found in several cereals, such as barley, oat, and rye, which all possess the A-type allomorph crystallinity. The structural type of these starches was therefore labeled "A:1" to denote the allomorph type of the granule and the structural type of the amylopectin, respectively. Type 2 amylopectins were found in both A- and C-type allomorph starches (the C allomorph is a mixture of A- and B-type crystals in the same granule (Buléon et al. 1998b)). To this structural type belong, e.g., maize, rice, and finger millet ("A:2") as well as kudzu and sago palm starch ("C:2"). (Finger millet is shown as an example in Fig. 1.2). In type 2 amylopectins, BS-chains are distinguished from BL-chains by a groove in the chromatograms at approximately DP 23 (because the size distribution of BS-chains is narrower compared to type 1), and BL-chains are more numerous than in type 1 amylopectins; the ratio of BS:BL-chains is approximately 4.4–6.8. B_{fp}-chains in both type 1 and type 2 amylopectins tend to appear as a clearly distinguishable peak in the internal chain profile. In some cereals, notably maize and rice, the B_{fp}-chains amount as much as about 20 % of all chains by number in the amylopectin, whereby they are as common as the major internal chain type (BS_{major}-chains).

Structural type 3 of amylopectin possesses somewhat more BL-chains than type 2, but notably less of B_{fp}-chains, so that these chains typically only possess a shoulder in the chromatograms instead of a clear peak as in type 2 (Bertoft et al. 2008). The BS:BL-chain ratio is 3.7–4.7. Examples of type 3 structure are arrowroot ("C:3") and tapioca starch ("A:3"). Type 4 amylopectins, finally, includes all B-type allomorph starches ("B:4"), such as potato and canna starch. This structural type is characteristic of a high content of BL-chains; especially B3-chains are found in larger number than in the other types. The ratio of BS:BL-chains is therefore low, around 2.3–3.0.

It should be noted that the division between the four types of amylopectin structures cannot be taken as absolute. The division was based on a collection of seventeen starches from diverse plant species (Bertoft et al. 2008), and some structural characteristics in other samples may overlap between the types. Indeed, Zhu et al. (2011a) found that amylopectin in sweet potatoes has a structure that is intermediate to types 2 and 3, and the structure of wheat amylopectin appears to be

intermediate between types 1 and 2 (Kalinga et al. 2013). Nevertheless, the results so far suggest a common, systematic division of the structure of amylopectins that is ubiquitous among plants with a normal starch anabolism. Amylopectin from mutant plants, in which one or more enzymes involved in the synthesis of starch are absent or inactive, might not fall into either of these structural types.

The internal B-chains are shorter than the original B-chains in the whole amylopectin (Yun and Matheson 1993; Klucinec and Thompson 2002; Bertoft et al. 2008; Zhu et al. 2011a), because the external segment is removed. Therefore, their original lengths are not exactly known. However, if it is assumed that the length of the external segments generally corresponds to the average ECL of the amylopectin (into which also the A-chains are included), one can theoretically reconstruct the original length of the B-chains by adding a segment to each B-chain that corresponds to ECL. This theoretical operation has been done for several samples, and it is generally found that the reconstructed profile of the B-chains fits rather well with the original unit chain profile at DP approximately ≥ 18 (Fig. 1.2) (Bertoft et al. 2008; Laohaphatanaleart et al. 2009; Zhu et al. 2011a). This suggests that in most cases the actual length of the external segment of the B-chains corresponds to ECL and that the majority of chains at DP ≥ 18 are B-chains in the amylopectin. In types 3 and 4 amylopectins, the reconstructions suggest that the peaks of the BS_{major}-chains and B_{fp}-chains correspond to a shoulder at DP 18–21 in the unit chain profiles of the whole amylopectins. In type 1 and 2 amylopectins, the BS_{major}-chains seem to correspond to DP 18 or 19 in the original chain profile (Bertoft et al. 2008). Especially in type 1 amylopectins, a clear shoulder is apparent at this position, whereas a weak shoulder at approximately DP 14 corresponds to the reconstructed position of the B_{fp}-chains. It was suggested that chains in the original amylopectin with approximate DP < 18 are mixtures of short B-chains (mostly B_{fp}-chains) and the A-chains (Bertoft et al. 2008).

Källman et al. (2013) analyzed the size distribution of the internal C-chain in barley. Like the size distribution of the C-chain in whole amylopectins (Hanashiro et al. 2002; Takeda et al. 2003), the internal C-chain distribution was unimodal with a peak around DP 30 (Källman et al. 2013). As the C-chain in whole amylopectin has peak DP ~40, this suggests that the length of the external segment of the C-chain is similar to that of the B-chains.

1.2.2 Clustered Arrangement of the Chains

The unit chains in amylopectin were originally suggested to be arranged as clusters by Nikuni in 1969. Soon thereafter, French (1972) and Robin et al. (1974) came to similar conclusions. A major contributor to these conclusions was the fact that the unit chain distribution shows that amylopectin consists of both long and short chains, of which the latter apparently are the clustered chains, whereas the former can be assumed to interconnect the clusters. Therefore, it appears that the ratio of S:L-chains in amylopectin is a measure of the size of clusters in the form of

the number of chains (NC) that at average is included in the clusters. Because A-type allomorph crystalline starches generally have higher S:L-chain ratio, their clusters are expected to be larger than in B-type allomorphs. Takeda et al. (2003) calculated the NC of clusters on this basis, and when also considering the average DP_n of amylopectin based on estimations of 2-aminopyridine-labeled samples, they concluded that up to 120 and 111 clusters build up the large amylopectin macromolecules in normal maize and rice, respectively (A-type allomorphs), but the small amylopectin molecules have only 5.0 and 4.2 clusters in these two starches, respectively. Large amylopectin molecules in potato (B-type allomorphs) have 117 clusters, whereas the small molecules consist of 15 clusters.

1.2.2.1 Isolation of Clusters

In order to perform a direct study of the structure of clusters in amylopectin, these structural units have to be isolated, which needs catalytic tools that break the long internal chains expected to be found between the clusters. Such tools are, unfortunately, difficult to find, and to date only a limited number of endo-acting enzymes have been used for this purpose. Bender et al. in 1982 hydrolyzed potato and maize amylopectin with cyclodextrin glycosyltransferase of *Klebsiella pneumoniae*. This enzyme form cyclodextrins by attack on the external chains of amylopectin, but it also attacks longer internal chain segments, whereby branched dextrins are released. These branched dextrins were further subjected to β-amylase digestion, and the remaining β-LDs were suggested to represent the clusters of the amylopectin. Bender et al. (1982) found that the size of the β-LDs of the clusters ranged between DP 40 and 140 and at average the clusters in potato were only slightly smaller (DP 75) than in maize (DP 80). The clusters from potato had longer B-chains (CL 17.8) than in maize (CL 14.1), and the degree of branching (DB) was 9.2–11.3 %, whereas maize clusters were more tightly packed with DB 12–14 %. The ratio of A:B-chains was slightly higher in maize clusters (1.22) than in potato clusters (1.06).

Finch and Sebesta (1992) used a maltotetraose-forming amylase from *Pseudomonas stutzeri* to isolate branched limit dextrins from the β-LDs of wheat and potato amylopectin. They suggested that the branched products corresponded to the units of clusters, which in wheat had a relative molecular mass of ∼7600 (corresponding to DP ∼47) and in potato ∼23,000 (DP ∼142). Thus, the size they found for the clusters in potato was nearly double compared to the value reported by Bender et al. (1982), who used cyclodextrin glycosyltransferase for the isolation. This suggests that the result is strongly dependent on the type of enzyme used in the investigation, because different enzymes apparently have different modes of action toward amylopectin. Interestingly, however, the results from both investigations showed that the structure of the amylopectin was a major factor that influenced on the results, because otherwise the results with a particular enzyme should have been similar regardless of the source of the amylopectin. Endo-acting enzymes have therefore an important potential to be used as tools for studying the structure of amylopectin.

An enzyme that has been much used by Bertoft and coworkers, and in a more systematic way, is the α-amylase of *Bacillus amyloliquefaciens* (known as the liquefying enzyme from *B. subtilis* in older literature). This enzyme was shown to have the most specific endo-catalytic action in comparison with several other α-amylases (Bijttebier et al. 2010; Goesaert et al. 2010). The enzyme has nine or ten subsites distributed unevenly around the catalytic site, so that three subsites are situated at the site where the reducing end side of the substrate binds and six subsites at the nonreducing side (Robyt and French 1963; Thoma et al. 1970). This specific structure results in a preferential production of maltohexaose from the nonreducing ends of amylopectin. Simultaneously with this action, the enzyme also performs endo-attack at internal chains (Robyt and French 1963; Bertoft 1989). Long internal chains are effectively attacked because all nine subsites on the enzyme become filled with the glucose residues in the chain (Robyt and French 1963). If the chain segment between branches is shorter than nine residues, the reaction rate slows down markedly, because all subsites cannot interact with the chain. Bertoft and coworkers have used this phenomenon to isolate clusters from a range of different starches by stopping the reaction at the time when the rate of hydrolysis slows down (Bertoft 1986, 1991, 2007b; Bertoft et al. 2011b; Bertoft et al. 1999; Bertoft et al. 2012a; Gérard et al. 2000; Kong et al. 2009; Laohaphatanaleart et al. 2010; Wikman et al. 2011; Zhu et al. 2011c). A consequence of using the enzyme from *B. amyloliquefaciens* is that a cluster will be defined as a group of chains in which the internal chain segments are shorter than nine residues (Bertoft 2007a).

The DP range of clusters in the form of φ,β-LDs obtained with the α-amylase from *B. amyloliquefaciens* and analyzed by GPC is generally broad: from approximately DP 10–15 up to 660–850 (Bertoft et al. 2012a). Average DP of clusters in different size fractions in potato ranged from 31 to 55 (Bertoft 2007b), which was smaller than the previously reported values by Bender et al. (1982) and Finch and Sebesta (1992). However, in comparison with the reported average size (DP 75) of clusters in waxy maize by Bender et al. (1982), the value obtained with *B. amyloliquefaciens* α-amylase was rather similar (DP 70.2). Wheat was reported to have clusters of average DP 82.4 (Kalinga et al. 2014), which is much higher than the value found by Finch and Sebesta (1992).

The average sizes of clusters isolated from different sources with the α-amylase from *B. amyloliquefaciens* are listed in Table 1.3. Interestingly, Bertoft et al. (2012a) found that the sizes generally follow the structural types of the amylopectins (discussed above in Sect. 1.2.1.3). Thus, clusters from types 1 and 2 amylopectin are larger than those from types 3 and 4. Also the average number of chains (NC) in the clusters in types 1 and 2 are larger than in type 4, whereas NC is intermediate in type 3. The degree of branches (DB) is around 15 % in types 1 and 2 and lower in types 3 and 4, which corresponds fairly well with the reported values for maize and potato, respectively, by Bender et al. (1982). Further, the average chain length (CL) is short in type 1–3 amylopectins and high in type 4. Moreover, ICL follows the same pattern (Table 1.3). As the division of amylopectin structural types is based on the internal unit chain profiles, it follows that the internal structure of amylopectin reflects the size and structure of the cluster units.

Table 1.3 Cluster structure of amylopectin from selected starches[a, b]

Starch source	Allomorph: amylopectin structure type[c]	NC	DP	DB	CL	ICL
Rye	A:1	11.5	70.1	15.0	6.1	4.0
Wheat[d]	A:1–2	14.2	82.4	16.0	5.8	3.6
Waxy maize	A:2	11.6	70.2	15.2	6.0	4.0
Rice	A:2	11.1	65.8	15.3	5.9	3.9
Arrowroot	C:3	9.2	56.3	14.6	6.1	4.2
Canna	B:4	8.2	56.3	12.7	6.9	5.2

[a]Values adapted from Bertoft et al. (2012a)
[b]*NC* number of chains per cluster, *DP* degree of polymerization, *DB* degree of branching, *CL* chain length, *ICL* internal chain length
[c]Crystalline structure of granule: amylopectin structural type
[d]Adapted from Kalinga et al. (2014)

1.2.2.2 Unit Chains in Clusters

The unit chain profile of clusters isolated with the α-amylase from *B. amyloliquefaciens* typically possesses less long chains than the original amylopectin, which shows that the long chains were cut by the enzyme (Bertoft 2007b; Bertoft and Koch 2000; Bertoft et al. 2011b; Gérard et al. 2000; Kalinga et al. 2014; Kong et al. 2009; Laohaphatanaleart et al. 2010; Zhu et al. 2011c). This suggests that the long chains are involved in the interconnection of the clusters. However, some long chains also remain in the isolated clusters, especially in clusters from amylopectins of structural type 4, but in smaller amounts also from the other structural types (Bertoft et al. 2012a). The profile of the short chains is mainly similar to the profile of S-chains in the amylopectin, which shows that the short chains build up the clusters in accordance with the cluster hypothesis (Bertoft et al. 2012a).

Many short chains in the isolated clusters are identical to those in the original macromolecule, albeit they are not experimentally distinguished from the new chains that are produced by the α-amylase by hydrolysis of the longer chains in amylopectin. As a result of the enzyme activity, new types of chains are found in the isolated clusters (Bertoft et al. 2012a). Notably, chains with DP around 18–27 were typically formed from all samples analyzed so far. The majority of the new C-chains of the isolated clusters were found to correspond to this DP range (Källman et al. 2013), which is expected because each cleavage of a longer B-chain by the α-amylase results in a new chain with a reducing end. The length of these new chains corresponds to the position of the groove in the chromatograms between S- and L-chains in amylopectin, and these chains are especially prominent in clusters from type 4 amylopectins (Bertoft 2007b; Bertoft et al. 2012a). In addition, isolated clusters from all types of starches have highly increased content of chains with DP 3 (Bertoft et al. 2012a). This suggests that there exists a certain conserved interconnection mode of the clusters in all types of starches that gives rise to this type of chains (Bertoft and Koch 2000).

Because the isolated clusters apparently contain new categories of chains, a new nomenclature for chains in these clusters was suggested, in which their names are assigned lowercase letters instead of the capital letters as for amylopectin (Bertoft et al. 2012a). Thus, a- and b-chains in isolated clusters correspond to A- and B-chains in amylopectin, but the size ranges are different. In φ,β-LDs of clusters, the majority of the a-chains correspond to maltosyl stubs. Several a-chains are maltotriosyl stubs, however, which depend on the fact that a small number of very short a-chains already are produced by the α-amylase and these are not attacked by either phosphorylase or β-amylase (Bertoft 2007b). These a-chains cannot be distinguished from the shortest b-chains with the same length. b0-chains have DP 4–6 and correspond largely to the internal B_{fp}-chains in amylopectin, but a lot of b0-chains, like all b-chains, are produced as a result of the action of the α-amylase (Bertoft et al. 2012a). b1-chains have a DP range of 7–18, b2-chains 19–27, and b3-chains DP ≥ 28. A detailed discussion of the involvement of these b-chain categories in the cluster structure is found in Sect. 1.3.2.2.

1.2.3 Building Blocks

The smallest, branched unit in amylopectin is the building block. These units are in practice limit dextrins that are produced by α-amylase (Bertoft et al. 1999; Zhu et al. 2011b). The composition of building blocks in amylopectin therefore depends on the mode of action of the enzyme, and different α-amylases might give rise to different compositions as a result of the distribution and number of subsites in the enzymes (Bijttebier et al. 2010; Derde et al. 2012). The structure of α-LDs of several α-amylases has been described in detail (Bines and Whelan 1960; Hall and Manners 1978; Hughes et al. 1963; Walker and Whelan 1960a). From the viewpoint of the structure of amylopectin, the works of Umeki and Yamamoto (1972a, b, 1975a, b) on the α-LDs formed by the saccharifying and liquefying α-amylases of *B. subtilis* (the latter being identical to the α-amylase of *B. amyloliquefacens*) are of special interest. They described the structure of singly and multiply branched limit dextrins from waxy rice in great detail and found that, by far, the most prominent dextrins are only singly branched. In LDs with three or more chains, they found that the branches are separated by one or two glucose residues (i.e., ICL is 1 or 2) and that branches never appear next to each other in amylopectin (Umeki and Yamamoto 1975a).

Later, Bertoft et al. (1999) also investigated the limit dextrins produced from isolated clusters of a waxy rice sample by the α-amylase of *B. amyloliquefaciens* and called them building blocks. This is thus the same enzyme as used for the isolation of clusters, but the reaction continues at a very slow rate and ultimately reaches a limit (Zhu et al. 2011b). In order to speed up the reaction rate, the isolated clusters are in practice treated with a 100- or 200-fold amount of the enzyme. The DP range of the branched building blocks is about 5–45 (Bertoft 2007a; Bertoft et al. 2012a; Bertoft et al. 2011a; Bertoft et al. 2010; Kalinga et al. 2014; Kong et al. 2009; Zhu et al. 2011b). The largest blocks correspond thereby to the smallest clusters in size – however, building blocks are much more densely branched than clusters: ICL

in building blocks is only 1–3. Branched building blocks have been size fractionated quantitatively by GPC and debranched (Bertoft 2007a; Bertoft et al. 2012b; Bertoft et al. 2011a; Bertoft et al. 2010). It was found that the smallest building blocks with DP 5–9 generally are singly branched, i.e., they consist of two chains and were named group 2 building blocks. Group 3 building blocks have three chains and cover generally the DP range 10–14, whereas group 4, with four chains, have DP 15–19 (Fig. 1.3). Group 5 building blocks consist of a more complicated mixture of α-LDs with between five and seven chains and DP approximately 20–35, whereas, finally, group 6 have DP > 35 and at average 10–12 chains. The same groups of building blocks appear to be universal among all starches, and, moreover, in all samples analyzed so far, group 2 building blocks are most abundant (they constitute roughly 50 % of all blocks by number), whereas group 3 is the second most abundant (25–30 %). Group 4 typically constitutes ∼10 % of the blocks, and groups 5 and 6 are found in only small amounts (Bertoft et al. 2012b). This surprising fact suggests a rather conservative architectural design of the amylopectin molecule throughout the plant kingdom.

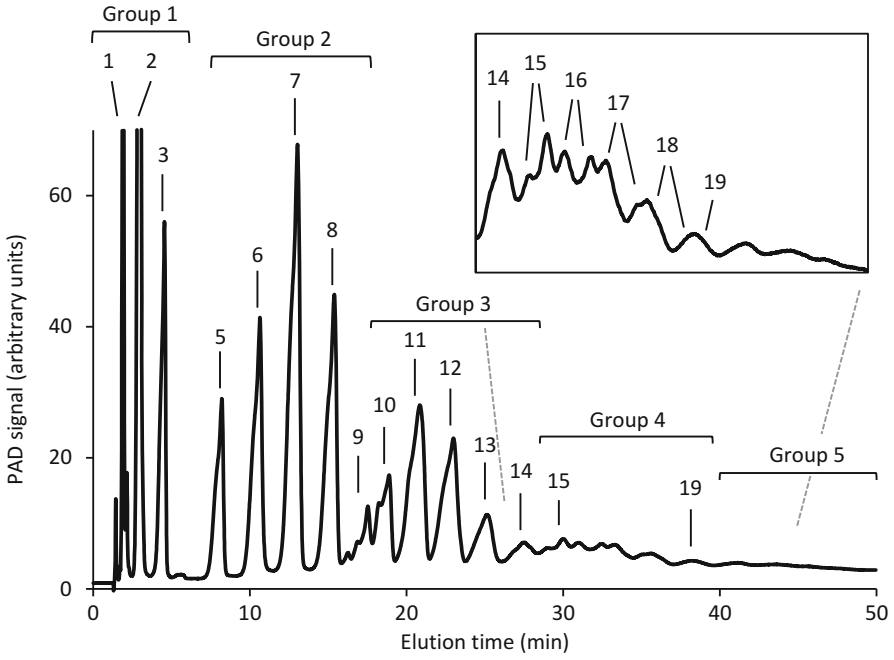

Fig. 1.3 Composition of building blocks in amylopectin from oat starch as obtained by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Groups of building blocks are highlighted and numbers indicate DP. Dextrins with DP 1–3 (group 1) are glucose, maltose, and maltotriose, respectively, and come from interblock segments. Inset shows an enlargement of the complex pattern of blocks belonging to group 4. Building blocks in groups 5 or larger cannot be distinguished as peaks by this chromatographic technique

The reducing end in isolated clusters from barley was labeled with 2-aminopyridine, after which the composition of fluorescence-labeled building blocks was analyzed (Källman et al. 2013). It was found that the size distribution of labeled blocks was practically identical to the distribution of all building blocks (labeled and nonlabeled blocks). This suggests that any of the structural types of blocks can be situated at the reducing end side of the clusters with equal probability and that the organization or sequence of building blocks within a cluster apparently is random.

The degree of branching (DB) in building blocks is high, and it increases typically with the size of the blocks (Bertoft et al. 2012b). Thus, in group 2 building blocks, DB is around 14–15 %, and in group 6 DB is 19–22 %. The average CL is generally short and increases slightly from DP ∼3.6 in group 2 to DP ∼4.4 in group 6. It follows that the size distribution of the chains is narrow: chains in group 2 building blocks have DP between 2 and 7 and the major chain has DP 5. The size distribution broadens with the size of the blocks, and generally the peak position is at DP 5–7. Building blocks in group 6 tend to possess a smaller, second peak at DP 8, and it was suggested that these very large blocks might represent "immature" building blocks with a longer chain interconnecting two smaller blocks, on which one or two chains are connected (Bertoft et al. 2012b). Hypothetically, these chains would normally be "trimmed" by debranching enzymes during starch biosynthesis. "Trimming" of amylopectin chains during biosynthesis is necessary in order to form normal amylopectin (Ball et al. 1996).

The ratio of A:B-chains in building blocks is very difficult to measure exactly because the chains are very short and there exists a considerable overlap of A- and B-chains at DP 3. Nevertheless, a ratio of chains with DP 2 to chains with DP ≥ 4 can be considered as reflecting the true ratio of A:B-chains. Interestingly, building blocks from cereals tend to have a lower apparent A:B ratio than blocks from other sources like roots, tubers, and trunks (Bertoft et al. 2012b). This suggests differences in the fine structure of the building blocks. In cereals, the blocks appear to have a more preferred so-called Haworth conformation of the chains, whereas the other starches have a more preferred Staudinger conformation (Fig. 1.4). In the extreme Haworth conformation, the ratio of A:B-chains reaches zero (Haworth et al. 1937),

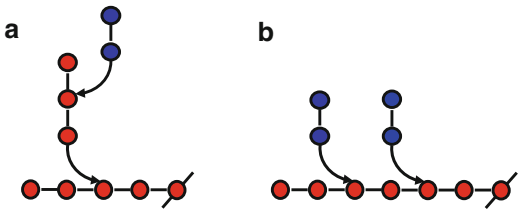

Fig. 1.4 Principal structure of a small building block with three chains with (**a**) Haworth conformation and (**b**) Staudinger conformation. *Blue circles* symbolize A-chains and *red circles* B-chains. In (**a**) the ratio of A:B-chains is 0.5 and for a very large molecule the ratio approaches zero. In (**b**) the ratio of A:B-chains is 2.0 and for a very large molecule the ratio approaches infinity