Barley Starch

Molecular Structure and Properties

Anna Källman
Faculty of Natural Resources and Agricultural Sciences
Department of Food Science
Uppsala

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Cover: Barley ears at 24 days after flowering.
(photo: Anna Källman)

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Barley Starch. Molecular Structure and Properties

Abstract

This thesis examined barley amylopectin structure and looked for correlations between the structure and physical properties of starch. The structure of amylopectin and gelatinisation and retrogradation of starch were studied in 10 different barley cultivars/breeding lines with differing genetic background.

Amylopectin is built up of thousands of chains of glucose monomers, organised into clusters. The detailed fine structure of amylopectin was studied by isolating clusters of amylopectin and their building blocks, which are the tightly branched units building up the clusters. Barley cultivars/breeding lines possessing the *amo1* mutation had fewer long chains of DP≥38 in amylopectin and more large building blocks. The structure of building blocks was rather conserved between the different barley cultivars/breeding lines studied and was categorized into different size groups. These different building blocks were shown to be randomly distributed in the amylopectin molecule. The C-chains in amylopectin can be of any length and are a category of chains different from the B-chains. The backbone in amylopectin consists of a special type of B-chains which, when cleaved by α-amylase, become chains of a similar type to C-chains.

Gelatinisation and retrogradation (recrystallisation of gelatinised starch) of barley starch was studied by differential scanning calorimetry. The *amo1* mutation resulted in a broader gelatinisation temperature range and a higher enthalpy of retrogradation. Other structural features were also found to influence the physical properties of starch. Small clusters and denser structure of the building blocks resulted in higher gelatinisation temperature. Fast retrogradation was observed in barley which had amylopectin with shorter chains and many large building blocks consisting of many chains.

Amylopectin structure was also studied in developing barley kernels. Three barley cultivars/breeding lines were grown in a phytotron and kernels were harvested at 9, 12 and 24 days after flowering. The results showed that amylopectin synthesized at later stages of development had a more tightly branched structure. Expression of the enzymes involved in starch biosynthesis is also known to change during endosperm development.

Keywords: barley, starch, amylopectin fine structure, clusters, building blocks, C-chain, gelatinisation, retrogradation

Author's address: Anna Källman, SLU, Department of Food Science,
P.O. Box 7051, SE-750 07 Uppsala, Sweden
E-mail: Anna.Källman@ slu.se

Dedication

Till min älskade underbara familj

The journey of a thousand miles begins with one step.
Lao Tzu

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Bertoft, E., Källman, A., Koch, K., Andersson, R., Åman, P. (2011). The cluster structure of barley amylopectins of different genetic backgrounds. *International Journal of Biological Macromolecules* 49(4), 441-453.

II Bertoft, E., Källman, A., Koch, K., Andersson, R., Åman, P. (2011). The building block structure of barley amylopectin. *International Journal of Biological Macromolecules* 49(5), 900-909.

III Källman, A., Bertoft, E., Koch, K., Åman, P., Andersson, R. (2013). On the interconnection of clusters and building blocks in barley amylopectin. *International Journal of Biological Macromolecules* 55, 75-82.

IV Källman, A., Vamadevan, V., Bertoft, E., Koch, K., Seetharaman, K., Åman, P., Andersson, R. Starch structure, gelatinization and retrogradation properties in barley samples with a high variation in carbohydrate composition (manuscript).

V Källman, A., Sun, C., Koch, K., Åman, P., Andersson, R. Starch structure in developing barley endosperm (manuscript).

The contribution of Anna Källman to the papers included in this thesis was as follows:

I Carried out experimental work and participated in evaluation of the results and in writing the manuscript.

II Carried out experimental work and participated in evaluation of the results and in writing the manuscript.

III Participated in planning the experimental work and in evaluation of results. Was responsible for the analytical work and for writing the manuscript.

IV Participated in planning the experimental work and in evaluation of the results. Was responsible for the majority of the analytical work and for writing the manuscript.

V Participated in planning the experimental work and in evaluation of the results. Was responsible for the analytical work and for writing the manuscript.

Abbreviations

2-AP	2-aminopyridine
ANOVA	Analysis of variance
a_w	Water activity
Bbl	Building block
CL	Average chain length
DAF	Days after flowering
DP	Degree of polymerization
DSC	Differential scanning calorimetry
ECL	External chain length
GBSS	Granule-bound starch synthase
GLM	General linear model
GPC	Gel permeation chromatography
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPSEC	High performance size exclusion chromatography
ICL	Internal chain length
IC-S	Inter cluster segment
PLS	Partial least squares
RVA	Rapid visco analyzer
SBE	Starch branching enzyme
SS	Starch synthase
TICL	Total internal chain length
WAXS	Wide angle X-ray diffraction

1 Introduction

Barley (*Hordeum vulgare)* is one of the most widely grown crops in Sweden (FAOSTAT, 2013) and is mostly used as animal feed and in malting and brewing (FAO, 2009). Only a small proportion is used as food, although barley has good nutritional values due to its high content of dietary fibre, especially β-glucan.

Starch is a cheap raw material and is widely used in industry, both in its native form and modified in different ways to fit industrial needs. Starch consists of the two polymers amylose and amylopectin. The properties of starch are influenced both by the ratio of these two molecules and by the structure of the amylopectin. Detailed knowledge of amylopectin structure is important in order to understand the biosynthesis of starch and, in the longer view, to genetically customize starch with specific properties. Diploid barley has few and large chromosomes and a high degree of self-fertility and is easy to hybridize. Therefore, barley is a good model plant for cereal genetics.

1.1 Starch in general

Starch is used in plants as an energy store and is synthesized in the amyloplasts in the form of granules. In barley, starch is the main component and constitutes ~60% of the grain (Åman & Newman, 1986; Oscarsson *et al*., 1998). The molecules of the main components in starch, the polysaccharides amylose and amylopectin, are built up from a number of glucose monomers, but their structure differs tremendously. Amylose is basically a linear molecule with a molecular weight of 10^5-10^6 Da, while amylopectin is a much larger, highly branched polymer with a molecular weight of 10^7-10^9 Da, in which thousands of chains of glucose are connected to each other through their reducing end (Buléon *et al*., 1998). Normal barley starch generally has an amylose content of 25-30%, although waxy types can have 0% amylase and high amylose varieties

more than 40% (Morrison, Milligan & Azudin, 1984). A transgenic barley was recently shown to contain 0% amylopectin (Carciofi *et al*., 2012).

1.2 Starch granules

Two types of granules exist in barley starch; large lenticular A-granules and small spherical B-granules (Burton *et al*., 2002; McDonald *et al*., 1991; Meritt & Walker, 1968; Schulman *et al*., 1994). The mean diameter is ~3 μm for the B-granules and ~10-13 μm for the A-granules (McDonald *et al*., 1991; Morrison, Scott & Karkalas, 1986; Schulman *et al*., 1994). A-granules constitute the majority (more than 90%) of starch by weight (McDonald *et al*., 1991) but less than 20% by number (Morrison, Scott & Karkalas, 1986, McDonald *et al*., 1991).

Barley A- and B-granules differ in terms of both amylopectin content and structure (Ao & Jane, 2007; Bathgate & Palmer, 1972; McDonald *et al*., 1991; Takeda *et al*., 1999; Tang, Watanabe & Mitsunaga, 2002). The amylose content is higher and the average chain length of amylopectin (CL) is longer in A-granules than in B-granules (Ao & Jane, 2007; McDonald *et al*., 1991; Takeda *et al*., 1999; Tang, Watanabe & Mitsunaga 2002). Furthermore, A-granules gelatinize at a lower temperature than B-granules (Ao & Jane, 2007; Bathgate & Palmer, 1972; Tang, Watanabe & Mitsunaga, 2002).

Starch granules are built up of blocklets forming alternating amorphous and semi-crystalline growth rings, as depicted schematically in Figure 1 (Gallant, Bouchet & Baldwin, 1997; Tang, Mitsunaga & Kawamura, 2006). These semi-crystalline growth rings consist of alternating crystalline and amorphous lamellae (Buttrose, 1960; Gallant, Bouchet & Baldwin 1997; Yamaguchi, Kainuma & French, 1979) with a 9 nm repeat distance (Jenkins, Cameron & Donald, 1993). The external chain segments of amylopectin, *i.e.* the part of the chains from their outermost branch point to the non-reducing end, form double helices that build up the crystalline lamellae, whereas the internal part of the molecule, containing the branch points of the chains, exist in the amorphous lamellae (Waigh *et al*., 2000). Depending on the organization of the double helices in the granules, three types of X-ray diffraction patterns appear, which classify starch into A-, B- or C-type (Buléon *et al*., 1998; Imberty *et al*., 1991). Cereal starch generally shows an A-type pattern, while the B-type pattern is found in root and tuber starch (Buléon *et al*., 1998). The C-type pattern is found in legume starch and is a mixture of A- and B-type crystallites. The crystals in B-type starch are less densely packed than those in A-type starch, and the B-type starch contains more water (Imberty *et al*., 1991). A V-type

pattern also exists in starch and comprises crystalline amylose inclusion complexes (Buléon *et al*., 1991).

It is still not clear where amylose is located in the granule, although it is suggested to be associated with the amylopectin chains (Vasanthan & Hoover, 1992; Jane & Shen, 1993; Jenkins & Donald, 1995; Kasemsuwan & Jane, 1994). Amylose has been suggested to be more concentrated at the periphery of the granules (Jane & Shen, 1993; Morrison & Gadan, 1987) as well as at the centre (Blennow *et al*., 2003).

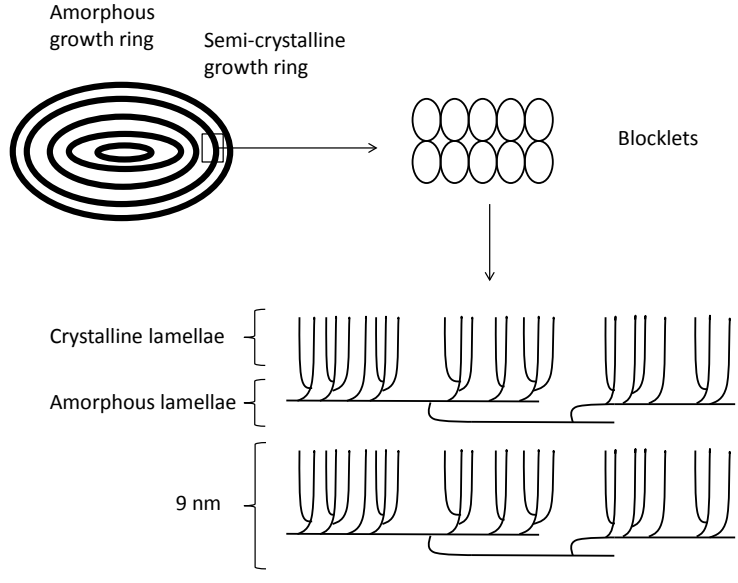

Figure 1. Schematic diagram of starch granule architecture.

1.3 Amylopectin structure

The amylopectin molecule is built up of thousands of chains of 1,4-linked α-D-glucose residues connected to each other by 1,6-linkages. There are three types of glucose chains in amylopectin, namely A- B- and C-chains (Peat, Whelan & Thomas, 1952). The first category, A-chains, carry no other chains, whilst B-chains can carry both A-chains and other B-chains. The C-chain is the only chain with a free reducing end and thus there is only one C-chain per amylopectin molecule and it carries all the other chains. Debranching of amylopectin with the enzymes isoamylase and pullulanase, which cleave the 1,6-linkages, reveals the chain length distribution (Figure 2a), which is an

important characteristic of amylopectin structure. Barley amylopectin has a chain length distribution with a peak at a degree of polymerization (DP) of 12, a distinct shoulder at DP 18-21, and a few long chains of DP>37. Chains of DP 6-8 in amylopectin have been shown to possess typical profiles depending on crop and are probably A-chains, so they are generally called fingerprint A-chains (A_{fp}-chains) (Bertoft, 2004).

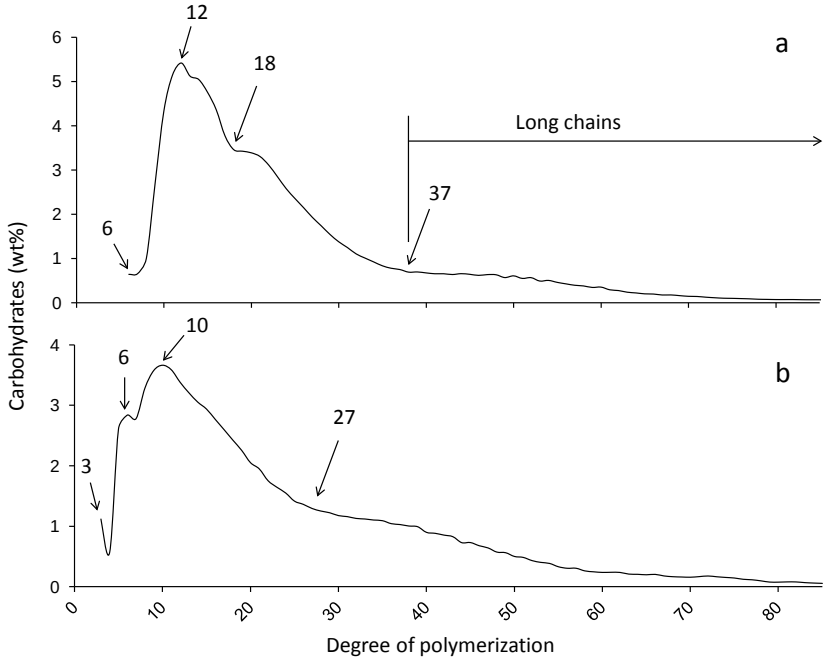

Figure 2. Chain length distribution of (a) barley amylopectin and (b) its φ,β-limit dextrin. Only the size distribution of B-chains is shown in (b).

The chains in amylopectin are organized into clusters and it is generally believed that short chains of up to DP ~35 constitute the cluster, while the longer chains of DP>35 interconnect them (Hizukuri, 1986). A cluster has been defined as a group of chains in which the internal chain length (ICL), *i.e.* the distance between branch points, is shorter than nine glucosyl residues (Bertoft, 2007b). This definition is based on the action pattern of α-amylase from *Bacillus amyloliquefaciens*, which has an active site that binds glucans with DP 9 (Bird & Hopkins, 1954). The size of the clusters and the number of chains they contain differ between crops (Bertoft, 2007a; Bertoft *et al*., 2011; Kong, Corke & Bertoft, 2009; Laohaphatanaleart *et al*., 2010; Zhu *et al*., 2011). In each cluster there are tightly branched units, referred to as building blocks, with an ICL of ~1-2 residues (Bertoft, Koch & Åman, 2012b). The smallest

blocks consist of only two chains and larger blocks contain an average of ~10-12 chains.

The exact mode of cluster interconnection is not known, however, and different alternative models currently exist (Figure 3). In the classical cluster model (Hizukuri, 1986), the chains are organized into clusters placed sequentially in one direction. This model is simply based on the periodicity of chain lengths. In an alternative model, the clusters, in the form of assemblies of building blocks, are oriented perpendicular to a backbone, which consists of long chains interconnecting the clusters (Bertoft, 2004).

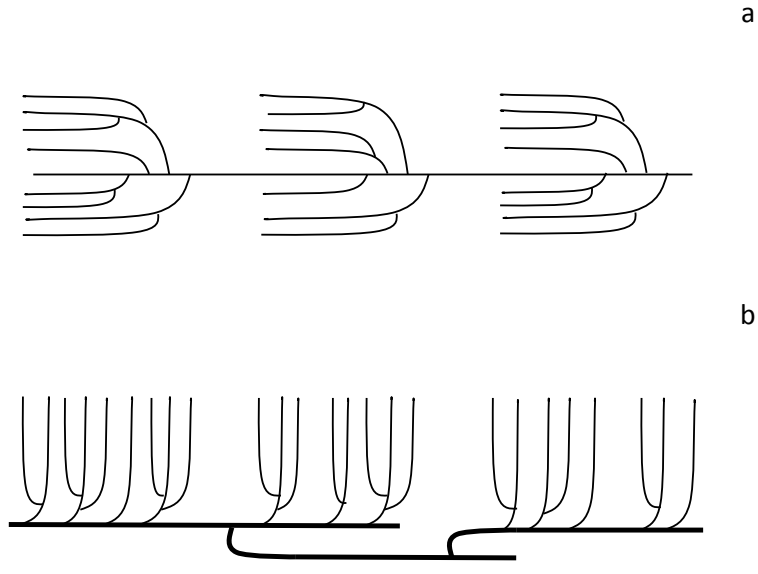

Figure 3. (a) The classical cluster model by Hizukuri, where clusters are organized in one direction, and (b) the two-directional backbone model, where clusters are oriented perpendicular to the backbone (thick lines).

1.3.1 Internal structure of amylopectin

The internal structure of amylopectin can be studied by degradation of the molecule with exo-acting enzymes, either both phosphorylase *a* and β-amylase or β-amylase alone, which leave all branch points intact and the external chains shortened to a great extent. Subsequent debranching reveals the internal chain length distribution of amylopectin (Figure 2b). Amylopectin internal structure has been shown to differ between crops (Bertoft *et al*., 2008). Barley typically has an internal chain length distribution with a peak at DP 10 and a shoulder at

DP ~30. The shortest B-chains of DP 3-7, are called fingerprint B-chains (B_{fp}-chains), since they have been shown to have different profiles between crops (Bertoft, 2004; Bertoft *et al*., 2008).

Degradation by β-amylase alone results in a β-limit dextrin, in which the A-chains are degraded into maltosyl or maltotriosyl stubs and the shortest possible B-chain has DP 3 (Bertoft, 2004). If amylopectin is successively degraded by phosphorylase *a* and β-amylase, resulting in a φ,β-limit dextrin, all A-chains are degraded into maltosyl stubs (Bertoft, 2004). This is because phosphorylase *a* degrades all A-chains into stubs with DP 4 (Walker & Whelan, 1960) and the subsequent treatment with β-amylase further degrades the A-chains into maltose (Bertoft, 2004). B-chains are also degraded by these enzymes and the shortest possible B-chain obtained has only one glucose residue outside the branching point, which results in a chain length of DP 3 (Bertoft, 1989), because the shortest internal chain length is DP 1 (Kainuma & French, 1969). Debranching of a φ,β-limit dextrin reveals the internal chain length of amylopectin and, since all A-chains appear as a maltose peak and the shortest B-chain as maltotriose, the ratio of A:B chains can be determined (Bertoft, 2004). The B-chain fraction actually consists of a mixture of B and C-chains, but the number of C-chains is negligible since there is only one C-chain per molecule. Hanashiro & Takeda (1998) have developed a method for labelling the reducing end of amylopectin, *i.e*. the C-chain, providing the possibility to distinguish C-chains from the other chains.

1.4 Starch biosynthesis

Starch granules grow by apposition (Badenhuizen & Dutton, 1956; Buttrose, 1960) and the amylose content increases during endosperm development (Banks, Greenwood & Muir, 1973; Fast Seefeldt *et al*., 2009; McDonald *et al*., 1991). Starch accumulates in the endosperm from 6 days after flowering (DAF) (Radchuk *et al*., 2009; Weschke *et al*., 2000) up to ~20-40 DAF, depending on growing conditions, after which its concentration remains constant (Banks, Greenwood & Muir, 1973; Fast Seefeldt *et al*., 2009; McDonald *et al*., 1991; Weschke *et al*., 2000). The A-granules are initiated at 5-10 DAF (Burton *et al*., 2002; Merritt & Walker, 1969), while the initiation of B-granules occurs at a later stage of granule development (Burton *et al*., 2002; MacGregor, LaBerge & Meredith, 1971; Merritt & Walker, 1969; Wei *et al*., 2008; Williams & Duffus, 1977).

A number of enzymes are involved in the biosynthesis of starch, namely granule-bound starch synthase (GBSS), multiple forms of starch synthases (SS: SSI, SSIIa and b, SSIII and SSIV), and starch branching enzymes (SBE: SBEI

and SBEII) (Tetlow, 2011). GBSS is responsible for the synthesis of amylose, while the SSs and SBEs are involved in amylopectin synthesis. The different enzymes are primarily responsible for synthesizing different chain categories. The expression of enzymes involved in barley starch synthesis changes during development (Radchuk *et al*., 2009; Sreenivasulu *et al*., 2004).

1.5 Physical properties

The pasting profile of starch can be obtained using a Rapid Visco-Analyzer (RVA), which measures the viscosity of a sample during heating and cooling. When starch is heated in excess water, the granules absorb water and amylose leaches out from the granules, which start to swell and lose their molecular order (Eliasson & Gudmundsson, 2006). This endothermic process is called gelatinization. Melting of the crystallites can be studied by differential scanning calorimetry (DSC). When gelatinized starch is cooled, amylose and amylopectin recrystallize, a process known as retrogradation.

The amylose/amylopectin ratio influences physical properties such as gelatinization temperature (Fredriksson *et al*., 1998) and retrogradation (Fredriksson *et al*., 1998; Jane *et al*., 1999; Zhu *et al*., 2010). Amylopectin fine structure also affects gelatinization properties (Bao *et al*., 2009; Jane *et al*., 1999; Kim & Huber, 2010; Kong *et al*., 2008; Singh *et al*., 2010; Vamadevan, Seetharaman & Bertoft, 2013; Zhu, Corke & Bertoft, 2011).

A higher proportion of short chains of DP 6-12 is correlated with lower gelatinization temperature (Bao *et al*., 2009; Kong *et al*., 2008; Singh *et al*., 2010; Zhu, Corke & Bertoft, 2011) and enthalpy (Kong *et al*., 2008; Singh *et al*., 2010). A higher proportion of chains of DP 13-24 is correlated with higher gelatinization temperature (Bao *et al*., 2009; Kong *et al*., 2008; Zhu, Corke & Bertoft, 2011). These short chains probably cause defects in the crystallites, resulting in their melting at a lower temperature (Genkina *et al*., 2007; Zhu, Corke & Bertoft, 2011).

2 Aims

The overall aim of this thesis was to obtain more information on the fine structure of amylopectin. Detailed knowledge of amylopectin structure is important in order to understand the biosynthesis of starch, as well as the relationship between structure and properties. The molecular structure of starch affects its properties, but it is not known how the organization of chains into clusters or the composition of building blocks affect a certain property, or how the structure is controlled by biosynthesis.

The specific aims of the work were to:

- Characterize the structure and composition of clusters in domains of barley amylopectin (Papers I-IV).
- Characterize the structure and composition of building blocks in domains and clusters of barley amylopectin (Papers II and IV).
- Investigate how building blocks and clusters are interconnected in barley amylopectin (Paper III).
- Study amylopectin structure and its physical properties in order to understand how they correlate (Paper IV).
- Study starch structure in developing barley endosperm in order to understand more about starch synthesis (Paper V).

3 Materials and methods

Barley starch structure and properties were studied in 10 different cultivars and breeding lines, provided either as isolated starch or kernels. Methods that underwent modification or which are of great importance for the results presented in this thesis are described in detail below, whilst only brief descriptions of other methods are presented, since they are described in detail in Papers I-V.

3.1 Barley cultivars/breeding lines

In Papers I-III, barley amylopectin structure was studied in detail. The barley starch studied in these papers were provided by SW Seed, Svalöv, Sweden, and originated from Cindy and Cinnamon, both possessing the *wax* mutation, the high amylose barley Glacier Ac38, which possessess the *amo1* mutation, and one double recessive breeding line, SW 49427, which possesses both the *wax* and *amo1* mutation (Table 1). These four barley samples were also included in Paper IV, together with six other barley cultivars/breeding lines, in an investigation of the relationship between amylopectin structure and physical properties of starch. These six additional barley samples were NGB 114602 (normal starch), KVL 301 (low β-glucan content), SW 28708 (Waxy), SLU 7 (with a high fibre and β-glucan content), Karmosé (a high amylose cultivar possessing the *amo1* mutation) and the normal feed barley Gustav. These six cultivars/breeding lines were all grown during summer 2009/10 in Vilcún, Chile, where the climate conditions are similar to those in Sweden.

The trials in Chile formed part of the BarleyFunFood research programme at the Faculty of Natural Resources and Agricultural Sciences, Swedish University of Agricultural Sciences (SLU), the general aim of which was to find and study mutants of barley with altered carbohydrate composition in order to identify barleys with potentially good nutritional and technological properties. Initially, 250 barley cultivars and breeding lines were collected and

screened with near infrared (NIR) techniques. Based on the results, 20 barleys which showed different NIR spectra and varied widely in terms of previously documented traits were chosen and grown in Vilcún during summer 2008/09. These barley cultivars/breeding lines were further characterized and six cultivars/breeding lines with differing carbohydrate composition were chosen and grown in Vilcún during summer 2009/10 and grain composition was studied in detail. In Paper V, starch structure during endosperm development was studied in three of the six barleys, namely SLU 7, Karmosé and Gustav, which were grown under controlled conditions in a phytotron.

Table 1. *Origin and characteristics of barley cultivars/breeding lines studied in this thesis*

Cultivar/breeding line	Provided by	Characteristics	Paper
Starch			
Cindy	SW Seed	Waxy	I – IV
Cinnamon	SW Seed	Waxy	I – IV
SW 49427	SW Seed	Waxy, amo1	I – IV
Glacier Ac38	SW Seed	Amo1, high amylose	I – IV
Kernels			
NGB 114602	NGB[a]	Anthocyanin rich, normal starch	IV
SLU 7	SLU	Shrunken endosperm, high β-glucan	IV, V
KVL 301	KVL[b]	Mutant in Bomi, low β-glucan	IV
SW 28708	SW Seed	Waxy, hulless	IV
Karmosé	SW Seed	Amo1, high amylose	IV, V
Gustav	SW Seed	Feed, normal starch	IV, V

[a] Nordic Genetic Bank.
[b] The Royal Veterinary and Agricultural University of Denmark.

3.2 Isolating barley starch

When studying the influence of genetic background on amylopectin structure, it is crucial to have a representative starch sample. This is the reason why the method of McDonald & Stark (1988), which was developed with the aim of minimizing the loss of B-granules, was chosen (with minor modifications) to isolate starch in Paper IV (Figure 4). In brief, barley flour was steeped in 0.02 M HCl in order to inactivate enzymes. After neutralization and wet-mixing, the slurry was wet-sieved through a 70 μm mesh cloth and the fibre residue was rubbed with a pestle and mortar. Crude starch and fibre residue were separately incubated with proteinase K. The fibre residue was wet-sieved through the 70 μm mesh cloth and the starch was pooled and treated once more with proteinase K. The starch was then mixed twice with toluene to remove protein

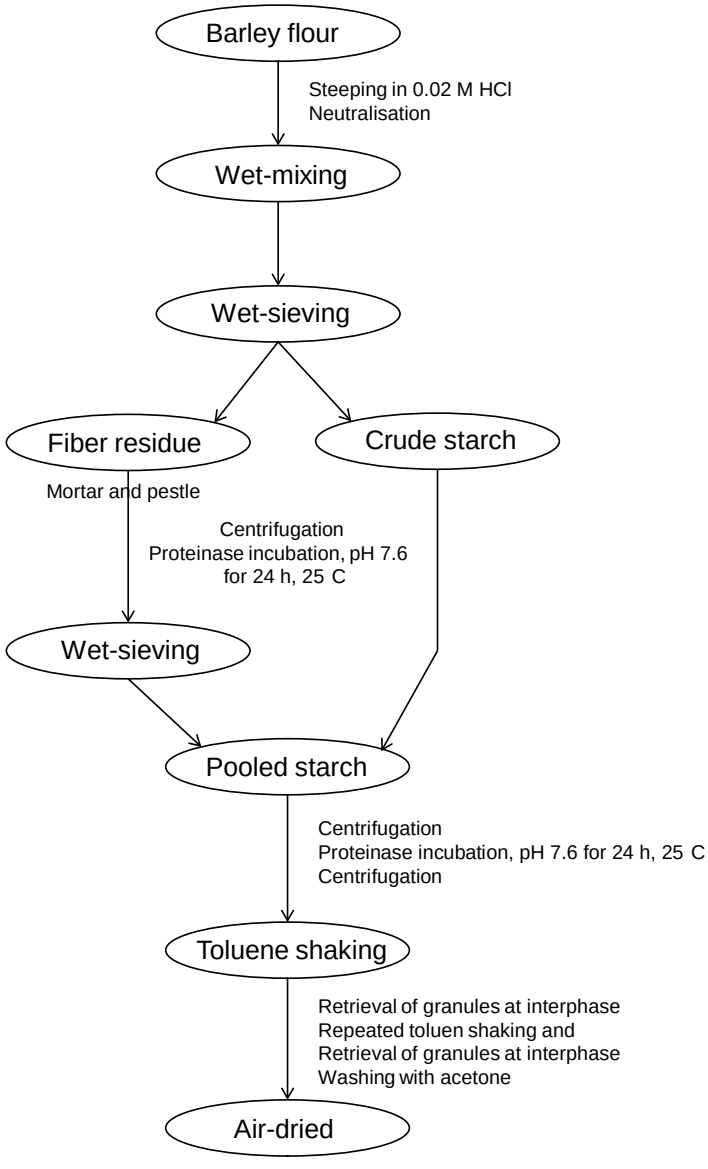

Figure 4. Flow chart of the starch isolation procedure used in Paper IV.

residues and any starch granules remaining in the toluene-aqueous interface were retrieved. The isolated starch was dried in acetone.

In Paper V, however, a simpler, quick method by Carciofi (2011) was modified and used (Figure 5). In the modified version, freeze-dried kernels were milled in a coffee grinder, mixed with 1% sodium dodecyl sulphate

containing 5 mM dithiothreitol and wet-mixed. The slurry was stirred for 30 minutes to inactivate enzymes and wet-sieved through a 70 µm mesh cloth. Starch was treated with lichenase to remove any β-glucan present, and air-dried in ethanol.

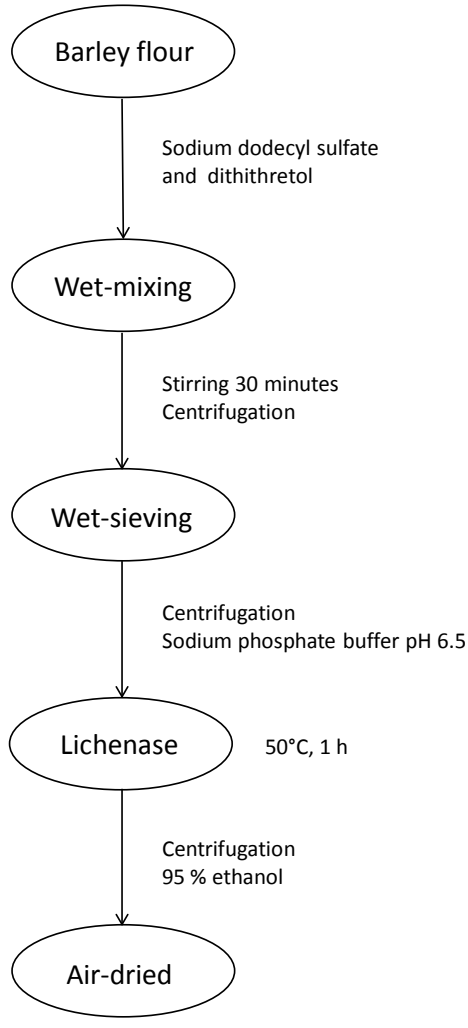

Figure 5. Flow chart of the starch isolation procedure used in Paper V.

3.3 Amylose content

In Papers I-IV, the amylose content of barley starch was determined by gel permeation chromatography (GPC) using a 90 × 1.6 cm Sepharose CL 6B column with 0.5 M NaOH as eluent. Starch was debranched with the enzymes isoamylase and pullulanase at pH 5.5 before being applied onto the column. Every second fraction of 0.5 mL was collected and the carbohydrate content analyzed by the phenol-sulphuric acid method (DuBois *et al.*, 1956). Waxy barley starch containing 0% amylose was used to determine the cut-off between amylose and amylopectin. Any carbohydrates in barley starches eluting before the waxy starch were considered to be amylose.

In Paper V, the amylose content was determined by a colorimetric method in which the iodine complex was stabilized with trichloroacetic acid. Lipids were removed after solubilization of starch according to Morrison & Laignelet (1983) and the amylose content analyzed according to Chrastil (1987).

3.4 Production of domains, clusters and building blocks

In Papers I-III, barley amylopectin structure was studied in detail in the four barley cultivars/breeding lines Cindy, Cinnamon, Glacier Ac38 and SW 49427. The amylopectin was fractionated according to Klucinec and Thompson (1998) and degraded with α-amylase for a short period of time into domains, *i.e.* groups of clusters (Figure 6), which were size-fractionated and each domain size fraction was further degraded into clusters (Paper I). Amylopectin, domains and clusters were transformed into φ,β-limit dextrins in order to study their internal structure. In Paper II, domains and clusters were degraded into building blocks (Bbl) by extensive treatment with α-amylase.

In Paper IV, aylopectin was also fractionated and its structure studied in detail in the barley cultivars/breeding lines NGB 114602, SLU 7, KVL 301, SW 28708, Karmosé and Gustav. However, in that case amylopectin was directly degraded into clusters and the clusters were further degraded into Bbl. The internal structure of clusters was studied as φ,β-limit dextrins.

The size distribution of domains and clusters was analyzed by GPC on a Sepharose CL 6B column (1.6 × 90 cm) with 0.5 M NaOH as eluent. Bbl are much smaller than clusters and their size distribution was therefore analyzed on a Superdex 30 column (1.6 × 90 cm) using 0.05 M NaCl as eluent. Fractions were collected from both columns and their carbohydrate content analyzed with the phenol-sulphuric acid method (DuBois *et al.*, 1956).

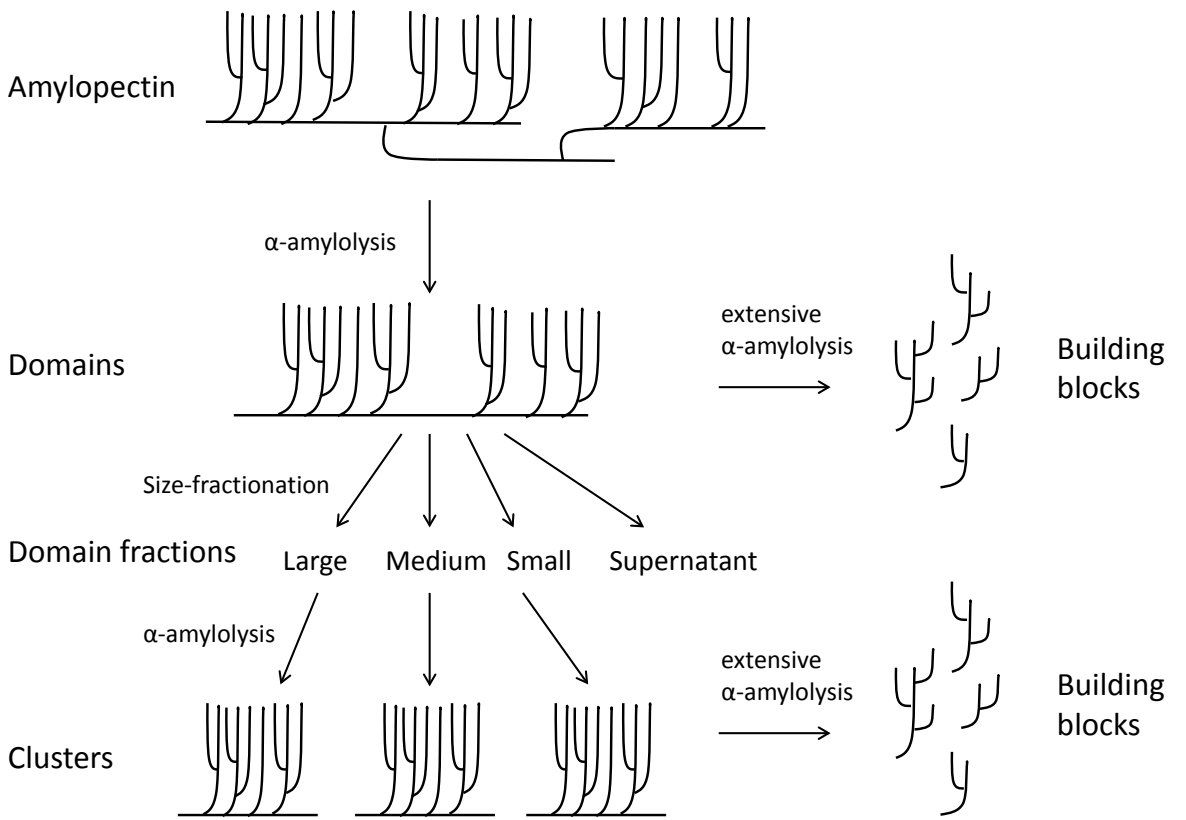

Figure 6. Schematic illustration of the degradation of amylopectin into domains, clusters (Paper I) and building blocks (Paper II) by α-amylase.

3.5 Labeling with 2-aminopyridine

In Paper III the reducing end of amylopectin, domains and clusters was labelled with 2-AP according to Hanashiro & Takeda (1998) in order to study how clusters are interconnected in barley amylopectin. Labelled amylopectin, domains and clusters were debranched with isoamylase and pullulanase and analyzed with high-performance size-exclusion chromatography (HPSEC). The system consisted of a SWXL guard column, one G3000 SWXL column and two G2000 SWXL columns (7.8 × 300 mm) connected in series and samples were detected with both refractive index and fluorescence. Treatment with β-amylase after debranching of labelled samples showed complete debranching.

Clusters and amylopectin were also labelled with 2-AP prior to extensive α-amylolysis in order to study the type of Bbl situated at the reducing end. Labelled Bbl was analyzed with HPSEC as described for the labelled and debranched samples.

3.6 Chain length size distribution

The chain length size distribution was studied in amylopectin (Papers I and IV), domains (Paper I), clusters (Papers I and IV) and whole starch (Paper V). The samples were debranched with isoamylase and pullulanase (only isoamylase was used in Paper IV) and analyzed with HPAEC-PAD by a Dionex system (Dionex, Sunnyvale, CA, USA). The column used was a CarboPac PA-100 column (4 × 250 mm) and the samples were eluted with a gradient of NaOAc in 0.15 M NaOH. In Paper III, amylopectin was labelled with 2-AP and the labelled and debranched samples were analyzed with HPSEC.

The internal chain length size distribution in Papers I, II and IV was analyzed by transforming samples into φ,β-limit dextrins using phosphorylase *a* and β-amylase before debranching and analysis with HPAEC-PAD. In Paper IV, the φ,β-limit dextrins were debranched using only pullulanase due to problems with impure enzymes. In Paper V, whole starch was transformed into β-limit dextrins and debranched with isoamylase and pullulanase.

3.7 Physical properties

The physical properties of starch were studied in Paper IV and correlated to structural parameters. Pasting properties of barley were determined by RVA (RVA, Newport Scientific, Warriewood, NSW, Australia). Barley flour (from

NGB 114602, SLU 7, KVL 301, SW 28708, Karmosé and Gustav) and starch (from Cinnamon, Cindy, Glacier Ac38 and SW 49427) were analyzed using method std1 defined by the manufacturer, with a total running time of 13 min.

Gelatinization and retrogradation parameters were analyzed with differential scanning calorimetry (DSC) using a Q1000 differential scanning calorimeter (TA Instruments, Universal Analysis 2000). The ratio of starch:water during gelatinization was 1:3 and the scanning temperature range was 20-120 °C at a heating rate of 2 °C/min. Retrogradation of barley starch was studied in gelatinized starch at a starch:water ratio of 1:2, stored at 4 °C for 3, 6 and 10 days. The scanning temperature range was 10-120 °C at a rate of 10 °C/min.

3.8 Statistical methods

In Paper II, the four barley cultivars/breeding lines were grouped based on their genetic background, with Glacier Ac38 and SW 49427 (both possessing the *amo1* mutation) constituting the *amo1* group and Cinnamon and Cindy the 'normal' group. Statistical tests were performed with the generalized linear model (GLM) procedure in Minitab 16 (State College, PA, USA). Differences between the two groups of barley and between domain and cluster fractions were analyzed by analysis of variance (ANOVA) using group, fraction (domain and cluster) and size fraction as factors. Tukey pairwise comparisons were used to study differences between size fractions.

The data obtained in Paper III were analyzed using the statistical software Minitab 16 (State College, PA, USA). The results were analyzed by regression using the GLM procedure.

The relationship between physical and chemical properties was evaluated by partial least square (PLS) regression (Paper IV). The models were validated by full cross-validation and uncertainty testing as defined by the Unscrambler software (CAMO Software AS, Oslo, Norway).

The results from Paper V were analyzed by ANOVA using the GLM procedure and Tukey pairwise comparisons in Minitab 16 (State College, PA, USA) to find differences between cultivars/breeding lines and between the different stages of maturity (9, 12 and 24 DAF).

4 Results and discussion

The fine structure of amylopectin from the 10 different barley cultivars/breeding lines studied and the impact of genetic background on amylopectin structure and on properties of starch are described in detail in Papers I-V.

Pasting properties of barley flour were studied by RVA in the six barley cultivars/breeding lines included in the BarleyFunFood project (Figure 7). The viscosity of a flour slurry depends on several factors, among which starch content is important, but *e.g.* protein and cell wall polysaccharides that also absorb water may contribute significantly. The different barleys showed distinct profiles, with the waxy barley SW 28708 having the lower pasting and peak temperature typical of waxy starch. KVL 301 did not gelatinize and SLU 7 showed a high final viscosity, probably due to its high content of β-glucan. The different pasting profiles showed that the barley cultivars/breeding lines had differing carbohydrate composition and were thus a good selection for studying the relationship between structure and properties.

The fine structure of barley amylopectin was studied by hydrolysis of the molecule with α-amylase into domains (Paper I), clusters and building blocks (Paper I, II, III and IV). For the 10 barley cultivars/breeding lines with different genetic background and amylose content studied (Table 2), the amylopectin fine structure was correlated to the physical properties of starch in Paper IV, and starch structure was studied during endosperm development in Paper V.

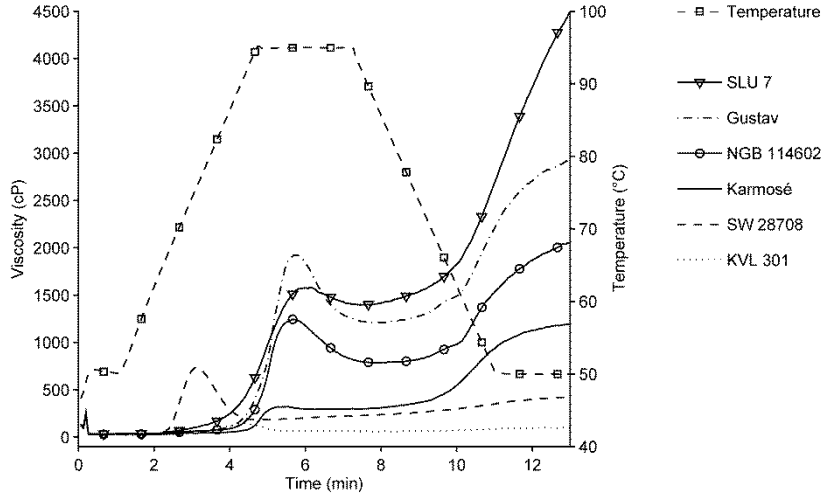

Figure 7. Pasting profiles of flour made from six different barley cultivars/breeding lines.

Table 2. *Genetic background, amylose content, and molar proportion of chain length categories in amylopectin from 10 different barley cultivars/breeding lines*

Sample	Genetic background	Amylose[1]	A_{fp}[2]	DP≥38
Cinnamon	*wax*	0.0	5.9	6.0
SW 28708	*wax*	0.6	6.6	5.9
Cindy	*wax*	10.2	5.5	6.1
NGB 114602		30.3	6.1	5.5
Gustav		31.2	6.3	5.5
KVL 301		31.5	5.0	5.6
SLU 7		32.7	5.6	4.8
SW 49427	*wax amo1*	3.7	6.1	4.1
Glacier Ac38	*amo1*	32.6	4.8	5.1
Karmosé	*amo1*	47.8	5.6	4.6

[1] Percentage amylose in starch.
[2] Fingerprint A-chains with DP 6-8.

4.1 Amylopectin structure

Three of the 10 barley cultivars/breeding lines studied (SW 49427, Glacier Ac38 and Karmosé) possessed the *amo1* mutation (Table 2), which resulted in an altered amylopectin structure. Barleys with the *amo1* mutation had fewer long chains of DP≥38 in amylopectin ($p<0.05$) (Papers I and IV; Table 2), confirming the work of others (Borén *et al*., 2008; Matsuki *et al*., 2008). The *amo1* mutation was also shown to result in a lower ($p<0.05$) molar proportion of long chains of DP≥38 in amylopectin in immature kernels (Paper V), where Karmosé had 4.9-5.6 % of this chain category and SLU 7 and Gustav had >6%.

Amylopectin structure was studied in detail in the barley cultivars/breeding lines Cinnamon, Cindy, Glacier Ac38 and SW 49427 by hydrolyzing the amylopectin with α-amylase into domains, which were size-fractionated and further hydrolyzed into clusters (Paper I). The ICL and total internal chain length (TICL) were lower (4.9 and 11.4-11.6, respectively) in amylopectin from the *amo1* mutants Glacier Ac38 and SW 49427 than in amylopectin from Cinnamon and Cindy (5.5-5.8 and 12.8-13.3, respectively) (Paper I). Immature starch from the *amo1* mutant Karmosé had a lower ($p<0.05$) ICL (~4.8) than Gustav and SLU 7 (>5) (Paper V). This suggests that barley possessing the *amo1* mutation has a denser structure.

The *amo1* mutation was further shown to result in more large clusters (Papers I and IV; Figure 8), and the clusters generally contained more B_{fp}-chains. The molar proportion of B_{fp}-chains was generally >16% in *amo1* mutants, compared with less than 15% in Cinnamon and Cindy (Paper I). No differences were seen for the other chain categories in the clusters and thus the molecular structures of clusters were probably very similar.

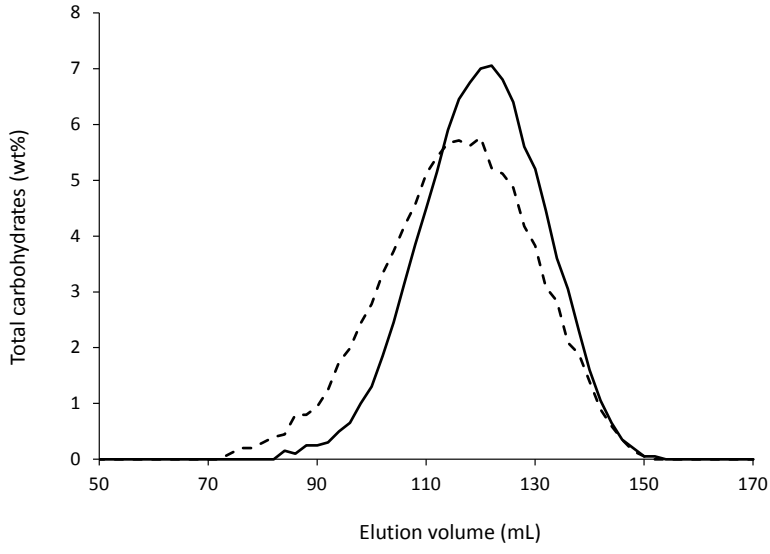

Figure 8. Size distribution of φ,β-limit dextrins of clusters from the *amo1* mutant Karmosé (- - -) and normal barley Gustav (─).

The φ,β-limit dextrins of barley domains were found to have few long chains of DP≥28 (Paper I), which are theoretically involved in the interconnection of clusters (Hizukuri, 1986). The number of long chains in the large domains was too few to be able to interconnect all of the clusters and therefore shorter B-chains are probably also involved in barley cluster interconnection. Potato and cassava are reported to have more long chains than expected from the number of clusters in their domains (Bertoft, 2007a; Laohaphatanaleart *et al*., 2010), suggesting differences in cluster structure, and thus in the cluster biosynthesis, between different plant species.

4.1.1 Building block structure

The size distribution of Bbl was similar in all barley cultivars/breeding lines studied in this thesis (Papers II and IV) and was also similar to the distribution in potato (Bertoft, 2007b), sweet potato (Zhu *et al*., 2011), wheat (Kalinga, 2013), cassava (Bertoft *et al*., 2010) and amaranth (Kong, Corke & Bertoft, 2009). This suggests a rather conserved structure of the basic structural unit in amylopectin from different plants (Bertoft, Koch & Åman, 2012b).

The structure of different size categories of Bbl (Paper II) was found comparable to that in potato (Bertoft, 2007b), sweet potato (Zhu *et al*., 2011),

cassava (Bertoft *et al*., 2010) and several other types of starch (Bertoft *et al*., 2012b). Bbl of group 2 consisted of two chains, that of group 3 of three chains, and that of group 4 of four chains. Groups 5 and 6 probably consisted of several types of blocks having an average of 6.5 and ~10 chains, respectively, as also shown by Bertoft *et al*. (2012b).

The *amo1* mutants had a higher proportion of large Bbl of group 6 ($p<0.005$) and a lower proportion of Bbl of group 2 ($p<0.05$) (Papers II and IV), as illustrated in Figure 9. Overall, the IB-CL of the barley amylopectins was shorter (~6.0) than in potato, sweet potato, amaranth and cassava, which have values of 6.8-8.5 (Bertoft, 2007b; Bertoft *et al*., 2010; Kong, Corke & Bertoft, 2009; Zhu *et al*., 2011). Thus, barley clusters have a more compact structure.

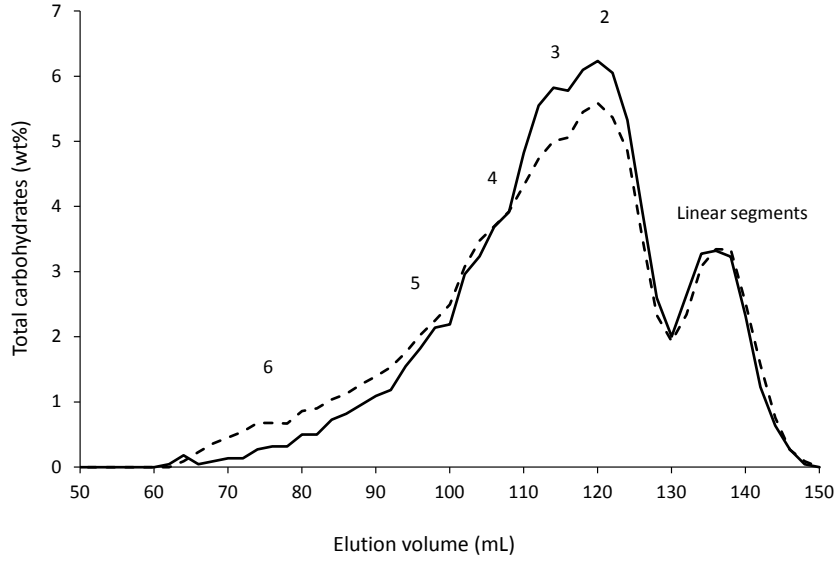

Figure 9. Size distribution of building blocks from the *amo1* mutant Karmosé (- - -) and normal barley Gustav (─), with numbers indicating the different size categories of building blocks.

4.1.2 C- and bc-chain size distribution

The interconnection mode of clusters was studied in the barley cultivars/breeding lines Cinnamon, Cindy, Glacier Ac38 and SW 49427 (Paper III) using the same samples as studied in Papers I and II. C-chain size distribution was studied by labelling φ,β-limit dextrins of amylopectin, large and small domains and their clusters with 2-AP according to Hanashiro &

Takeda (1998). Since all A-chains are degraded into maltosyl stubs and the B-chains have a DP of 3 or more (Bertoft, 2004), it was possible to compare the internal part of the C- and bc-chains (new C-chains formed by α-amylolysis of amylopectin) with the B-chain size-distribution.

The φ,β-limit dextrins of amylopectin of all four barley cultivars/breeding lines possessed a broad size-distribution of C-chains and the longest C-chains (C_{max}) had a DP>100 (Paper III; Table 3). This is in agreement with Hanashiro *et al*. (2002), who reported a C-chain size distribution in whole amylopectin from different cereals and tubers ranging from DP ~10 to ~130, with a peak at DP ~40. The shorter CL and C_{max} found in Paper III (Table 3) suggest that the C-chains generally possess an external segment, which has been removed in the φ,β-limit dextrins.

The size distribution of bc-chains in domains and clusters was also broad, with an average bc_{max} of DP 95 and 59 in large and small domains, respectively, and 53 and 46 in large and small clusters, respectively (Table 3). This possibly reflects the wide DP range of the molecules in each fraction. The narrower chain length distribution of bc-chains in clusters compared with domains suggests that the longer chains found in domains were hydrolyzed by the α-amylase.

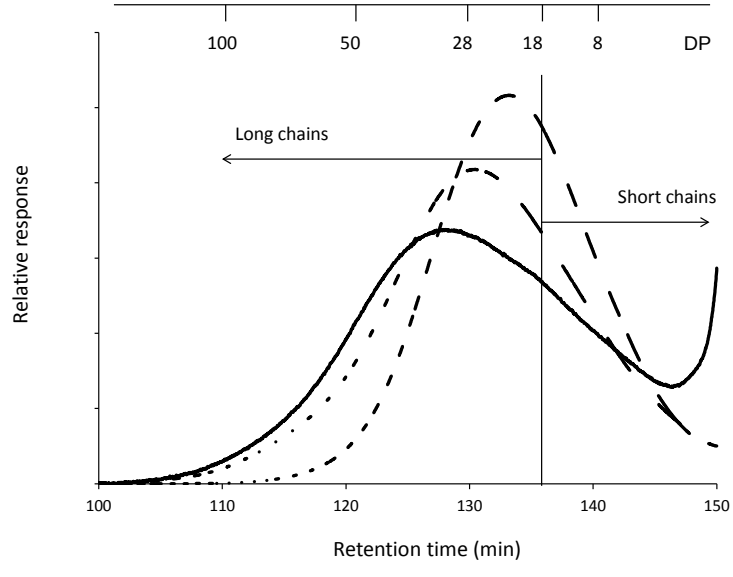

Figure 10. Chain length distribution of C- and bc-chains in φ,β-limit dextrins of amylopectin (─), large domain (- - -) and large cluster (···) of the barley cultivar Cindy.

In a limit dextrin, the size distribution of B-chains is bimodal, but that of both C- and bc-chains is unimodal (Figure 10). We found that the CL of C- and bc-chains was similar in the different barley cultivars/breeding lines, but differed markedly from the CL of B-chains (Paper III; Table 3). In amylopectin, the CL of C-chains was 32-35, while the CL of the B-chains was only 17-18. In domains and clusters, the CL of bc-chains and B-chains was on average 24-32 and 15-16, respectively.

We sub-divided the C- and bc-chains into short chains of DP 8-18 and long chains of DP>18, based on a recent study suggesting that internal B-chains of ~8-18 interconnect two Bbl, while chains of DP>18 interconnect three or more Bbl (Bertoft, Koch & Åman 2012a). In amylopectin from the barley cultivars/breeding lines studied in this thesis, the ratio of long to short (L:S) C-chains was 9-11 times higher than the corresponding ratio of B-chains (Table 3). In domains and clusters, the L:S ratio of bc-chains was on average 9-15 and 8-9 times higher, respectively, than the L:S ratio of B-chains (Paper III).

The unimodal size distribution of C- and bc-chains, and the difference in CL and L:S ratio compared with B-chains, implies that C- and bc-chains are not simply a special type of B-chain, but in fact comprise a specific group of their own.

Table 3. *B-, C- and bc-chains in φ,β-limit dextrins in amylopectin, domains and clusters from four cultivars/breeding lines of barley*

	C- and bc-chains			B-chains	
Sample	CL[1]	C_{max} or bc_{max} [2]	L:S[3]	CL[1]	L:S[3]
SW 49427[4]	32	101	3.6	17	0.4
Glacier Ac38[4]	35	121	4.3	17	0.4
Cinnamon[4]	33	105	4.4	18	0.5
Cindy[4]	33	102	4.6	18	0.5
Large domains[5]	32	95	4.5	16	0.3
Small domains[5]	27	59	3.4	16	0.4
Large clusters[5]	25	53	2.6	15	0.3
Small clusters[5]	24	46	2.3	15	0.3

[1] Average chain length of C- and bc-chains.
[2] DP of longest C- or bc-chain detected.
[3] Molar ratio of long chains of DP>18 to short chains of DP 8-18.
[4] φ,β-limit dextrins of amylopectin.
[5] Average values of large and small domains and clusters from all four barley cultivars/breeding lines.

4.1.3 Bbl at the reducing end

The size distribution of building blocks situated at the reducing end (C-Bbl) in amylopectin and clusters was almost identical in all four barley cultivars/breeding lines studied and their size distribution was similar to the distribution of all building blocks (Paper III; Table 4). Glacier Ac38 and SW 49427 (both possessing the *amo1* mutation) were found to have a higher molar proportion of large building blocks compared with Cinnamon and Cindy (Paper II), and also had more of the larger C-Bbl of groups 5 and 6 ($p<0.01$) compared with Cinnamon and Cindy (Paper III). Thus, it appears that any type of building block can be situated at the reducing end of amylopectin and the clusters, implying a random distribution of building blocks adjacent to an inter cluster segment (IC-S), *i.e*. a chain segment separating clusters, thus having an ICL of more than 9 residues.

Table 4. *Relative number distribution (%) of all building blocks and C-building blocks in barley amylopectin and clusters*

	amo1[1]			Normal[2]		
Sample	AP	Large clusters	Small clusters	AP	Large clusters	Small clusters
Group 2						
All Bbl	n.a.[3]	51.1	54.4	n.a.	54.6	56.1
C-Bbl	47.6	53.7	56.0	52.0	53.3	54.8
Group 3						
All Bbl	n.a.	26.4	26.0	n.a.	26.8	26.4
C-Bbl	25.6	24.5	24.4	25.25	27.0	26.1
Group 4						
All Bbl	n.a.	9.7	9.2	n.a.	8.9	8.9
C-Bbl	11.2	10.4	9.7	10.6	10.5	10.2
Group 5						
All Bbl	n.a.	9.2	8.0	n.a.	7.7	7.7
C-Bbl	10.3	9.1	8.2	8.3	8.0	7.7
Group 6						
All Bbl	n.a.	3.6	2.5	n.a.	2.0	1.5
C-Bbl	5.5	2.5	1.8	4.0	1.4	1.2

[1] Average values of Glacier Ac38 and SW 49427.
[2] Average values of Cinnamon and Cindy.
[3] Not analyzed.

4.1.4 Cluster interconnection

The traditional cluster model by Hizukuri (1986) and the backbone model (Bertoft, 2007b) use different definitions of a cluster. In the cluster model by Hizukuri, chains with length up to approximately DP 28 build up a single cluster of amylopectin, whereas in the Bertoft backbone model clusters are defined as groups of chains with ICL<9. Thus, according to the cluster model, in a φ,β-limit dextrin of a cluster, the bc-chain would most likely be shorter than DP 28. However, in this thesis the φ,β-limit dextrins of clusters possessed many bc-chains longer than this (Paper III; Figure 10).

In the backbone model, long chains form an amorphous backbone to which short chains are attached and form the crystalline lamella, and the clusters can be interconnected by both long and short B-chains. New bc-chains created by the α-amylase can be either long or short depending on the distribution of IC-S. Thus, the backbone model provides an explanation for the high amount of long bc-chains found in clusters, since in this model there is no upper limit for the length of the bc-chain. Since the *amo1* mutation did not affect the C- and bc-chain size distributions, it is possible that the more compact structure in *amo1* barley is the result of more chains being attached to the C- and bc-chains and thus more side chains on the backbone. This would give rise to larger Bbl and clusters, while the length of the C- and bc-chains would remain the same.

The large difference in L:S ratio between C- or bc-chains and B-chains suggests that C- and bc-chains more frequently interconnect several building blocks and clusters. It is thus possible that bc- and B-chains have different structural roles. The backbone in amylopectin possibly consists of the C-chain and a certain type of B-chains with a high frequency of IC-S, whereas other B-chains mostly exist as side chains to the backbone. The α-amylase would then preferentially attack the IC-S in the backbone and new bc-chains created by the α-amylase would be of the same category as the C-chain.

4.2 Influence of amylopectin structure on physical properties

In Paper IV the physical properties of starch were studied in all 10 barley cultivars/breeding lines used in this thesis. Gelatinisation was studied by DSC at a starch:water ratio of 1:3. The barley cultivars SW 49427, Glacier Ac38 and Karmosé, all possessing the *amo1* mutation, had a broader gelatinisation temperature range ($p<0.001$) due to higher mean peak ($p<0.001$) and conclusion temperature ($p<0.05$) (Figure 11), which is in agreement with previous studies (Matsuki *et al*. 2008). The peak temperature of gelatinisation was ~60-64°C and the conclusion temperature ~69-74°C in the *amo1* barleys, compared with ~55-61°C and ~62-66°C, respectively, in the other barley

cultivars/breeding lines. The broader temperature range of gelatinisation in the *amo1* barleys suggests that they possess more heterogeneous crystals.

The peak temperature of gelatinisation was modelled by PLS and the regression coefficients showed that it was affected by the size of the clusters in amylopectin and the density of the molecule (Paper IV). Large clusters with many chains contributed to a lower peak temperature ($p<0.1$). Shorter chains in the building blocks ($p<0.05$), few building blocks in the clusters and short ICL in building blocks ($p<0.1$) resulted in a higher peak temperature. Thus, within the granule, more perfect crystals are formed, resulting in a higher peak temperature of gelatinisation, if the amylopectin clusters are small and their building blocks have short chains and a dense structure.

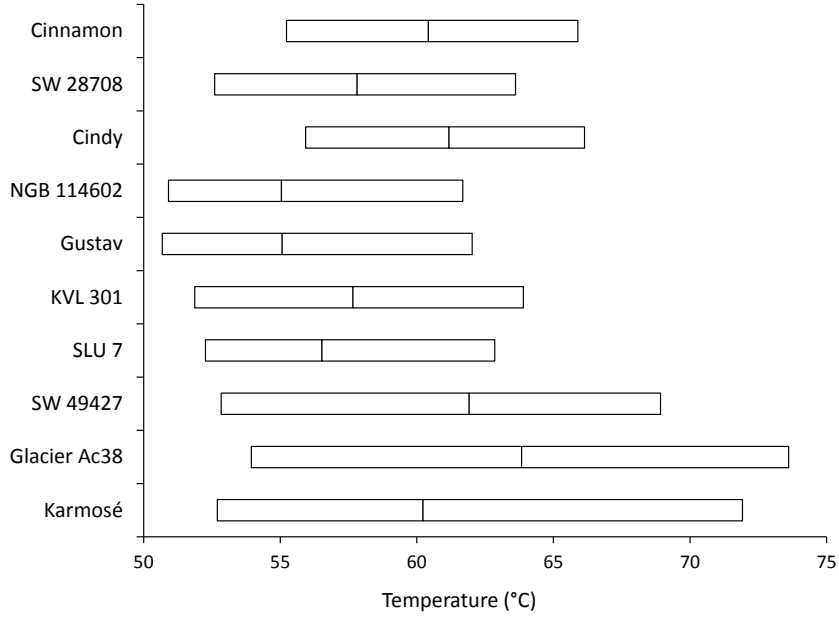

Figure 11. Gelatinisation onset, peak and conclusion temperatures of the different barley cultivars/breeding lines.

Barley starch was also gelatinised at a starch:water ratio of 1:2 and stored at 4°C for 3, 6 and 10 days to study retrogradation (Paper IV). The waxy barleys Cinnamon and SW 28708 did not show any retrogradation until after 10 days of storage, and the waxy barley Cindy did not show any retrogradation until after 6 days (Figure 12). All other barleys had retrograded already after 3 days at 4°C. No systematic difference was found in the melting temperature of retrograded starch between the different days of storage. The onset of melting

temperature was ~41-51°C, the peak ~54-59°C and the conclusion temperature ~63-74°C for all barley cultivars/breeding lines at the different number of days of storage (Figure 12). The markedly higher

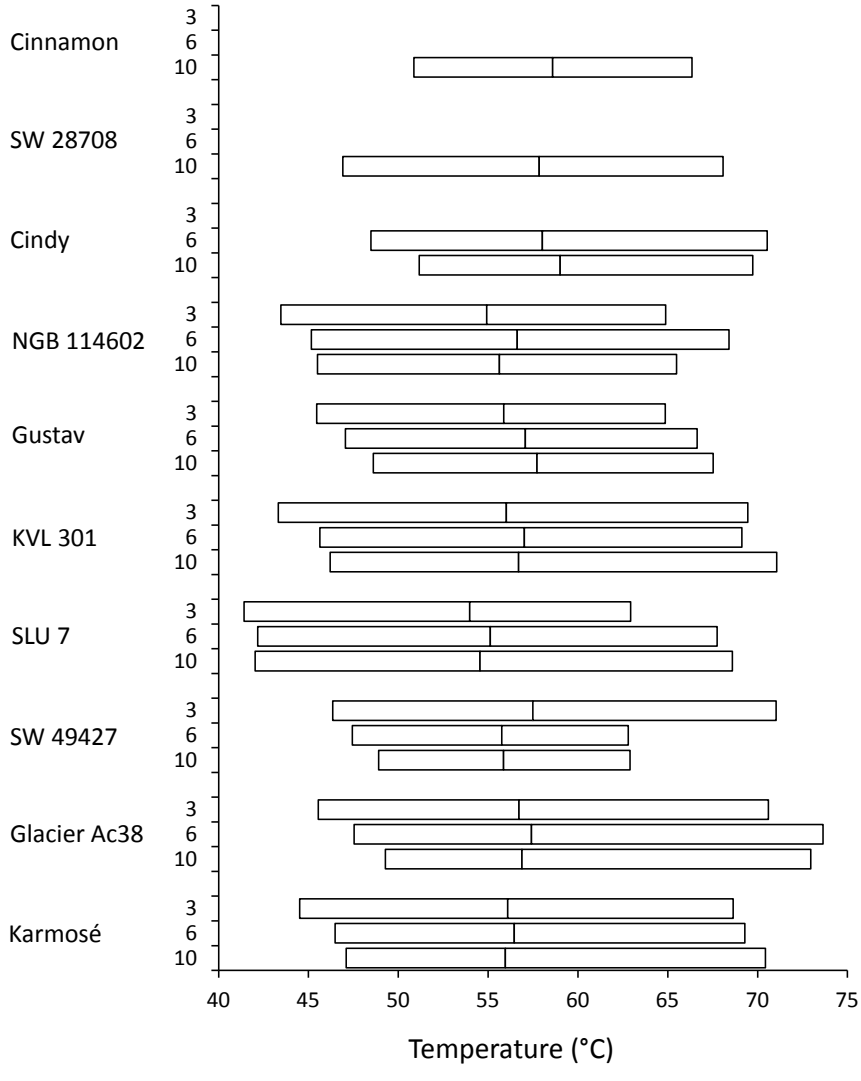

Figure 12. Retrogradation onset, peak and conclusion temperatures of the different barley cultivars/breeding lines after 3, 6 and 10 days at 4°C.

conclusion temperature for SW 49427 at 3 days compared to 6 and 10 days was due to difficulties determining the temperature.

Although no significant effect of the *amo1* mutation was seen on the melting temperature of retrograded starch, barley possessing the *amo1* mutation had a higher enthalpy than the other cultivars/breeding lines ($p<0.001$), indicating that more crystals were formed.

The percentage of crystallinity in the granule did not have an impact on gelatinisation, but it contributed significantly to the PLS model of starch retrogradation. Granules with a high percentage of crystallinity in native form had a high peak temperature and lower enthalpy of melting of retrograded starch ($p<0.05$). Thus, starch from those granules formed less, but more perfect, crystals during retrogradation.

Amylose content also contributed to the model of amylopectin retrogradation, which is in agreement with previous findings by Zhou *et al*. (2011), with a higher amylose content resulting in a lower peak temperature of melting ($p<0.05$) and a higher enthalpy ($p<0.1$). Thus, more crystals were formed, but they were less perfect. However, it is possible that the correlation between amylose and retrogradation is caused by a correlation between amylopectin structure and amylose, since the barley cultivars with the highest amylose content also possess the *amo1* mutation, which affects amylopectin structure. The fact that the waxy barley SW 49427, possessing the *amo1* mutation, retrograded as if it were a high amylose barley supports this suggestion. Long chains of amylopectin resulted in more perfect crystals, although fewer crystals were formed.

Amylopectin molecules which had larger clusters, consisting of more chains, formed less perfect crystals. The prersence of many large building blocks in the clusters also resulted in faster retrogradation, and thus, in less perfect crystals being formed.

4.3 Starch structure in developing barley endosperm

The structure of starch in developing barley endosperm was studied in the cultivars/breeding lines Gustav, Karmosé (possessing the *amo1* mutation) and SLU 7 (with a high content of β-glucan) (Paper V). These barleys were grown in a phytotron and their spikes harvested at 9, 12 and 24 DAF. The dry weight of the kernels increased with maturity ($p<0.05$), which is in agreement with previous findings by Fast Seefeldt *et al*. (2009). The recorded increase was from 19.4 to 39.6 mg for SLU 7, from 19.8 to 46.9 mg for Karmosé and from 16.6 to 45.3 mg for Gustav (Figure 13). The starch content increased with maturity ($p<0.05$) and was lowest in SLU 7 ($p<0.05$).

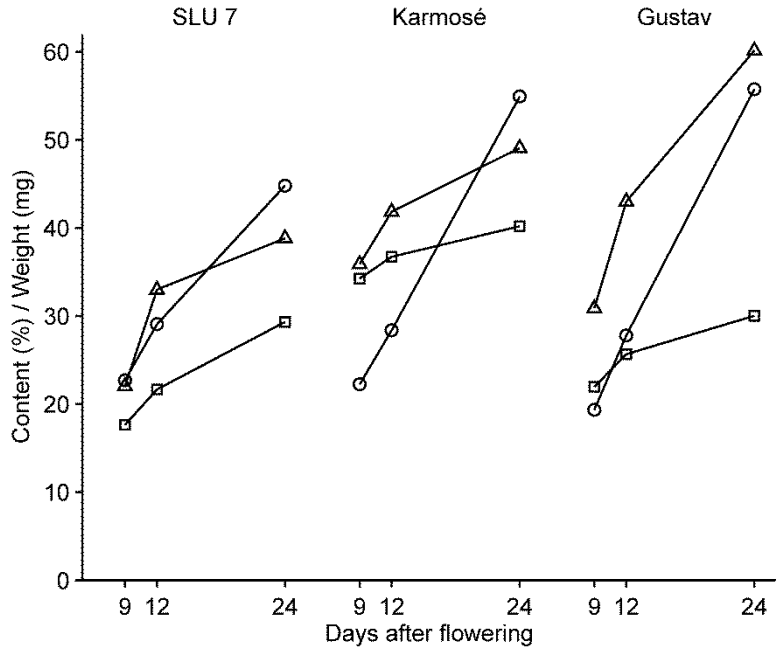

Figure 13. Kernel dry weight in mg (○), starch content as a percentage of dry matter (Δ), and amylose content as a percentage of starch (□) in barley varieties SLU 7, Karmosé and Gustav at 9, 12 and 24 days after flowering (DAF).

Starch was isolated by wet-sieving through a 70 μm mesh cloth and treated with lichenase to remove any remaining β-glucans. The purity of the isolated starch varied between 89.5 and 93.6 %. In all three barley cultivars/breeding lines, the amylose content increased with maturity ($p<0.01$) (Figure 11), confirming the work of others (Banks *et al.*, 1973; McDonald *et al.*, 1991; Fast Seefeldt *et al.*, 2009). This can be explained by the fact that the granule-bound starch synthase (GBSS), which synthesizes amylose (Patron *et al.*, 2002), is expressed more strongly late in development (Radchuk *et al.*, 2009). The amylose content was significantly higher ($p<0.001$) in the *amo1* mutant Karmosé than in Gustav and SLU 7 (Paper V), which is in agreement with previous findings on barley with the *amo1* mutation (Borén *et al.*, 2008; Li *et al.*, 2011; Matsuki *et al.*, 2008).

Amylose and amylopectin were not separated from each other when studying starch structure in the developing endosperm (Paper V). However, amylose chains are too long to be detected by HPAEC-PAD, and thus the chain length distribution represents the size distribution of chains in amylopectin. In all barley cultivars/breeding lines, the molar proportion of chains with DP 22–37 decreased with maturity ($p<0.01$): from 20.4 to 18.9 % in SLU 7, from 20.8

to 18.6 % in Karmosé, and from 20.7 to 19.5 % in Gustav (Table 5). A recent study on starch structure during endosperm development in wheat (Waduge, 2012) showed an increase in A_{fp}-chains and a decrease in chains of DP 18-26 during pre-physiological maturity, *i.e.* up to 35 DAF (Dupont & Altenbach, 2003). In our study, the molar proportion of A_{fp}-chains also tended to increase, although the differences were not statistically significant (Paper V).

The internal structure was studied by transforming starch into β-limit dextrin. In a β-limit dextrin, maltose and maltotriose originate from the A-chains, with only a minor part of maltotriose originating from B-chains (Bertoft, 2004). The barley amylopectin studied in Paper I contained 48-49% A-chains (Bertoft *et al*., 2011), while in the starch from immature kernels studied in Paper V, maltose and maltotriose together constituted ~51-53% of the chains in the β-limit dextrins (Table 5). The slightly higher values in immature samples may be attributed to the fact that we analyzed β-limit dextrins instead of φ,β-limit dextrins, resulting in somewhat more of maltotriose originating from B-chains, which could have overestimated the number of A-chains (Bertoft, 2004). The molar proportion of B_{fp}-chains (DP 4-7) increased with increasing maturity ($p=0.05$) while the molar proportion of BS_{major}-chains of DP 15-27 decreased ($p<0.005$) (Table 5). No differences were seen for the other chain categories. This differs from wheat, where all B-chain categories are reported to increase during development (Kalinga, 2013).

Starch branching enzymes (SBEs) are expressed later in development than starch synthases (SS) (Radchuk *et al*., 2009). SBEI, in particular, is expressed late in barley endosperm development (Mutisya *et al*., 2003), suggesting that the rearrangement of chains in amylopectin by SBE occurs more frequently at later stages of maturity. Higher SBE activity would result in a more tightly branched structure of the amylopectin synthesized later in endosperm development. The higher proportion of B_{fp}-chains and decreasing proportion of BS-chains of DP 15-27 seen in the barley cultivars/breeding lines during development might be due to cleavage of the BS_{major}-chains, giving rise to more B_{fp}-chains and thus a more tightly branched structure.

Table 5. *Molar composition (%) of chain categories*[1] *and lengths of chain segments in starch and β-limit dextrins from three barley cultivars/breeding lines*

Cultivar/breeding line		SLU 7			Karmosé			Gustav		
DAF		9	12	24	9	12	24	9	12	24
Starch	A_{fp}	5.2	5.1	5.3	4.7	5.0	5.6	5.1	5.5	6.2
	DP 9–17	54.0	54.4	55.9	55.6	55.1	56.8	54.4	54.2	53.7
	DP 18–21	13.8	14.1	13.9	13.9	14.1	14.0	13.1	13.3	13.6
	DP 22–37	20.4	20.0	18.9	20.8	20.3	18.6	20.7	20.2	19.5
	DP≥38	6.7	6.3	7.8	5.1	5.6	4.9	6.8	6.8	7.0
β-LD	DP 2 + 3	51.3	53.1	52.3	51.8	51.4	51.5	53.3	52.1	52.6
	B_{fp}	14.2	14.1	14.8	14.6	15.4	15.5	12.6	13.2	14.2
	DP 8–14	17.4	16.8	17.7	17.7	17.8	17.9	17.1	17.1	16.9
	DP15–27	11.2	10.4	10.0	11.5	11.0	10.5	11.2	11.2	10.3
	BL	5.9	5.6	5.2	4.5	4.4	4.6	5.8	6.4	6.0

[1] Chains were divided as: A_{fp} = DP 6-8; B_{fp} = DP 4-7; BL = DP ≥ 28. BS_{major}-chains were sub-divided into dp 8–14 and dp 15–27.

5 Conclusions

This thesis examined the fine structure of barley amylopectin, knowledge of which is important in order to better understand the biosynthesis of starch. Barley amylopectin was degraded into domains, clusters and building blocks, and the *amo1* mutation was found to result in altered amylopectin structure. Barley cultivars/breeding lines with the *amo1* mutation had a higher molar proportion of large building blocks and lower proportion of long chains of DP≥38.

The *amo1* mutation also affected the physical properties of starch. Barley cultivars/breeding lines possessing the *amo1* mutation had a broader gelatinisation temperature range, suggesting the crystals in the granule are more heterogeneous in these barleys. Overall, small clusters with a compact structure had more ordered crystals in the granule, resulting in a higher gelatinisation temperature. Fast retrogradation was promoted by short chains in amylopectin and large building blocks. However, amylopectin that retrograded rapidly formed less perfect crystals, seen as a lower peak temperature of melting. More perfect crystals in retrograded starch were found when the amylopectin had more long chains and small building blocks.

The *amo1* mutation did not affect the size distribution of C-chains. The C-chains in amylopectin can be of any length and thus differ from the B-chains with their unimodal size distribution, suggesting that they are biosynthesized differently from B-chains. When amylopectin is hydrolysed by α-amylase, new C-chains are created, named bc-chains. The length of these bc-chains depends on the distribution of IC-S (the chain segments long enough for the enzyme to hydrolyse) in amylopectin. The size distribution of bc-chains is also unimodal and thus the backbone in amylopectin consists of a special type of B-chains which, when cleaved by α-amylase, become bc-chains of a similar type to C-chains. Any type of Bbl can be situated at the reducing end of amylopectin and any type of Bbl can be associated with the IC-S.

The structure of amylopectin synthesised at later stages of endosperm development was different from the amylopectin synthesised at early stages. The external chain length did not change during development, suggesting that changes only occurred in the internal part of amylopectin. Amylopectin synthesised at later stages of development was more tightly branched, as reflected in a higher proportion of B_{fp}-chains and fewer short B-chains of DP 15-27 in the β-limit dextrin.

6 Future research

The aim of this thesis was to study the fine structure of amylopectin and to find correlations between amylopectin structure and the physical properties of starch. Detailed knowledge of amylopectin structure would also make it possible to better understand the biosynthesis of starch, and thus in the future could lead to new possibilities to create tailor-made starch *in planta*. However, many questions still remain unanswered and further work needs to be carried out, for example:

- Studies on the different roles of enzymes and enzyme complexes involved in biosynthesis and interactions between these.
- Investigations of how altered expression/activity of these enzymes affects the structure of amylopectin.
- Simultaneous studies on the fine structure of amylopectin during endosperm development and expression of enzymes involved in biosynthesis.
- Further investigations of how amylopectin structure affects the properties of starch.
- Studies on the correlation between altered amylopectin structure and the physical properties of starch.

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