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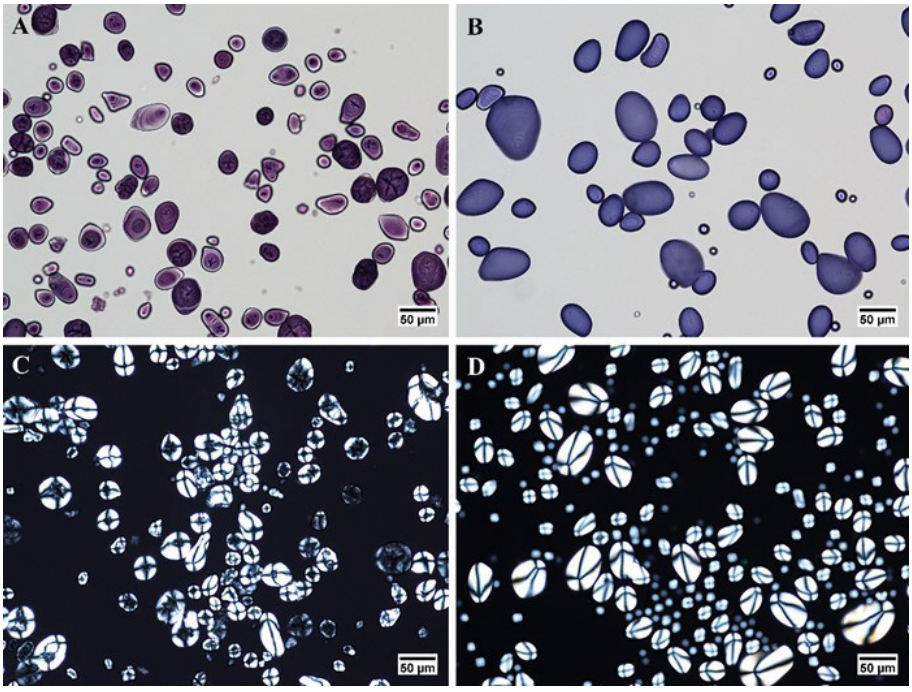
Novel potato starch

New structure and beneficial qualities

Xue Zhao



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New structure and beneficial qualities

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Cover: Microscopic images of potato starch granules

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Abstract

This thesis presents a simplified method for determining the internal molecular structure of whole starch without prior amylopectin isolation. The structure of potato and barley whole starches, the thermal properties of starch from potato lines with different genetic backgrounds and the relationship between molecular structure and functional properties of starch were examined in the thesis.

The internal B-chain distribution and building block composition of amylopectin were characterised effectively by degrading starch into β-limit dextrins (β-LDs), α-limit dextrins (α-LDs) and building blocks. Great variations in internal structure were observed for starches from different plant sources and genetic backgrounds. The general composition of intermediate and large building blocks and the proportion of fingerprint B-chains (B_{fp}-chains), in size order, were determined for starches with decreasing amylose content.

Thermal properties (gelatinisation and retrogradation) of potato starches were investigated using differential scanning calorimetry. Amylopectin lines with a high degree of mutations in multiple genes showed a broader gelatinisation temperature range and lower enthalpy of gelatinisation and retrogradation. Various internal structure parameters were found to affect the thermal properties of potato starch. A dense structure of building blocks led to higher gelatinisation temperatures and enthalpy, while retrogradation was found to be favoured by more large building blocks and many short internal chains.

The high-amylose potato line T-2012 was shown to have higher levels of resistant starch and dietary fibre than the parental variety after cooking. The level of resistant starch increased further after one extra day of cold storage. T-2012 had a very large fraction of long outer amylopectin chains and intermediate-sized inner amylopectin chains, and more intermediate and large building blocks, than the parental potato. The unique amylopectin structure of T-2012 starch favoured formation of recrystallised amylopectin that did not split as easily as ordinary potato starch and was resistant to enzyme digestion.

Keywords: potato, starch, amylopectin internal structure, building blocks, resistant starch, dietary fibre, gelatinisation, retrogradation

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Ny potatisstärkelse

Sammanfattning

Avhandlingen beskriver en förenklad metod för att bestämma den interna molekylära strukturen hos stärkelse utan tidigare isolering av amylopektin. Strukturen hos potatis- och kornstärkelse, de termiska egenskaperna hos potatislinjer med olika genetisk bakgrund och sambandet mellan molekylär struktur och stärkelsens funktionella egenskaper studerades i avhandlingen.

Den interna fördelningen av B-kedjornas längd samt förgreningarnas densitet hos amylopektinet karakteriserades effektivt genom att bryta ned stärkelse till β-LD och α-LD, dvs den interna strukturens byggstenar. Stora variationer i stärkelsens interna struktur erhölls från olika växtkällor och olika genetiska bakgrunder. Det visade sig att andelen halvstora och stora byggstenar samt andelen sk fingeravtryck-kedjor minskade för stärkelserna med minskande amyloshalt.

Termiska egenskaper (gelatinisering och retrogradering) av potatisstärkelser undersöktes med hjälp av differentiell scanningskalorimetri. Amylopektinlinjerna med en hög grad av mutationer i flera gener gelatiniserade i ett bredare temperaturintervall och hade en lägre entalpi för såväl gelatinisering som retrogradering. Olika detaljer i den interna strukturen av potatisstärkelse visade sig påverka stärkelsens termiska egenskaper. Tät struktur hos byggstenarna ledde till högre gelatiniseringstemperaturer och entalpi. Retrogradering visade sig gynnas av fler stora byggstenar och många korta interna kedjor.

I avhandlingen visade sig att högamylospotatislinjen T-2012 hade högre nivåer av resistent stärkelse (RS) och kostfiber efter tillagning, jämfört med ursprungssorten. RS-nivån ökade ytterligare efter ett extra dygn i kylförvaring. T-2012 hade en mycket stor fraktion långa yttre kedjor och medelstora inre kedjor i amylopektinet, och mellanstora såväl som stora byggstenar jämfört med ursprungssorten. Denna unika amylopektinstruktur gynnade bildningen av retrograderat, dvs kristalliserat, amylopektin efter tillagning. Den kristalliserade stärkelsen bryts inte ned lika lätt som vanlig potatisstärkelse och blir delvis resistent mot enzymatisk hydrolys.

Nyckelord: potatis, stärkelse, inre struktur av amylopektin, byggstenar, resistent stärkelse, kostfiber, gelatinisering, retrogradering

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Dedication

To my beloved wonderful family

“The more I learn, the more I realise how much I don’t know.”

Albert Einstein

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List of publications

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

I. **Zhao, X.***, Andersson, M. & Andersson, R. (2018). Resistant starch and other dietary fibre components in tubers from a high-amylose potato. *Food Chemistry* 251, 58-63.

II. **Zhao, X.***, Andersson, M. & Andersson, R. (2021). A simplified method of determining the internal structure of amylopectin from barley starch without amylopectin isolation. *Carbohydrate Polymers* 255, 117503.

III. **Zhao, X.**[#], Jayarathna, S.[#], Turesson, H., Fält, A-S., Nestor, G., González, M.N., Olsson, N., Beganovic, M., Hofvander, P., Andersson, R. & Andersson, M.* (2021). Amylose starch with no detectable branching developed through DNA-free CRISPR-Cas9 mediated mutagenesis of two starch branching enzymes in potato. *Scientific Reports* 11, 4311.

IV. **Zhao, X.**, Hofvander, P., Andersson, M., & Andersson, R. Amylopectin internal structure and thermal properties of potato starches with a wide variation in amylose content (manuscript).

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[#]First authorship shared.

The contribution of Xue Zhao to the papers included in this thesis was as follows:

I. Designed the experiments together with the supervisors, conducted the experimental work, collected and analysed the data, and wrote and revised the manuscript.

II. Planned the study together with the supervisor and conducted all the experiments. Was responsible for data collection and evaluation. Wrote and revised the manuscript.

III. Participated in designing and conducting the characterisation of starch structure and evaluating the results. Had responsibility for writing and revising the manuscript.

IV. Designed the experiments, carried out the experimental work and performed the statistical analysis. Was responsible for writing the manuscript.

Abbreviations

ANOVA	Analysis of variance
DF	Dietary fibre
DM	Dry matter
DMSO	Dimethylsulphoxide
DP	Degree of polymerisation
DSC	Differential scanning calorimetry
GBSS	Granule-bound starch synthase
GLM	General linear model
HPAEC	High performance anion exchange chromatography
HPSEC	High performance size exclusion chromatography
LD	Limit dextrin
MALLS	Multiple-angle laser light scattering
PAD	Pulsed amperometric detection
PLS	Partial least squares (regression analysis)
RS	Resistant starch
SBE	Starch branching enzyme
SS	Starch synthase
UDMSO	Urea-dimethylsulphoxide

1. Introduction

Potato (*Solanum tuberosum*) is one of the most important, nutritious, high-yielding and starch-rich staple crops worldwide (Birch *et al*., 2012). Potatoes comprise a major part of the human diet in the Nordic countries and potato consumption has also increased greatly elsewhere in the world in recent decades (Eriksson *et al.*, 2016).

Potato is also grown as a crop for starch production. Starch is an inexpensive raw material and is widely used for food and non-food applications. Starch is the most abundant food energy source for the human population and animals globally. However, native starch has some drawbacks when it is used in manufactured foods. In order to overcome these disadvantages, much effort has been devoted to physical and/or chemical modification of native starch in food processing. However, the chemical and physical modifications devised to date are money-, time- and energy-consuming, as well as labour- and chemicals-intensive.

Work is underway to make starch production and downstream processing more sustainable, and one part of the solution could be to use modern breeding technologies to develop new starch qualities in a crop. Therefore, increased understanding of the effect of genetic modification on molecular structure and functional properties of starch is vitally important. The ability to tailor starch at the genetic level with desired functional properties for multiple applications, without the need for further chemical or physical modification, would provide an environmentally and economically friendly and sustainable approach for developing novel desirable starches in the near future.

1.1 Starch composition

Starch is primarily extracted from plant tubers, cereal grains and legume seeds. In potato, starch comprises about 15-20% of the tuber by fresh weight and over 80% of the dry matter content (Bertoft & Blennow, 2016). Therefore, starch is considered a major factor affecting the functional properties of potato products developed in the food industry.

Starch consists of two main components, amylose and amylopectin, which are built up of a number of glucose monomers. The molecular weight of amylose and amylopectin is in the order of 10^5-10^6 Da and 10^7-10^9 Da, respectively. In native potato starch, the ratio of amylose to amylopectin is approximately 1:4 (Zeeman *et al*., 2010). However, certain lines known as waxy potato contain very little or no amylose (Vamadevan & Bertoft, 2015) and some high-amylose potato lines have an amylose content of up to 80% (Menzel *et al.*, 2015).

1.2 Starch granule

Starch molecules are organised into highly ordered granules (Figure 1), the morphology and size of which differ between plant species. The diameter of potato starch granules is within the range 10-100 μm, and potato starches generally have larger and smoother granules than cereal starches (Jane *et al*., 1994). The starch granules from native potato have alternating amorphous and semi-crystalline rings, and characteristic Maltese crosses can be seen in polarised light under the microscope (Jenkins *et al*., 1993).

The organisation of the alternating rings in potato starch granules is still not well known. However, it is generally believed that amylose is primarily located in the amorphous parts of the granules, since the majority of amylose is in the amorphous state in granules. Amylopectin is mainly responsible for the architecture of the semi-crystalline rings, although components of both amylose and amylopectin can be found in the amorphous and semi-crystalline rings inside the granules (Vamadevan & Bertoft, 2015). The semi-crystalline rings of starch granules comprise stacks of alternating amorphous and crystalline lamellae with a general repeat distance of ~9 nm (Jenkins *et al*., 1993).

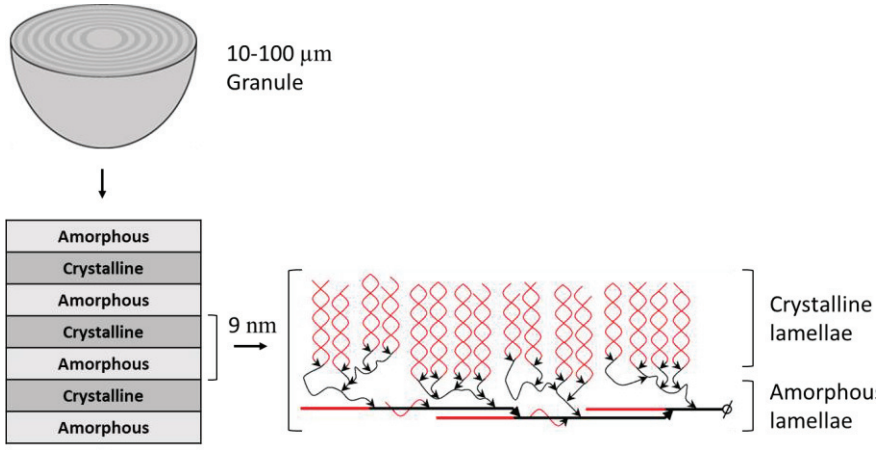

Figure 1. Schematic diagram of starch granule architecture. Source: adapted from *Carbohydrate Polymers* 57 (2004), 211-224.

The external part of amylopectin is the part of chain segments from the non-reducing end to their outermost branch points (Figure 2). These external amylopectin chains form double helices that make up the crystalline lamellae (Perez & Bertoft, 2010). The internal part of amylopectin is the part of chain segments from the outmost branches to the reducing end (Figure 2). This part contains most of the branching points and is involved in the conformation of the amorphous lamellae of starch granules (Perez & Bertoft, 2010). According to the building block backbone model (Bertoft, 2004), the backbone of amylopectin is located here. The stacks of the lamellae are built up of layers of amylopectin molecules (Bertoft, 2013).

Depending on the organisation of the double helices in starch granules, three types of X-ray diffraction patterns are displayed and are used to classify starch into A- B- or C-type (Buléon *et al*., 1998; Imberty *et al*., 1991). Tuber and root starches generally show a B-type pattern, while the A-type pattern is found in cereal starch (Buléon *et al*., 1998). The C-type pattern, which is a mixture of B- and A-type crystallites, is found in legume starch (Buléon *et al*., 1998). The crystals in A-type starch are more densely packed than those in B-type starch, and less water is contained in A-type starch (Imberty *et al*., 1991). Complexes of crystalline amylose-lipid show a V-type pattern (Buléon *et al*., 1998).

1.3 Starch

Starch is one of the most complex materials in nature, with characteristic structures that differ between granular populations and within single granules. Knowledge of starch molecular structure is of vital importance in order to further biosynthesis of starch and understand its structure-property relationships.

1.3.1 Amylose

Amylose is essentially a very long linear molecule consisting of α-(1,4)-linked D-glucosyl chains with degree of polymerisation (DP) in the range of 2000-5000 residues (Hoover, 2001). A minor proportion of branches can also be found in the amylose molecule (Zhu *et al*., 2013).

1.3.2 Amylopectin

Amylopectin is a large and highly branched molecule with thousands of α-(1,4)-linked D-glucosyl chains connected to each other through α-D-(1,6)-branches. These chains are divided into three types, *i.e.* A-, B- and C-chains (Peat *et al*., 1952). One amylopectin molecule contains only one C-chain with a free reducing end and the C-chain carries B-chains and A-chains. B-chains can carry both other B-chains and A-chains, while A-chains do not carry other chains. The chain-length distribution of starch is primarily represented by the chain-length distribution of amylopectin after debranching by the enzymes pullulanase and isoamylase. The chain-length distribution is an important characteristic of starch molecular structure. Amylopectin in a native potato starch has an average amylopectin chain length of DP35, with a peak at DP13 (Menzel *et al*., 2015). Similar results for native potato amylopectin have been reported in earlier studies (Jane *et al*., 1999; Koch *et al*., 1998).

The backbone model assumes a variable and flexible arrangement of the amylopectin chains (Bertoft, 2004). In the model, clusters are oriented perpendicular to a backbone and external segments of clustered chains form double helices (Figure 2). This model, which indicates the crystalline and amorphous lamellae inside starch granules, offers an alternative solution to explore how amylopectin structure influences starch properties and to better understand the biosynthesis of starch. Within the model, the long chains of amylopectin form a backbone where building blocks are bound (Bertoft,

2013). In cereal starches extensive branches connect to the backbone, whereas in tuber starches, like potato, the backbone is probably also bound with a few, shorter branches (Bertoft *et al*., 2012a, 2012b).

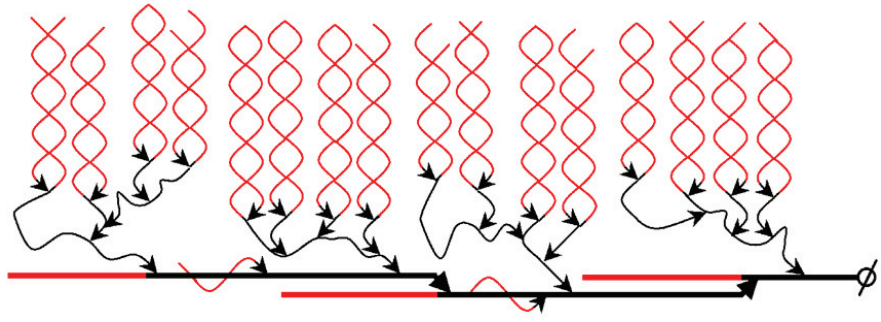

Figure 2. The building block backbone model of amylopectin structure. External chains are shown in red and internal chains are shown in black. The black part is the φ, β-limit dextrin (φ, β-LD). Source: structure adapted from *Carbohydrate Polymers* 57 (2004), 211-224.

1.3.3 External molecular structure of amylopectin

The external part of amylopectin is the part of chain segments from the non-reducing end to their outermost branch points (Figure 2). These external amylopectin chains include all A-chains and the external parts of the B-chains. They form double helices that constitute the crystalline lamellae of starch granules (Perez & Bertoft, 2010).

1.3.4 Internal molecular structure of amylopectin

The internal part of amylopectin is the part from outmost branches to the reducing end. This part is involved in the conformation of the amorphous lamellae of starch granules (Bertoft, 2017). In recent years, multiple studies have revealed the importance of amylopectin internal structure in determining physicochemical properties of starch (Zhu, 2018).

Internal chain-length distribution

The internal chain-length distribution of starch is mainly the internal B-chain distribution of amylopectin and it can be determined by debranching β-limit dextrins (β-LDs) or φ, β-LDs with pullulanase and isoamylase. However, the

internal chain-length distributions in the amylose fraction may also make a slightly different contribution, because of the increased amylose content when de-branched β-LDs are studied in whole starch samples.

In order to study the internal B chains, starch scientists have divided them into different categories with different naming systems and varying ranges of DP, depending on different botanical sources, starch genotypes and properties (Bertoft, 2004; Källman *et al*., 2015; Zhu, 2018). Therefore, it is very important to carefully define the categories of internal B-chains using additional information, such as DP range or elution interval, so as to avoid confusion over the actual molecular structure referred to in different studies.

Based on differences in the composition of internal B-chains, the amylopectins can be summarised into four groups (Bertoft *et al*., 2008). Amylopectins in group one have the highest amount of short internal B-chains and the lowest amount of long internal B-chains, while those in group four have the lowest amount of short internal B-chains but the highest amount of long internal B-chains. The second and third groups of amylopectins are structurally intermediate between the first and fourth groups. Starches containing group one amylopectins have an A-type polymorph, whereas those containing group four amylopectins have a B-type polymorph (Bertoft *et al*., 2008).

Building blocks

The amylopectin branches are concentrated in the form of building blocks in the internal part of amylopectin. Building blocks are conventionally obtained by partial α-amylolysis and successive extensive hydrolysis with the enzymes β-amylase and phosphorylase (Bertoft *et al*., 2011a, 2011b). Building blocks, as one of the basic internal structural units, provide information on aspects of starch structure and have a major impact on the physicochemical properties of starch (Källman *et al*., 2015). Building blocks can be categorised into five groups based on differences in their size, from the most common smallest building blocks carrying two chains per block to the largest carrying an average of about 10-12 chains per block (Bertoft *et al*., 2012a, 2012b). Building blocks have tight branching patterns and the distance between branching points is no longer than DP 3 (Bertoft *et al*., 2012b).

It is estimated that only 1-2% of total branches in whole starch may originate from the amylose fraction (Zhu *et al*., 2013). Thus, the branched

amylose fractions in normal starches might not interfere with the results when studying the composition of building blocks of whole starch.

1.3.5 Starch structure characterisation

Most of the conventional methods used to date for characterising amylopectin structure involve many time-consuming and labour-intensive steps, including amylopectin isolation and partial α-amylolysis of amylopectin into domains, clusters and building blocks at the end (Bertoft *et al*., 2011a, 2011b, 2012a, 2012b; Källman *et al.*, 2013). These steps in total require a relatively large sample amount to obtain enough material for final structure characterisation at the level of building blocks (Lemos *et al*., 2019; Zhu *et al*., 2013). Furthermore, it has been shown that α-amylolysis of β-LD or amylopectin (*i.e*. the order of enzyme addition of β-amylase and α-amylase) gives practically the same result (Bertoft, 1989). Zhu *et al*. (2013) made an attempt to reduce the workload in analysing amylopectin internal structure in maize starch without separating amylopectin. However, the whole starch sample was still hydrolysed in two steps by partial α-amylolysis, with clusters produced in the first step and then hydrolysed again in the second step with α-amylase and β-amylase to produce building blocks.

Therefore, it is very important to have a good method for effective characterisation of starch internal structure in order to better explore the relationships between the complex structure and properties of starch components. The internal structure of whole starch can be studied after extensive removal of the external chains using an exo-acting enzyme β-amylase only, or in combination with phosphorylase. The resulting β-LDs or φ, β-LDs contain all intact branch points, while all A-chains and the external parts of B-chains are degraded into maltotriosyl or maltosyl stubs (Bertoft, 2004).

Examining the structural features of whole starch is important, since it can provide an alternative viewpoint and a broader picture for better understanding the relationships of structure-synthesis and structure-function.

1.4 Genetically modified potato starches

Starch biosynthesis involves many enzymes, including granule-bound starch synthase (GBSS), several types of starch synthases (SS1, SS2, SS3, SS4, SS5 and SS6) and starch branching enzymes (SBE1 and SBE2) (Sarka &

Dvoracek, 2017). GBSS is the only starch synthase isoform in amylose synthesis, while SSs and SBEs act in amylopectin biosynthesis. SSs are involved in glucan chain extensions by transferring ADP-glucose residues to the growing glucan chains at the non-reducing end. SS1 catalyses the formation of chains of amylopectin with DP 8-12 from chains with DP 6-7. SS2 catalyses the synthesis of amylopectin chains with DP of about 6-10 to form chains with DP 12-25. SS3 catalyses the synthesis of chains with DP 25-35 or longer. SS4 is suggested to regulate the initiation of starch granules and SS5 is a novel starch synthase isoform found in cereals (Sarka & Dvoracek, 2017). SS5 and SS6 are also found in potato, but they have not yet been thoroughly characterised (Van Harsselaar *et al*., 2017). The enzyme SBE1 is involved in the formation of branches leading to B-chains, while SBE2 is involved in the biosynthesis of A-chains. Both SBE1 and SBE2 synthesise α-D-(1,6)- linkages that form the amylopectin branches (Sarka & Dvoracek, 2017).

There are generally no major differences in amylose content between potato cultivars and the ratio of amylose to amylopectin cannot be changed through conventional cross-breeding, but only through mutational breeding or genetic tools (Karlsson *et al*., 2007). Potatoes with an increased amylose content and/or altered starch molecular structure have been developed through targeting two SBEs using conventional gene silencing technologies (Andersson *et al*., 2006; Schwall *et al*., 2000) and most recently CRISPR-Cas9 (Tuncel *et al.*, 2019; Zhao *et al*., 2021). Likewise, potatoes that synthesise pure amylopectin starch have been obtained by eliminating GBSS activity using conventional mutagenesis, antisense, RNA-interference (RNAi) and genome editing (Andersson *et al*., 2003; Andersson *et al*., 2017; Jacobsen *et al*., 1991; Visser *et al*., 1991).

1.5 Nutritional properties

Potato is rich in starch and starch is the main component of the energy in most human and animal diets. Unfortunately, potato starch is digested quickly and results in a high blood sugar level when eaten. A potato with more dietary fibre (DF) or a potato with starch that is partly resistant to digestion (resistant starch, RS) or digested more slowly, would be beneficial for human health and would help control glycaemic index and body weight.

1.5.1 Dietary fibre

The health benefits of DF are well-known, *e.g*. reducing calories in foods, decreasing insulin and glucose responses, boosting faecal output and transition, and promoting the production of short-chain fatty acids and the growth of beneficial gut bacteria (Brown *et al*., 2001). Dietary fibre is the fraction of plant material that is retained in the small intestine after enzymatic digestion. It contains RS, cellulose, hemicellulose, pectin, gums, mucilages and lignin. These components can be chemically determined as RS, non-starch polysaccharide residues and Klason lignin (Theander *et al*., 1995).

1.5.2 Resistant starch

Resistant starch forms part of DF. It is determined as the sum of starch and degradation products which cannot be absorbed by healthy people in their small intestine (Englyst & Cummings, 1984; Englyst *et al.*, 1982). In the large intestine, resistant starch is fully or partly fermented. Resistant starch (RS) is classified as five types: I) inaccessible starch, II) resistant granules, III) retrograded amylose, IV) chemically modified starch and V) the complex of amylose and lipid (Hasjim *et al*., 2013). Type II RS consists of resistant granules which are found in raw potatoes. Cooking and then cooling starches can transform ordinary starch to RS through the processes of gelatinisation and retrogradation. When starch is heated in excess water, the granules absorb water and amylose leaches out from the granules (Figure 3), so the granules start to swell and lose their molecular order (Srichuwong *et al*., 2005; Vamadevan & Bertoft, 2015). When gelatinised starch is cooled, amylose and amylopectin recrystallise and become ordered again (Figure 3). The recrystallised amylose is resistant to human α-amylase (type III RS).

The content of RS in food products is reported to be affected mainly by the amylose content, amylopectin structure and some other factors like starch granule phenotypes and different temperature treatments (Karlsson *et al*., 2007; Leeman *et al*., 2006; Lehmann & Robin, 2007; Tian *et al*., 2016).

1.6 Thermal properties

Gelatinisation and retrogradation, two commonly investigated thermal properties analysed using differential scanning calorimetry (DSC), are very important for various industrial applications of starch (Srichuwong & Jane, 2007). These thermal properties vary considerably between different plant

samples and between varieties/lines within species, depending on the starch composition and the molecular structure of the starch (Vamadevan & Bertoft, 2015).

When starch is heated in excess water, the granules undergo an order-disorder phase transition called gelatinisation (Srichuwong *et al*., 2005; Vamadevan & Bertoft, 2015). The gelatinisation process involves water uptake by the amorphous region of starch granules, loss of birefringence with swelling of the starch granules due to water hydration and loss of crystalline order as a result of dissociation of double helices and amylose leaching by taking up heat (Srichuwong *et al*., 2005; Vamadevan & Bertoft, 2015). When gelatinised starch is cooled, disorganised amylose and amylopectin recrystallise and become ordered (Figure 3), a process known as retrogradation (Srichuwong *et al*., 2005).

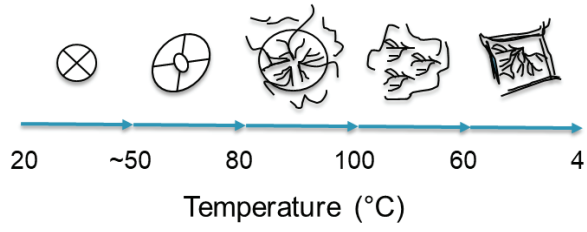

Figure 3. Changes in starch granules during gelatinisation and retrogradation.

It is generally known that retrogradation occurs more easily in starches containing more amylose or longer glucan chains (long amylopectin external chains or intermediate material) (Sasaki *et al*., 2000). The molecular structure of starch also influences its thermal properties. Amylopectin with longer internal chains tends to form a more ordered packing structure of double helices in the granules, which gives higher thermal stability. Longer internal amylopectin chains have also been found to favour the formation of recrystallised amylopectin with higher thermal stability, due to the more ordered structure (Zhu, 2018). Studies on the retrogradation properties of starch have also found that the long chains (DP >~37) and intermediate-sized chains (DP 17-20) in amylopectin promote retrogradation, as indicated by high ΔH measured using DSC (Silverio *et al*., 2000). Very short chains (DP <12 or 10) have the opposite effect (Silverio *et al*., 2000). Amylopectin with large building blocks has a dense branching structure that apparently

facilitates recrystallisation (Källman *et al*., 2015). Previous studies have also shown that the composition of internal amylopectin chains determines the polymorph type of starch (Bertoft *et al*., 2008). Polymorph type is known to greatly influence the physicochemical properties of starch (Zhu, 2018). Thus, starch internal structure indirectly influences the properties of starch in terms of granular structure (Zhu, 2018).

1.7 Starch of interest

Barley starch and potato starch were the main types studied in this thesis. Barley has many genetic variations with varying molecular structure available for research, and therefore barley starch is ideal for use in development of methods for structure characterisation.

Potato is an important crop in Sweden and potato starch was selected for study in the thesis due to its economic value and research interest. Potato starch is widely used in industry. In particular, it is preferred in the food industry because of its neutral flavour and clear pastes due to its low content of lipids and protein compared with cereal and legume starches. Moreover, potato starch is known to contain a high level of phosphate. The high phosphate content of starch granules promotes larger granule size and increased susceptibility to gelatinisation, and is important in potato starch rheology (Bergthaller, 2004). Potato starch is also preferred in the paper industry, owing to good suspensibility of its amylopectin for coating printing paper and the high molecular weight of its amylose for barrier film in packaging paper.

2. Aims

Much is known about the effect of the molecular structure of starch on its properties, but there is still a significant gap in current knowledge about genetically tailored starches, knowledge that is essential when seeking to alter the genome to reach desirable functionality. The overall aims of this thesis were to develop and optimise a method for effectively characterising starch internal structure and to apply the optimised method to a wide range of potato starches, developed using conventional genetic modification or targeted mutagenesis, in order to further explore the relationships between molecular structure and functional properties of starch.

Specific objectives of the work were to:

- Investigate nutritional properties of tubers from a high-amylose potato line T-2012 (Paper I).

- Develop a simplified method without amylopectin separation for effectively determining the internal structure (internal B-chain distribution and building block composition) of starch (Paper II).

- Characterise the molecular structure (chain-length distribution) of starch and the crystalline type and molecular organisation of starch granules from “amylose potato lines” (Paper III).

- Apply the optimised method to “amylose and amylopectin potato starches” in analysis of their internal structure (IV).

- Study the effect of genetic modification on starch structure and the relationship between molecular structure and functional properties of starch (Papers I, III and IV).

3. Materials and methods

The structure and properties of potato starch were investigated in 22 different potato lines and three parental varieties, provided either as tubers or isolated starch. Methods of great importance for the results presented in the thesis are explained in detail below. Other methods are presented with only brief descriptions, but are described in detail in Papers I-IV.

3.1 Potato varieties and developed lines

All potato lines studied in the thesis were developed at the Department of Plant Breeding, Swedish University of Agricultural Sciences (SLU) (Alnarp, Sweden). The wild-type potato varieties Dinamo (Paper I), Desiree (Papers III and IV) and Kuras (Paper IV) were studied as reference.

T-2012 line

In Paper I, the nutritional properties of potato tubers from a high-amylose line (T-2012) were investigated and the results were compared with those of the parental potato cultivar Dinamo. The potato line T-2012 was developed through genetic modification, by down-regulating two starch branching enzymes genes (*SBE1* and *SBE2*) using RNA-interference (RNAi). This potato line was also included in the analyses in Papers III and IV.

"Amylose lines"

Thirteen potato lines (82007, 82050, 82079, 104011, 104032, 104001, 104005, 104006, 104016, 104018, 104034, 104010 and 104023) with an increased amylose content were developed by DNA-free genome editing in Paper III, by inducing mutations in *SBE1* alone or in combination with *SBE2*, using the CRISPR-Cas9 technique. Some representative "amylose lines" were also included in Paper IV (Table 1).

"Amylopectin lines"

In Paper IV, eight potato lines (L1, L2, L3, L4, L5, L6, L7 and L8) with a decreased amylose content were developed through DNA-free genome editing, by inducing mutations in *GBSS* alone or in combination with one or two starch synthase enzymes genes (*SS2* and *SS3*), using the CRISPR-Cas9 technique.

Based on the allelic dosage of mutations in the different genes, the potato lines analysed in this thesis (except for T-2012) were divided into eight groups. Detailed information on these groups is provided in Table 1.

Table 1. *Origin and mutations of potato lines investigated in this thesis*

Line	Parent	Group	Mutations (CRISPR-Cas9)			Paper
"*Amylose lines*"			*SBE1*		*SBE2*	
82007	Desiree	Group 1	×		-	III
82050	Desiree	"	×		-	III, IV
82079	Desiree	"	×		-	III, IV
104011	Desiree	"	×		-	III
104032	Desiree	"	×		-	III
104001	Desiree	Group 2	×		partial	III
104005	Desiree	"	×		partial	III
104006	Desiree	"	×		partial	III, IV
104016	Desiree	"	×		partial	III
104018	Desiree	"	×		partial	III, IV
104034	Desiree	"	×		partial	III
104010	Desiree	Group 3	×		×	III, IV
104023	Desiree	"	×		×	III, IV
"*Amylopectin lines*"			*GBSS*	*SS2*	*SS3*	
L1	Kuras	Group 4	partial	-	-	IV
L2	Kuras	Group 5	×	-	×	IV
L3	Kuras	Group 6	×	partial	partial	IV
L4	L3	Group 7	×	×	partial	IV
L5	L3	"	×	×	partial	IV
L6	L1	Group 8	×	×	×	IV
L7	L1	"	×	×	×	IV
L8	L1	"	×	×	×	IV

"×" = full mutation, "-" = no mutation, "partial" = partial mutation.

3.2 Potato tubers and starch preparation

Tuber cube preparation

To analyse nutritional properties of a high-amylose potato (Paper I), potato tubers from line T-2012 and its parental variety Dinamo were peeled and cut into about 1-cm^3 cubes. The cubes were fully cooked using an autoclave for 5 min at 120 °C. After cooking, the potato cubes were stored cold at 4 °C for 0, 1 or 2 days before analysis. Uncooked potato cubes were prepared similarly, but without cold storage.

Starch isolation

When studying the effect of genetic modification on the molecular structure of whole starch, it is important to achieve good isolation of starch samples. In this thesis, starch of the RNAi line T-2012 (Papers I, III and IV), all "amylose potato lines" (Papers III and IV), and the parental variety Desiree (Papers III and IV) was isolated from mature tubers (Larsson *et al*., 1996), with minor modification of the duration of each buffer washing step and sedimentation after extraction overnight to ensure that small granules were retained. Starch of "amylopectin lines" (Paper IV) and the parental varieties Dinamo (Paper I) and Kuras (Paper IV) was kindly provided by a Swedish starch producer (former Lyckeby Starch AB, Kristianstad, Sweden).

In Paper II, the starch from 10 barley cultivars and breeding lines was used for optimisation of a method for effectively characterising starch internal structure from whole starch samples without prior amylopectin and amylose separation. The barley starches were the same samples studied using conventional methods in a previous doctoral thesis produced within the research group (Källman, 2013).

3.3 Nutritional properties of potato tubers

Resistant starch and total starch

Nutritional properties of potato tubers from line T-2012 were studied in Paper I. The content of RS was analysed according to AOAC Method 2002.02 (McCleary & Monaghan, 2002), using a K-RSTAR 09/14 kit from Megazyme (Bray, Ireland). This method is aimed at determining "physiologically RS", since the analytical conditions correspond to those in the human small intestine. Physiologically resistant starch is

determined as the part of starch not digested after 16 h incubation with amyloglucosidase and pancreatic α-amylase at 37 °C, while non-resistant starch is determined as the part of the starch that has been hydrolysed. The sum of RS and non-RS is the content of total starch.

Dietary fibre

Dietary fibre constituents were analysed based on AOAC Method 994.13 (Theander *et al*., 1995), after removal of non-resistant starch by amyloglucosidase and thermostable α-amylase. The content of DF constituents was then chemically determined as the sum of sugar residues, uronic acid residues and Klason lignin. The sugar residues included rhamnose, arabinose, xylose, mannose, galactose and glucose.

Type III resistant starch

Based on the assumption that the content of type III RS in uncooked tubers was zero, any increase in glucose residues quantified in DF analysis after cooking was taken as the type III RS content in cooked potato tubers.

3.4 Amylose content of starch samples

In Paper I, the amylose content in potato starch from the high-amylose line T-2012 and the parental cultivar Dinamo was measured using a colorimetric assay, by determining the absorbance of starch-iodine complex according to a standard curve with defined amylose contents (Morrison & Laignelet, 1983). The amylose content in potato starch from the "amylose lines", the parental cultivar Desiree and the RNAi line T-2012 (Paper III) was also analysed colorimetrically, but based on stabilised starch-iodine complex formation with trichloroacetic acid (Chrastil, 1987). In brief, the isolated starch was dissolved in urea-dimethylsulphoxide (UDMSO), and then 0.01 N KI-I_2 solution, alone or in combination with 0.5% trichloroacetic acid, was added for incubation before the absorbance analysis. The amylose content in Paper III was double checked using an enzymatic assay with an amylose/amylopectin kit (Megazyme, Bray, Co, Ireland) (Paper III).

In Paper IV, the amylose content of potato starch from the "amylopectin lines" was carefully measured using high performance size exclusion chromatography (HPSEC) (Sveriges stärkelseproducenter, Kristianstad, Sweden).

3.5 Characterisation of starch granules

Crystalline type

The X-ray pattern of potato starch from line T-2012 (Papers I and III) and Dinamo (Paper I), all the "amylose lines" and Desiree (Paper III) was determined using a PANalytical X'Pert alpha1 powder X-ray diffractometer. After spreading the starch sample onto 1.5 x 2 cm^2 silicon wafers, the starch was scanned between diffraction angle (2θ) 5° and 40°.

Microstructural analysis

The phenotype of starch granules from line T-2012 (Papers I and III), Dinamo (Paper I) and the representative "amylose lines" and Desiree (Paper III) was studied using light microscopy after staining the purified starch with iodine solution (Paper I) and Lugol's solution (Paper III). A Nikon Eclipse Ni-U light microscope (Nikon, Tokyo, Japan) was used in Paper I and a Leica DMLB light microscope (Leica Microsystems, Wetzlar, Germany) in Paper III. The images of starch granules were documented with a Nikon DS-Fi2-U3 camera in Paper I and an assembled Leica DFC450C camera in Paper III.

The birefringence of starch granules from line T-2012 and Dinamo (Paper I), line 104016 (Group 2), line 104010 (Group 3) and Desiree (Paper III) was studied. A starch/water dispersion (50 mg/mL) was freshly prepared and then the starch granules were visualised with a 20× objective under polarised light. In Paper I, the Nikon Eclipse Ni-U light microscope referred to above was applied. In Paper III, a light microscope (Leica DMLB, Wetzlar, Germany) equipped with an infinity X-32 digital camera (DeltaPix, Samourn, Denmark) was used.

3.6 Production of β-limit dextrin, α-limit dextrin and building blocks

In Paper II, a method for effectively characterising the internal molecular structure of barley whole starch was developed and optimised. In Paper IV, this optimised method was applied in determining the internal molecular structure of potato starch with a wide range of amylose contents, originating from different gene (*SBE1, SBE2, GBSS, SS2, SS3)* mutations.

Without prior amylopectin fractionation, the whole starch samples from barley and potato were solubilised in 90% aqueous dimethylsulphoxide (DMSO) by heating and stirring. The dissolved starch was then treated with

barley β-amylase for 1 h (Figure 4). After β-amylolysis, the whole starch was degraded into β-LDs and maltose, and the amylose without branching was removed (Figure 5). Finally, the β-LDs were precipitated by ethanol through cold storage, followed by centrifugation (Figure 4).

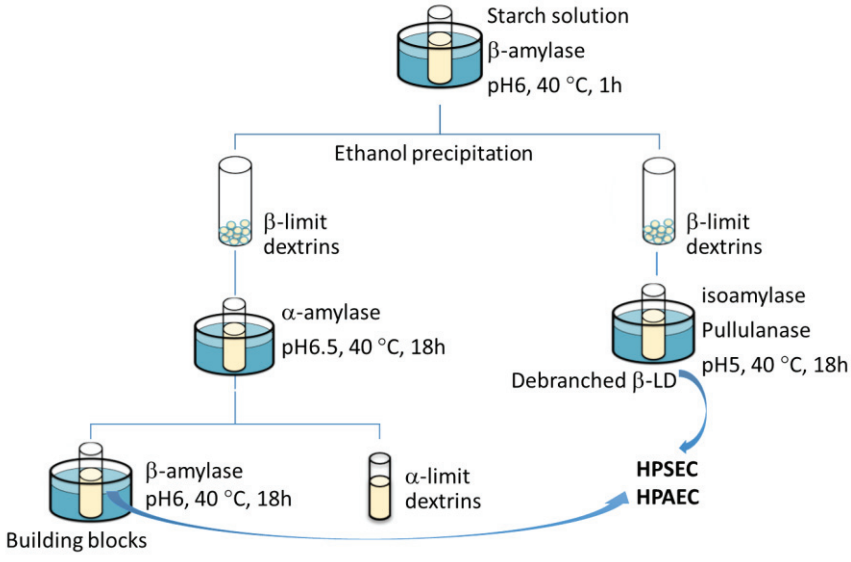

Figure 4. Flowchart of experimental production of β-limit dextrins, α-limit dextrins and building blocks from whole starch.

The β-LDs obtained were re-dissolved in water with the help of heating, stirring and scraping. Extensive treatment with α-amylase was then applied overnight to produce α-limit dextrins (α-LDs) (Figure 4).

Finally, in order to remove all interconnecting chains, the α-LDs were further hydrolysed into building blocks from the amylopectin fraction by extensive β-amylolysis overnight (Figure 4). By the end of the process, the amylose fraction had been degraded into mainly maltotriose, maltose and glucose, together with a minor proportion of very small building blocks originating from the branched amylose (Figure 5).

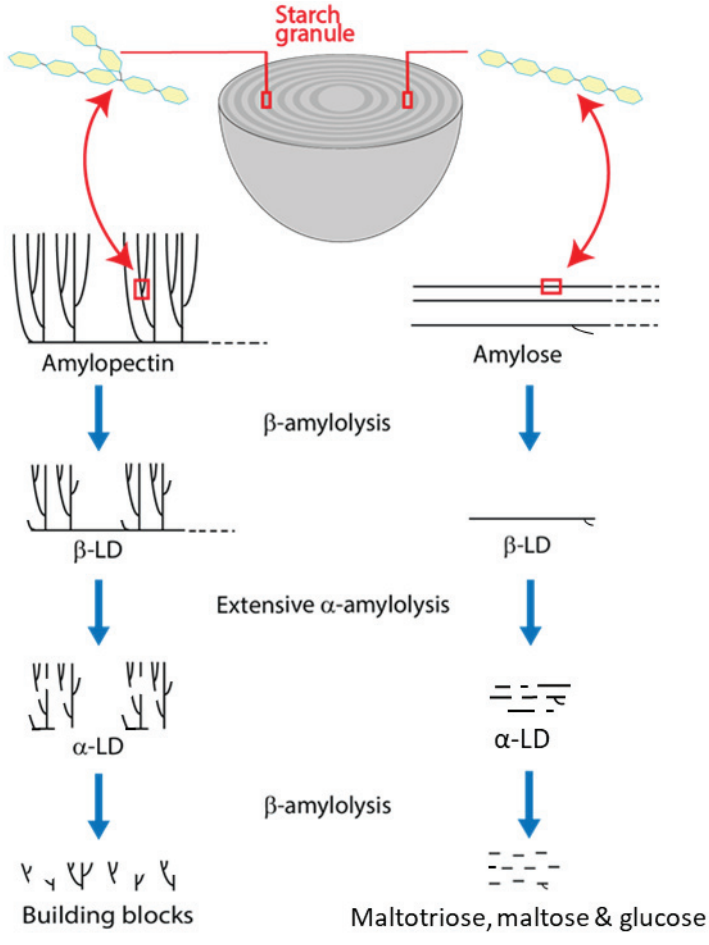

Figure 5. Schematic illustration of degradation of starch into β-limit dextrin (β-LD), α-limit dextrin (α-LD) and building blocks by β-amylase and α-amylase (Paper II).

3.7 Chain and building block distribution of starches

The chain-length distribution of whole starch from the potato "amylose lines" was studied in Paper III. The whole starch samples were dissolved in UDMSO (0.6 M urea in 90% aqueous DMSO) and then directly debranched by isoamylase and pullulanase before analysis of the chain-length distribution, using HPSEC and high performance anion exchange chromatography (HPAEC).

The internal chain-length distribution of whole starch from the barley samples (Paper II) and the potato "amylose lines" and "amylopectin lines" (Paper IV) was studied based on β-LDs. The whole starch samples were first degraded into β-LDs and then debranched with isoamylase and pullulanase. The internal chain-length distribution was analysed by HPSEC and HPAEC.

The composition of building blocks from whole barley and potato starches was investigated in Papers II and IV, respectively, using HPSEC and HPAEC, after transforming the whole starches into β-LDs, α-LDs and final building blocks by β-amylase, α-amylase and then β-amylase again (see Figure 5).

The HPSEC analysis was carried out according to a previous study (Andersson *et al*., 2009), with minor modifications. The samples were eluted at 35 °C using 0.1 M $NaNO_3$ with 0.02% NaN_3 at a flow rate of 0.5 mL/min, through the serially connected guard column and two OHpak SB-802.5 HQ columns (Shodex, Showa Denko KK, Miniato, Japan). Multiple-angle laser light scattering (MALLS) and refractive index detectors (Wyatt Technology Corp., Santa Barbara, CA) were used. The molecular weight was determined using ASTRA software (Wyatt Technology Corp., Santa Barbara, CA), with a dn/dc ratio of 0.147 (Andersson *et al*., 2009).

An HPAEC Series 4500i (Dionex Corp., Sunnyvale, CA, USA) was used for the analyses. A pulsed amperometric detector (PAD) and a BioLC gradient pump were incorporated into the HPAEC system. A CarboPac PA-100 (4×250 mm) analytical column (Dionex, Sunnyvale USA) was used for the separation and a guard column was also coupled with. A 25 μL portion of sample was injected at 25 °C and the eluents 0.15 M NaOH (A) and 0.50 M NaOAc+0.15 M NaOH (B) were eluted at a flow rate of 1 mL/min. The de-branched whole starches (Paper III) and β-LDs (Papers II and IV) were separated with the following gradient: 0-15 min, 15-28% eluent B; 15-45 min, 28-55% B; 45-75 min, 55-70% B; and 75-80 min 70-15% B. The PAD response of de-branched whole starches and β-LDs was converted to molar percentage (M%) and then normalised by total molar weight (Koch *et al*., 1998). The building blocks were separated with a shorter gradient: 0-20 min, 15-28% eluent B; 20-35 min, 28-50% B; 35-45 min, 50% B; and 45-50 min 50-15% B. The PAD response of building blocks was directly reported as relative peak area, without conversion.

3.8 Thermal properties of potato starches

Gelatinisation and retrogradation properties of potato starches were investigated by differential scanning calorimetry (DSC) with a modulated DSC250 (TA Instruments, New Castle, DE, USA). A starch:water ratio of 1:3 was used during gelatinisation and the scanning temperature range was 20-120 °C, with a heating rate of 4 °C/min. Retrogradation of potato starch was studied in gelatinised starch at a starch:water ratio of 1:2, after cold storage at 5 °C for three days. The scanning temperature range was -5-120 °C, with a heating rate of 10 °C/min.

3.9 Statistical methods

In Paper I, the content of total starch, resistant starch, other dietary fibres and its constituents was compared between the RNAi line T-2012 and the parent variety Dinamo. Statistical analyses were performed using the general linear model (GLM) procedure in Statistical Analysis System (Version 9.4; SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was applied for uncooked potato tubers and two-way ANOVA was applied for cooked potato tubers, since storage time was included as another factor. Tukey's multiple comparison test was used to determine the statistical significance of any differences observed.

In Papers II and IV, differences in starch structure based on HPAEC results between the groups of barley lines and between the groups of potato lines, and differences in gelatinisation and retrogradation parameters of potato starch between the groups of potato lines, were analysed by one-way ANOVA, using Minitab 18 (State College, PA, USA). Tukey comparisons (Paper II) and Fisher comparisons (Paper IV) were applied in the statistical analysis.

The relationship between molecular structure and thermal properties of potato starch (Paper IV) was evaluated by partial least squares (PLS) regression analysis, using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden).

4. Results and discussion

The structure and properties of potato starch were investigated for 22 different potato lines and three wild-type varieties, provided either as tubers or isolated starch, in Papers I, III and IV. The effects of genetic modification on starch structure and on functional properties were evaluated and assessed in detail in those papers.

A simplified analytical method was developed for effective characterisation of the internal molecular structure of whole starch without amylopectin separation (Paper II). By applying this method, structural information on internal chain-length distribution and building block composition of whole starch from 14 potato samples was obtained in Paper IV. The internal starch structure was also correlated to thermal properties in Paper IV.

The starch granular structure, starch and amylopectin chain distribution of potato lines with increased amylose content were studied and compared with those of the parental variety in Paper III.

The nutritional properties of potato tubers of the genetically modified RNAi line T-2012 and the effect of cooking and storage treatment on these nutritional properties were studied in Paper I.

4.1 Amylose content

In Paper I, the apparent amylose content of field-grown T-2012 starch and the parental variety Dinamo was found to be 57% and 41%, respectively, based on a spectrophotometric iodine-based method. The high-amylose potato line T-2012 was produced through transgenesis, by down-regulating two starch branching enzymes involved in building amylopectin. When the

branching of amylopectin was down-regulated, the proportion of amylose was successfully increased.

In Paper III, the amylose content in starch from lines in Group 3 (see Table 1) and from the RNAi line T-2012 was found to be 98% and 40%, respectively, based on an enzymatic method. However, lines in Groups 1 and 2 and the parental variety all had an amylose content of around 25%, with no significant differences according to the enzymatic method. The amylose content was also measured using a colorimetric method in Paper III. The results indicated an amylose content of 159-168% in lines in Group 3, 40-48% in lines in Group 2, 31-35% in lines in Group 1 and 38% in the parental variety. The RNAi line was found to have an amylose content of 87%, which is similar to the first published value of 89% for T-2012, obtained using the same colorimetric method (Menzel *et al*., 2015). The colorimetric method was found to overestimate the amylose content, while it was also influenced by variations in the chain-length distribution of the amylopectin molecule.

In Paper III, none of the Group 1 and 2 lines were found to have as high an amylose content as that found in the RNAi line T-2012 included in the analysis. Some previous studies on potato have shown that antisense suppression of SBE1 alone does not influence the amylose content (Safford *et al*., 1998). Lines with completely mutated *SBE1* alleles together with two to three mutated *SBE2* alleles (Group 2) only displayed a slightly increased amylose ratio based on the colorimetric method. Thus, mutations in all alleles of both *SBE1* and *SBE2* (Group 3), using the genome-editing method, were necessary in order to develop a starch with a significant increase in amylose content detectable using the applied methods.

The amylose content of potato starch in almost all "amylopectin lines" was below the detection limit of 0.5%, according to the HPSEC method. However, a line with a partial mutation in *GBSS* was found to have an amylose content (14.5%) close to that of the parental variety Kuras (19.2%) (Paper IV). Hence, full knockout of *GBSS* was needed to develop a waxy potato, while to develop a short-chain amylopectin starch *SS2* and *SS3* also needed to be mutated.

4.2 Starch granular structure

Most of the potato "amylose lines" displayed the B-type X-ray diffraction pattern (Figure 6a), with diffraction peaks at 15° (broad), 17° (strong) and a

doublet at 22-24° 2θ, which is the common pattern for tuber starches (Hizukuri *et al*., 2006). However, the diffraction intensity of starch from the Group 3 lines decreased for most of the peaks compared with the other lines. Moreover, the Maltese crosses of starch granules from Group 3 were not visible under polarised light, but were evident in starch from the parent variety Desiree and line 104016 from Group 2 (Figure 6b).

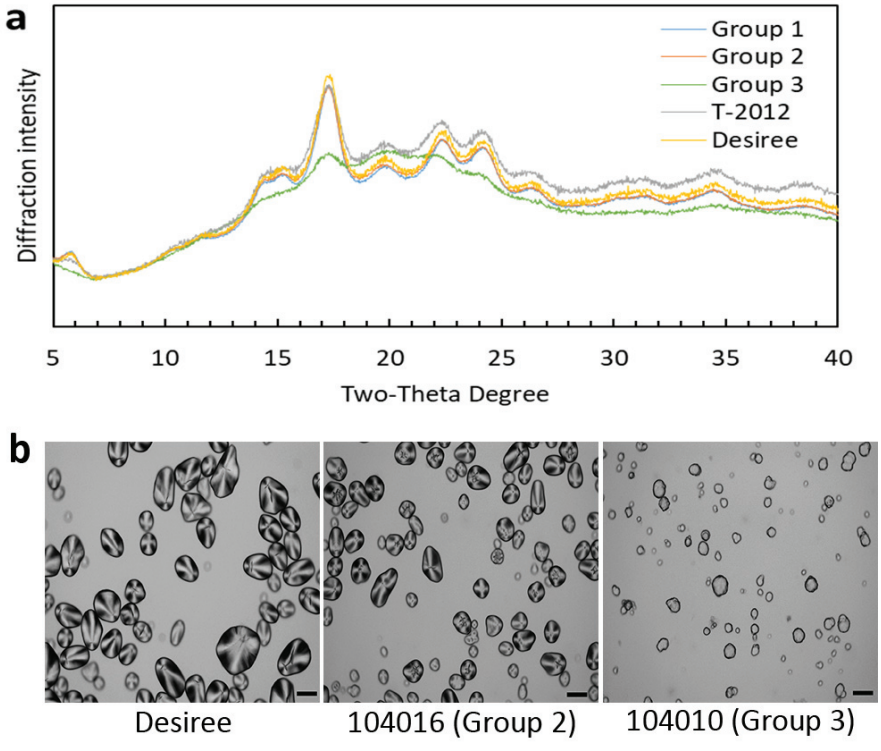

Figure 6. a) X-ray diffraction patterns of potato lines from Groups 1, 2 and 3. Average diffraction intensity is shown for each group, with the parental variety Desiree and the high-amylose line T-2012 included for comparison. *b)* Images of selected potato starches using polarised light microscopy. Scale bar = 40 µm.

Compared with the parent potato Dinamo, starch granules from RNAi line T-2012 displayed more triangle- or rod-shaped and smaller granules with deep fissures and irregular surfaces (Figure 7A, 7B). Similar results have been reported previously (Menzel *et al*., 2015). Under polarised light, the Maltese crosses of T-2012 starch granules overlapped and some granules

showed less birefringence than those of the parental starch Dinamo (Figure 7C, 7D). The altered appearances of the Maltese cross in the RNAi T-2012 starch may indicate some changes in molecule order within the starch granule.

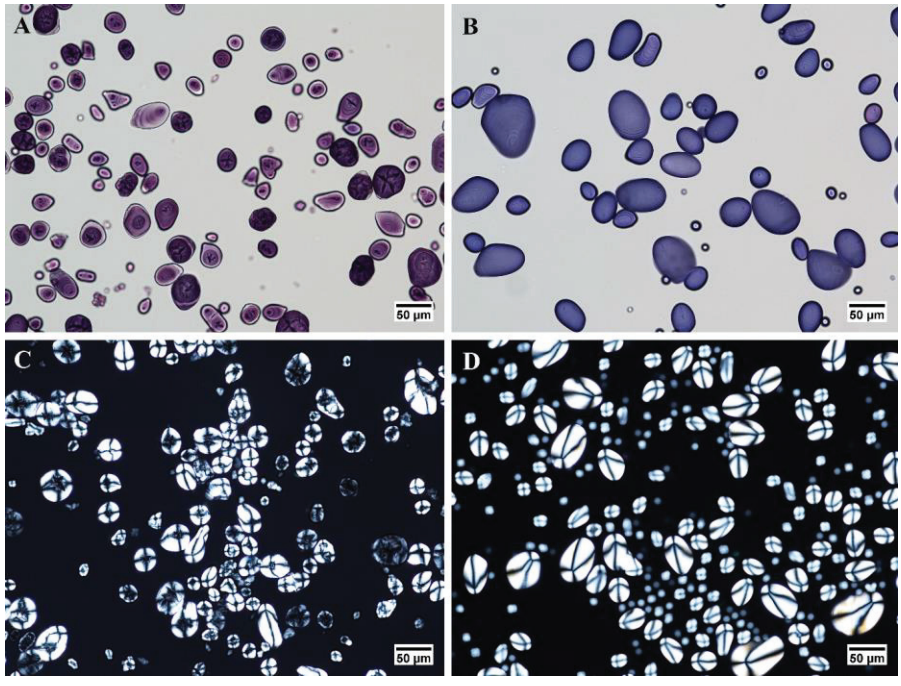

Figure 7. Microscopic images of potato starch granules (Paper I). *A)* T-2012 stained with iodine, *B)* Dinamo stained with iodine, *C)* T-2012 under polarised light, *D)* Dinamo under polarised light).

X-ray diffraction of starch is correlated with crystalline organisation of double helices within the starch granules, while the amylopectin fraction mostly contributes to the crystallinity of granular starches (Hizukuri *et al.*, 2006). Thus, the considerably reduced X-ray diffraction pattern of the Group 3 starches was probably attributable to the major loss of the amylopectin fraction in starches from that group. Moreover, the amylose content inversely affects the level of crystallinity (Cheetham & Tao, 1998). Hence, the high amylose content of the Group 3 starches might also be the reason for the lower level of crystallinity in those potato lines. However, the RNAi line T-2012, with high amylose content, showed a similar X-ray diffraction pattern

to the parental variety. This is probably because there was no real increase in amylose molecules, just an increase in long-chain amylopectin.

The Maltese crosses of starch granules generally indicate a highly ordered granular structure, with molecules arranged in a radial pattern (Bertoft, 2017). It is also known that granular thickness, the degree of crystallinity and the orientation of the crystallites all affect the apparent intensity of birefringence (French, 1984). Therefore, the complete disappearance of Maltese crosses in the Group 3 starches was probably due to a deviation in crystallite orientation from their radial arrangement. The polarised light microscopy and X-ray results both revealed that the Group 3 starches have the ability to organise into granules with a poorly ordered arrangement of crystalline amylose, even with the essential loss of the amylopectin fraction. These results are in line with findings reported for amylose-only barley starch (Carciofi *et al*., 2012).

4.3 Starch molecular structure

4.3.1 Starch chain distribution (Paper III)

Compared with the wild-type variety Desiree, changes in the chain distribution pattern were observed in both the amylopectin and amylose fractions based on the HPSEC results (Paper III), in the order: "amylose lines" of Group 1, Group 2, T-2012 and Group 3 (Figure 8). Following this order, the amylopectin fraction decreased gradually to almost absence. No amylopectin fraction was detected in the Group 3 starches when analysed by HPAEC. However, the total amount of the amylose fraction increased constantly, with a shift from a more short-chain amylose fraction to a more long-chain amylose fraction. Moreover, the T-2012 RNAi line showed a unique fraction around elution volume 13 mL with amylose-like long glucan chains (Figure 8).

The starch chain distribution suggested that mutation of *SBE1* alone or combined with *SBE2* had different effects on starch molecular structure, as has been suggested in previous studies (Andersson *et al*., 2002; Andersson *et al*., 2002; Blennow *et al*., 2005; Brummell *et al*., 2015; Rydberg *et al*., 2001; Tuncel *et al*., 2019). The reason is believed to be that the potato SBE isoforms play different roles (Rydberg *et al*., 2001) during amylopectin construction (Kossmann & Lloyd, 2000). It has been shown that suppression

of both SBEs in potato induces drastic effects on starch molecular structure (Hofvander *et al*., 2004; Schwall *et al*., 2000; Tuncel *et al*., 2019). This is consistent with the HPSEC results obtained in this thesis, which showed that the Group 3 starches experienced a dramatic increase in amylose fraction and lost a fraction of amylopectin chains.

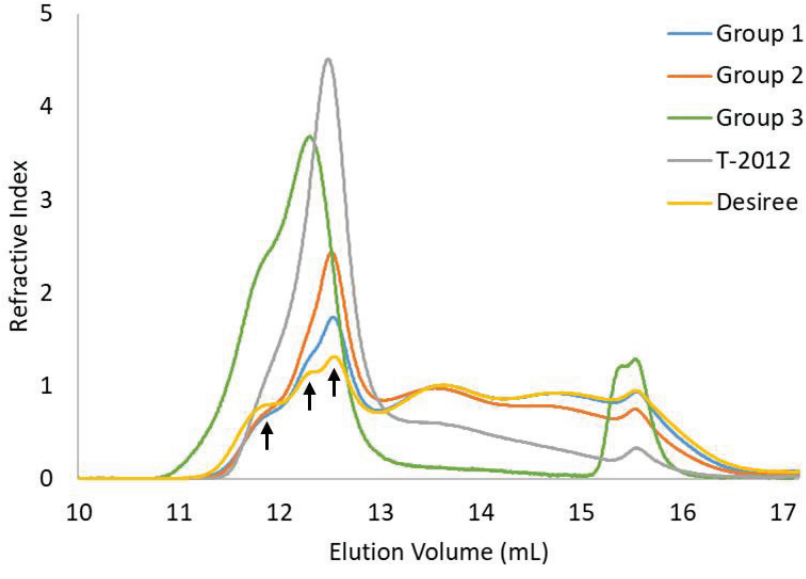

Figure 8. Chain distribution of debranched starches in potato lines from Groups 1, 2 and 3 after normalisation for the peak area, analysed with HPSEC. The parental variety Desiree and the high-amylose line T-2012 are included for comparison. The elution volume of 13 mL is a breakpoint, before which the primary amylose fraction eluted and after which the main amylopectin fraction eluted. The arrows from left to right indicate the three populations of amylose chains, *i.e*. the long-chain, intermediate and short-chain amylose fraction, respectively.

4.3.2 Amylopectin chain distribution (Paper III)

Besides the overall structural features of potato whole starch, the molar proportion distribution of amylopectin chains of debranched starches was analysed in detail by HPAEC (Figure 9).

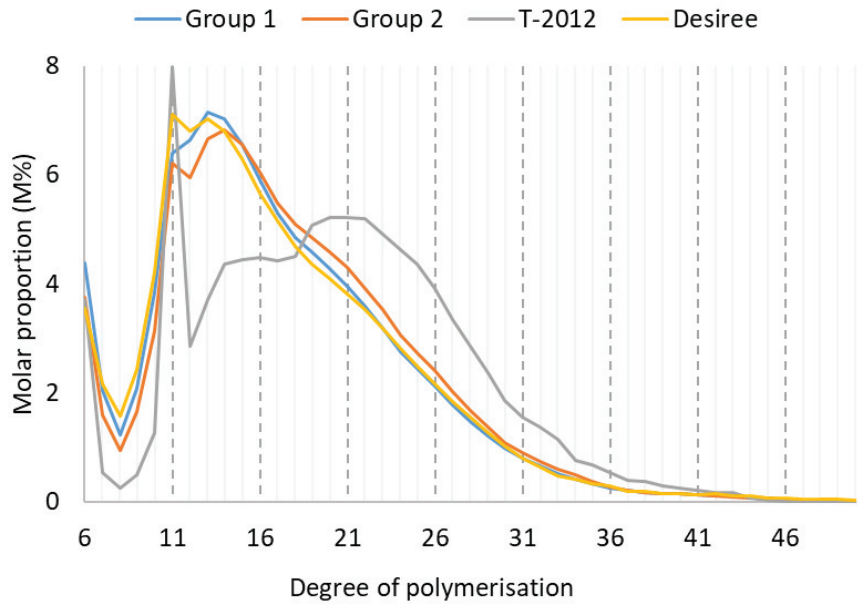

Figure 9. Chain distribution of debranched starches on a relative molar basis (M%) with degree of polymerisation (DP) 6-50, based on HPAEC analysis with averages of potato lines from Groups 1 and 2. No peak was detected for the starches from Group 3. The chain-length distribution from the HPAEC analysis primarily represented chains originating from the amylopectin fraction. The parental variety Desiree and the high-amylose line T-2012 are included for comparison.

Starch from the wild-type potato Desiree had a predominant peak at DP13 and a shoulder peak at DP11. Compared with the wild-type potato starch, the starch from T-2012 showed a sharp and high peak at DP11 and a large increase in chains of DP19-42, but a large decrease in chains of DP7-10 and DP12-18 (Figure 9). A similar distribution of amylopectin chains in T-2012 potato starch has been reported previously (Menzel *et al.*, 2015), with the peak shifted to DP22 and the proportion of chains of DP6-18 decreasing in particular. In general, the chain distribution of starch from Group 1 lines was similar to that of starch from the parental variety Desiree, with only slightly increased proportions of DP6 and DP12-21 chains and decreased proportions of DP7-11 and DP22-33 chains (Figure 9). In contrast, the starch from Group 2 lines analysed in Paper III had a reduced proportion of short chains (DP≤13), but an increased proportion of DP14-33 chains compared with the

parental variety (Figure 9). Short chains with DP6-8 are suggested to be the amylopectin outermost chains and these chains probably have characteristic profiles depending on the crop and different genotypes (Bertoft, 2004).

Many studies on cereals and some previous studies on potato have reported that suppression of a single SBE1 does not affect, or only slightly affects, the molecular structure of starch (Safford *et al*., 1998). This was supported by the HPSEC results obtained in this thesis, which indicated that the chain distribution of amylopectin fraction in Group 1 starches was similar to that in the parental variety. However, an altered amylopectin chain distribution was found in the Group 1 starches in analyses with higher resolution using HPAEC. This indicates that complete knockout of *SBE1* alone affected the starch structure somewhat.

4.3.3 Starch internal chain distribution

The pattern of internal chain distribution of potato whole starch was not similar to that of barley whole starch. Potato whole starch had a remarkably high proportion of very long internal chains which eluted before elution volume 13 mL (Figure 10). Moreover, the proportion of long internal B-chains was greater in the potato amylopectin fraction compared with in barley amylopectins, while potato whole starch lacked the dominant proportion of fingerprint B-chains (B_{fp}-chains) which elute between elution volume 16.5 mL and 18 mL in barley whole starch (Figures 10 and 11). It has been reported previously that tuber starches with a B-type polymorph contain the lowest amount of short internal B-chains and the highest amount of long internal B-chains, while cereal amylopectins with an A-type polymorph contain the highest amount of short internal B-chains and lowest amount of long internal B-chains (Bertoft *et al*., 2008).

Internal chain distribution of potato starch (Paper IV)

In the HPSEC analysis, the debranched β-LDs of potato "amylose lines" from Group 1, Group 2, T-2012 and Group 3 were found to have more (or even an almost full proportion) of the very long internal B-chains from the amylose fraction (eluted before elution volume 13 mL) than the wild-type potato Desiree (Figure 10). Correspondingly, there was a lower proportion of internal B-chains originating from the amylopectin fraction in starch from potato lines from Group 1, Group 2, T-2012 and Group 3 than in starch from the variety Desiree (Figure 10). Compared with the parental variety Kuras,

the debranched β-LDs of potato starch from "amylopectin lines" showed a decreased proportion of very long internal B-chains originating from the amylose fraction, while the degree of mutation increased gradually (Figure 10). Consequently, more internal B-chains that eluted around elution volume 14 mL and between elution volume 15 and 16.5 mL were present in starch from "amylopectin lines" with an increased degree of mutation.

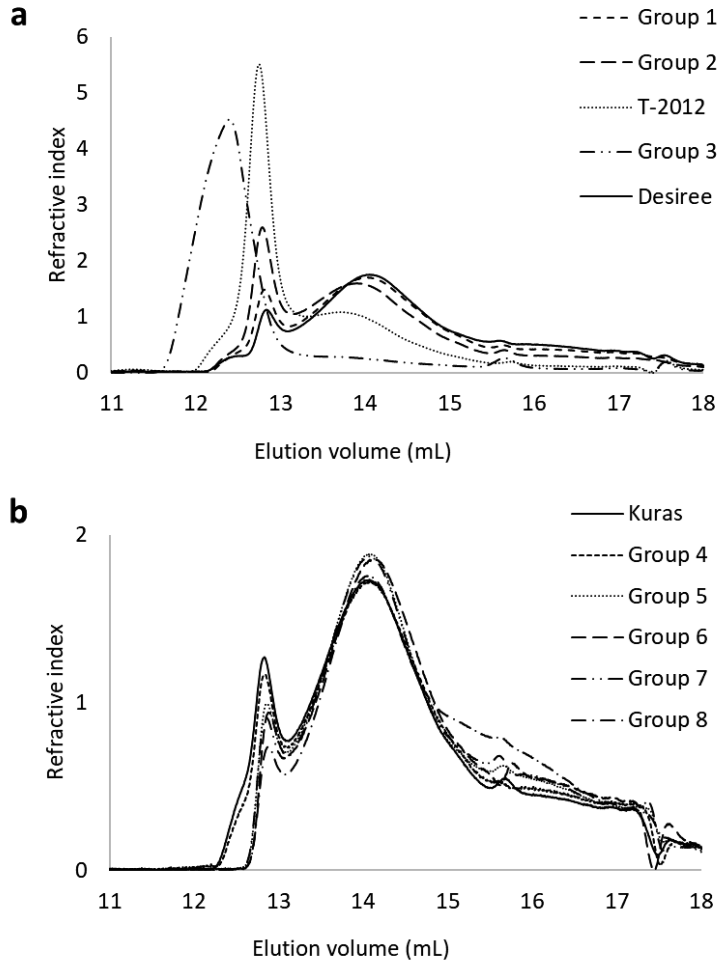

Figure 10. Mean chain distribution of de-branched β-limit dextrins (β-LDs) in starches from potato lines with the *SBE1*, *SBE2*, *GBSS*, *SS2*, and *SS3* mutation, analysed with HPSEC after normalisation of the peak area from elution volume 11 mL to 18 mL (nothing eluted before 11 mL). Wild-type potatoes (Desiree and Kuras) and T-2012 are included for comparison. For group information on the mutation lines, see Table 1.

On studying the internal chain distribution of whole potato starch samples by HPSEC analysis, major variations were found for the amylose fraction between and within the "amylose lines" and "amylopectin lines" compared with the wild-type potato starch (Figure 10). The proportion of very long internal chains was correlated with the amylose content of the starch samples. Moreover, some variations in long internal chains in T-2012 starch may be explained by the content of debranched intermediate material, which can vary from 9% to 4% in high-amylose and native starches (Tang *et al.*, 2001).

Internal chain distribution of barley starch (Paper II)

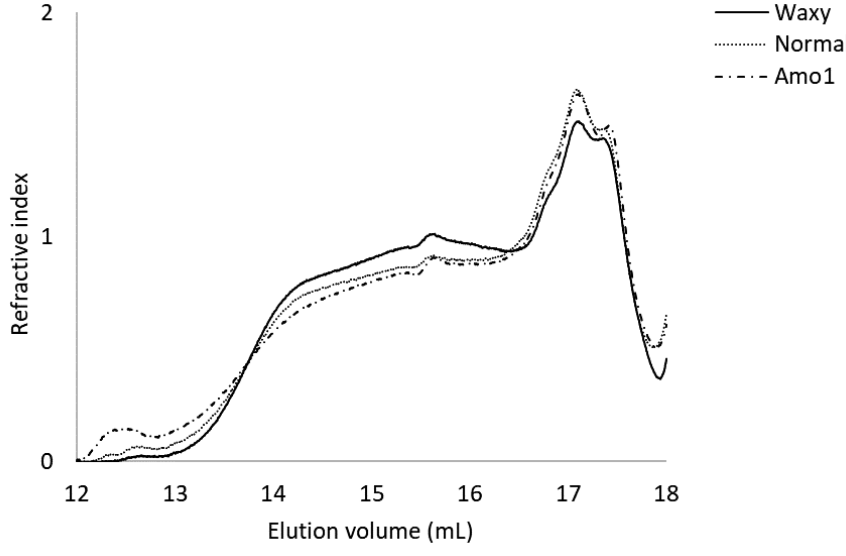

Figure 11. Mean distribution of de-branched β-limit dextrins (β-LDs) in starch from barley varieties with the *wax* mutation, starch from barley varieties with the *amo1* mutation and normal starch, analysed with HPSEC after normalisation of the total area from elution volume 12 mL to 18 mL (nothing eluted before 12 mL).

Despite differences in patterns, the HPSEC results for the debranched β-LDs of barley starch revealed a similar structural feature in starch internal chain distribution to that of potato starch. Starch from barley varieties with the *amo1* mutation had a higher proportion of very long internal B-chains which

eluted before 13 mL and a lower proportion of intermediate internal B-chains which eluted between elution volumes 13.7 and 16.5 mL than starch from normal barley and waxy barley varieties (Figure 11).

4.3.4 Amylopectin internal chain distribution

Significant variations in amylopectin internal chains of whole starch samples from potato were found between different genotype groups, based on the distribution analysis of different chain categories using HPAEC. The results are shown in Table 2.

Internal chain distribution of potato amylopectin (Paper IV)

The molar percentages of B_{fp}-chains from potato whole starch samples in the form of β-LDs varied from 16.9% ("amylopectin line") to 29.8% ("amylose line"), whereas that of long internal B-chains (DP 42-51) ranged from 1.1% (high-amylose line T-2012) to 2.6% ("amylopectin line") (Table 2). However, starch from T-2012 showed a very different pattern in chain-length distribution, with a high proportion of internal B-chains with DP 21-24 and a considerably lower amount of internal B-chains with DP ≥25 compared with all other starch samples (Table 2).

Table 2. *Mean composition of chain categories of β-limit dextrins (β-LDs) with degree of polymerisation (DP) 4-51, based on relative molar composition analysed by HPAEC, in potato starch of lines from Group 1 (n=2), Group 2 (n=2), Group 7 (n=2), Group 8 (n=3) and wild type (n=2). Results for T-2012 are included for comparison*

Sample	DP 4-7	DP 8-12	DP 13-20	DP 21-24	DP 25-41	DP 42-51
Group 8	16.9^{c}	27.3^{b}	28.6^{a}	7.1^{a}	17.5^{ab}	2.6^{a}
Group 7	19.9^{c}	27.4^{ab}	23.8^{b}	5.9^{b}	20.5^{a}	2.4^{a}
Wild type	25.1^{b}	28.7^{ab}	20.6^{c}	5.7^{b}	18.1^{ab}	1.7^{b}
Group 1	29.8^{a}	30.4^{a}	19.4^{c}	5.6^{b}	13.5^{b}	1.3^{b}
Group 2	28.7^{a}	30.1^{ab}	19.8^{c}	6.0^{b}	13.6^{b}	1.7^{b}
T-2012	26.1	27.7	22.6	9.7	12.8	1.1

Different superscript letters indicate statistically significant differences (p<0.05).
B_{fp}-chains are chains of DP4-7.
The starch of potato lines from Group 3 has almost no amylopectin, and no internal amylopectin chains were detected.
Values for Groups 4-6 are not included in the statistics because there was only one sample per group.

The potato starch of "amylopectin lines" from Group 8, with the highest degree of mutations in *GBSS*, *SS2* and *SS3*, contained the lowest proportion of B_{fp}-chains ($p \leq 0.05$) and the highest proportion of chains with DP 13-24 ($p \leq 0.001$) of all the potato starches (Table 2). In contrast, the potato starch of "amylose lines" from Group 1 and Group 2 showed the highest proportion of B_{fp}-chains ($p < 0.05$) of all the potato starches. The potato starch of "amylopectin lines" from Group 7 had the second lowest proportion of B_{fp}-chains ($p \leq 0.05$). It also had the second highest proportion of chains with DP 13-20 ($p < 0.01$) (Table 2) among all the potato starches analysed except that of line from Group 5 (Paper IV). The potato starch of "amylopectin lines" from Group 7 showed a higher proportion of internal B-chains with DP 25-41 than the starch from the "amylose line" potatoes ($p < 0.05$). For the long internal B-chains with DP 42-51, the potato starch of "amylopectin lines" from Group 7 and Group 8 had a significantly higher proportion than the starch from the wild-type and the "amylose line" potatoes ($p < 0.05$). For the "amylose line" starches, no significant difference was found between the lines from Group 1 and Group 2 or with the starch of wild-type potatoes in the chain categories except for B_{fp}-chains (Table 2).

In general, different combinations of mutated *GBSS, SS2* and *SS3* resulted in different effects on amylopectin internal chain distribution. Clear significant variations were found in the molar proportion of B_{fp}-chains that correlated with the phenotype and genetic background of the potato lines (Table 2). In descending order, the ranking was "amylose lines" from Group 1 and Group 2, wild type, and "amylopectin lines" from Group 7 and Group 8.

4.3.5 Building block structure

Building block composition of potato starch (Paper IV)

The differences in building block groups revealed by HPAEC and the differences in composition of building blocks from potato whole starch samples were related to the genetic background of potato lines (Table 3). In general, the proportion of intermediate (G4) and large (G5 and G6) building blocks increased gradually in the starch of potato lines in the order: Group 8, Group 7, wild type, Group 1 and Group 2. The proportion of small (G2 and G3) building blocks declined gradually following the same order. The starch

of “amylose lines” from Group 1 and Group 2 had significantly more (p<0.01) intermediate (G4) and large (G5 and G6) building blocks, and fewer (p<0.05) small (G2 and G3) building blocks, than the starch of “amylopectin lines” from Group 7 and Group 8 (Table 3). For building blocks G3, G4 and G5, the starch of lines from Group 1 and Group 2 also showed significant differences (p<0.05).

However, a very different distribution of building blocks was found in the starch of T-2012, where building blocks G2 (42.2%) and G4 (34.6%) were the major components, whereas the starch from all the other samples mostly contained G2 and G3 building blocks (Table 3). As a consequence, the starch of T-2012 had the highest proportion of intermediate (G4) and large (G5 and G6) building blocks, and the lowest proportion of small (G2 and G3) building blocks, of all the starch samples analysed in this thesis (Table 3).

Table 3. *Mean composition of building blocks in five different size groups (G2-G6), based on relative peak area analysed by HPAEC, in potato starch samples of lines from Group 1 (n=2), Group 2 (n=2), Group 7 (n=2) and Group 8 (n=3) and in the wild type (n=2). Results for T-2012 are included for comparison*

Sample	G2	G3	G4	G5	G6
Group 8	63.4^{a}	28.0^{a}	6.5^{d}	1.7^{d}	0.4^{b}
Group 7	62.2^{a}	26.8^{b}	8.5^{cd}	2.0^{cd}	0.5^{b}
Wild type	61.3^{ab}	23.9^{c}	11.3^{bc}	2.7^{bc}	0.7^{b}
Group 1	57.2^{bc}	22.8^{d}	14.9^{b}	3.7^{b}	1.5^{a}
Group 2	54.9^{c}	19.0^{e}	19.4^{a}	4.9^{a}	1.8^{a}
T-2012	42.2	12.6	34.6	7.9	2.7

Different superscript letters indicate statistically significant differences (p<0.05).
The starch of potato lines from Group 3 has almost no amylopectin and no building blocks were detected. Values for Group 4-6 are not included in the statistics because there was only one sample per group.

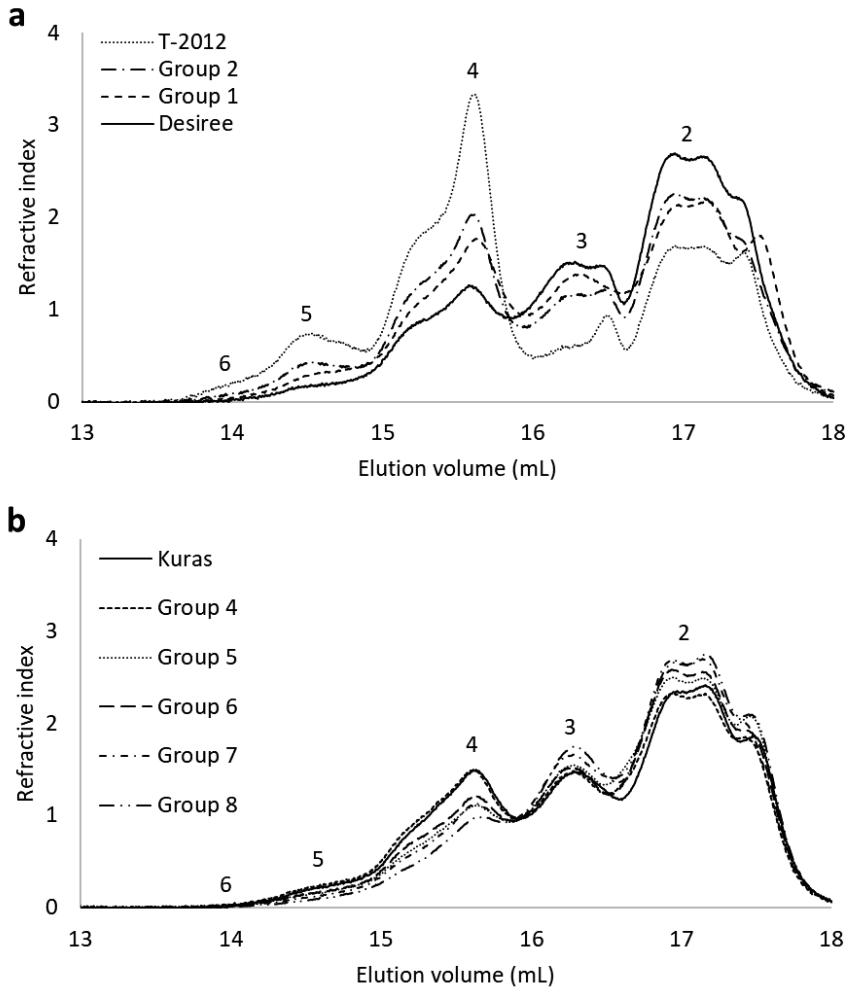

Figure 12. Mean distribution of building blocks with two and more chains in starch from potato lines with the *SBE1*, *SBE2*, *GBSS*, *SS2* and *SS3* mutation, determined by HPSEC analysis after normalisation of the peak area from elution volume 13 mL to 18 mL (nothing eluted before 13 mL). Numbers 2-6 indicate building block size groups G2-G6. The wild-type potato starches and T-2012 are included for comparison. For full information on the mutation lines, see Table 1.

The HPSEC results of building block composition for the potato starch samples (Figure 12) agreed very well with the distribution of building block analysed by HPAEC (Table 3). The proportion of intermediate (G4) and

large (G5 and G6) building blocks increased gradually in the starch of potato lines with the order Group 8, Group 7, Group 6, Group 5, Group 4, wild type, Group 1, Group 2 and T-2012. The proportion of small (G2 and G3) building blocks declined gradually following the same order. The pattern of building block composition of potato whole starch and barley whole starch was generally similar, with the slight difference that potato whole starch had a comparatively larger proportion of intermediate (G4) building blocks than barley whole starch (Figures 12 and 13).

Building block composition of barley starch (Paper II)

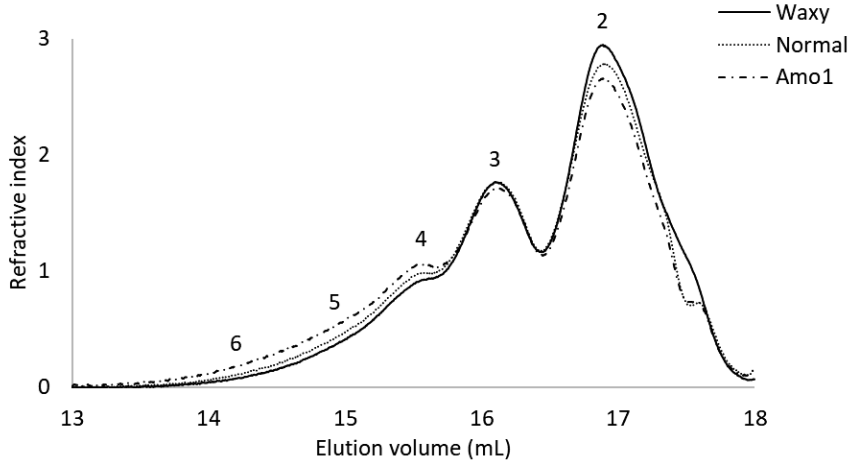

Figure 13. Mean distribution of building blocks with two and more chains in starch from barley varieties with the *wax* mutation, starch from barley varieties with the *amo1* mutation and normal starch, determined by HPSEC analysis after normalisation of the total area from elution volume 13 mL to 18 mL (nothing eluted before 13 mL). Numbers 2-6 indicate building block size groups G2-G6, which were assigned according to the old division of building blocks (Bertoft *et al*., 2011a).

4.4 Nutritional properties of potato line T-2012

In Paper I, the nutritional properties of potato tubers from the high-amylose line T-2012 were investigated and compared with those of the parental potato cultivar Dinamo. T-2012 had a slightly lower total starch content (65-68% of dry matter (DM)) than the parental potato Dinamo (72-76% of DM). This supports findings in a previous study that high-amylose potato lines generally have a reduced starch content, but an increased content of free sugars (such as fructose and glucose) (Hofvander *et al*., 2004). However, the content of DF excluding RS in T-2012 (10-14% of DM) was significantly (p<0.001) higher than that in Dinamo (5-7% of DM), irrespective of whether it was cooked or not.

The RS in uncooked tubers is type II RS, where starch granules resist enzyme digestion (Brown *et al*., 1995). T-2012 contained less RS (30% of DM) than the parent potato Dinamo (56% of DM), which could be due to the altered starch granular structure in T-2012 (Figure 7). It is known that besides amylose content, granular structure also has a strong impact on enzyme resistance (Themeier *et al*., 2005). Native potato starch granules are less accessible to enzymes, due to the homogeneous, compact and large granules, and the smooth surfaces of these granules (Lehmann & Robin, 2007). The starch granules in T-2012 were much smaller, with a more irregular shape and surface and deep fissures (see Figure 7). This probably renders the granules more accessible to enzymes, and therefore the type II RS content decreased in raw tubers of this high-amylose potato line.

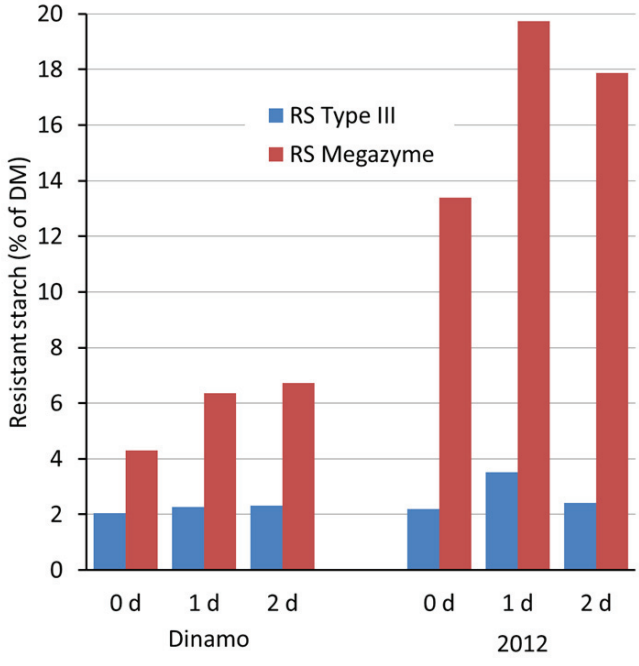

Figure 14. Content of resistant starch (RS) in boiled potato tubers of the parent potato Dinamo and the high-amylose line T-2012 (Paper I).

T-2012 had much higher level of physiological RS (13-20% of DM) than the parent potato (4-7% of DM) after cooking (Figure 14). This was most probably due to the higher amylose content in T-2012, since a high level of amylose usually gives a high level of RS in cooked food (Leeman *et al*., 2006; Lehmann & Robin, 2007). After cold storage, the level of RS in T-2012 increased further, but the level of type III RS (*i.e*. recrystallised amylose) did not show significant differences between T-2012 and the parent, or between different storage durations. When the level of recrystallised amylose did not differ, part of the increased level of RS after cold storage might be recrystallised amylopectin, which needs some time to form. Recrystallised amylopectin is generally not resistant to enzyme degradation, since it has a much lower melting point (35-80 °C) than recrystallised amylose (120-170 °C) (Silverio *et al*., 2000; Sievert & Pomeranz, 1990). However, genetic modification was found to have a major effect on amylopectin structure, with the RNAi line T-2012 having a very large fraction of long outer starch chains and intermediate-sized inner

amylopectin chains, and more intermediate and large building blocks, compared with the wild-type potato. Longer internal amylopectin chains have been found to promote formation of recrystallised amylopectin with higher thermal stability, due to a more ordered structure (Zhu, 2018). This unique amylopectin has properties that are similar to amylose. After cooking and cooling, the modified amylopectin recrystallises and thereafter is not as easily split as ordinary potato starch. Therefore in total, T-2012 had almost three-fold higher content of RS than the parental potato after cooking and cold storage.

The cell wall composition of T-2012 was also altered indirectly by the genetic modification, giving a greater amount of glucose residues (cellulose) and a smaller amount of other polysaccharides than in the parent (Figure 15).

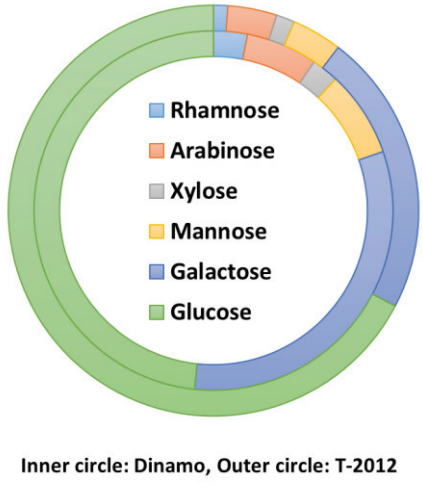

Figure 15. Composition of the cell walls of the parent potato Dinamo and the high-amylose line T-2012, as revealed by dietary fibre analysis of uncooked potato tubers.

4.5 Influence of starch structure on thermal properties

In Paper IV, the thermal properties of starch in the 15 potato lines and two wild types included in the thesis were investigated. Gelatinisation was examined using DSC with a starch:water ratio of 1:3. The starch of the high-amylose potato line T-2012 and "amylopectin lines" from Group 8 had a

broader gelatinisation temperature range. T-2012 had the highest peak (75.8 °C) and endset (87.4 °C) temperature, whereas the "amylopectin lines" from Group 8 had the lowest mean onset ($p \leq 0.001$) and peak temperature ($p < 0.01$) (Figure 16). The onset temperature for gelatinisation was ~50-54 °C and the peak temperature was ~59-61 °C in the potato lines from Group 8, compared with ~60-65 °C and ~64-76 °C, respectively, in the other potato lines. These results are in line with previous findings that starch with more heterogeneous crystals leads to a broader temperature range of gelatinisation (Källman *et al*., 2015).

The relationship between gelatinisation and structural features was studied by PLS regression analysis. Structural parameters were able to explain most of the variance with the first two components in onset, peak and endset temperatures and gelatinisation enthalpy (Paper IV). The onset temperature of gelatinisation and the gelatinisation enthalpy were positively correlated with short internal B-chains (DP 4-7 and DP 8-12), and large and intermediate building blocks (G6, G5 and G4). The peak and endset temperatures of gelatinisation were positively affected by intermediate and large building blocks (G4, G5 and G6), and short internal B-chains of DP 4-7 and intermediate B-chains of DP 21-24.

The mean gelatinisation enthalpy of potato starch from the different lines gradually increased from "amylopectin starch" (15 J/g in Group 8), to wild type starch (18.7 J/g), and finally to "amylose starch" (19.2 J/g in Group 2) (Paper IV). The PLS regression coefficients showed that the thermal properties were influenced by the size of the building blocks in amylopectin. Presence of more large building blocks and more short internal B-chains resulted in higher gelatinisation enthalpy ($p < 0.05$). A previous study concluded that more perfect crystals are formed within the granule, contributing to a higher peak temperature of gelatinisation, if the amylopectin building blocks have short chains and a dense structure (Källman *et al*., 2015).

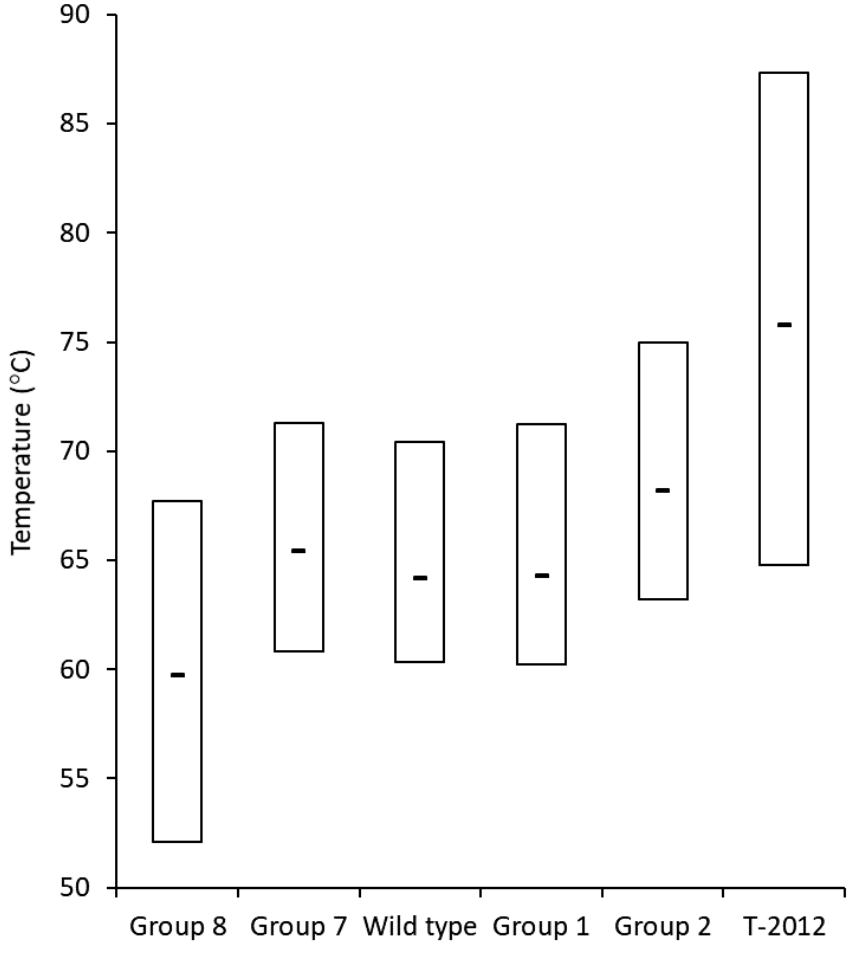

Figure 16. Gelatinisation onset, peak and endset temperatures of starch from the different groups of potato lines.

In Paper IV, the retrogradation of potato starch was also investigated after gelatinisation at a starch:water ratio of 1:2 and storage at 5 °C for three days. There were no clear differences in the onset and peak melting temperatures of retrograded starch between the different potato lines. The onset and peak of melting temperature was ~39-40 °C and ~64-66 °C, respectively, for all potato starches (Figure 17). However, T-2012 had the lowest endset (70.6 °C) temperature, whereas the "amylose lines" from Group 2 had the highest

mean endset (84.2 °C) temperature (p<0.05). As a result, T-2012 had the narrowest melting temperature range, while the amylose lines from Group 2 had the widest range (Figure 17).

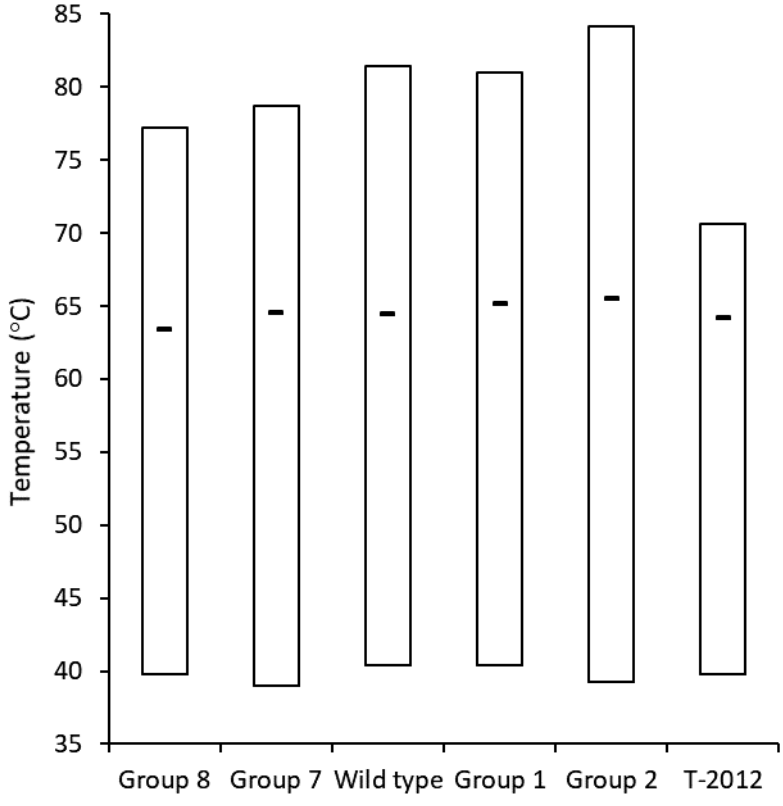

Figure 17. Retrogradation onset, peak and endset temperatures of starch from the different groups of potato lines.

Although no significant effect of structural parameters was seen for the onset, peak and endset melting temperatures of retrograded potato starch, the starch of potato lines from Group 8 (3.1 J/g; p<0.001) and Group 7 (6.0 J/g; p≤0.001) had lower retrogradation enthalpy than the starch from other lines (~8 J/g), indicating that fewer crystals were formed. When the retrogradation enthalpy of the potato starch was modelled by PLS analysis, the regression coefficients showed that the retrogradation enthalpy was influenced by the different chain categories, size of the building blocks in amylopectin and the

amylose content. More short internal B-chains (DP 4-7 and 8-12) and more large and intermediate building blocks (G6, G5 and G4) resulted in higher retrogradation enthalpy. This indicates that molecular network re-formation in gelatinised starch is favoured by a high density of branching and low levels of long internal chains.

Amylose content also contributed to the amylopectin gelatinisation and retrogradation properties of the different starches. The enthalpy of potato starch gelatinisation and the retrogradation and gelatinisation temperatures were positively affected by the amylose content. It is also possible that the correlation between starch thermal properties and amylose content is attributable to a correlation between starch structure and amylose level. The starch of potato lines from Group 8, with a lack of amylose and the most affected amylopectin, also had the highest degree of mutations, with the lowest levels of short internal B-chains and large building blocks found in this type of starch.

However, the starch of T-2012 line, with high amylose content, resulted in lower retrogradation enthalpy but the highest peak temperature of gelatinisation. This might be because oven cooking at 105 °C was not sufficient to achieve full gelatinisation of the starch in T-2012. A previous study indicated that the starch of T-2012 needs higher gelatinisation temperature, since the starch showed no granule swelling and viscosity under the conditions applied in rapid viscosity analysis with the standard method (up to 95 °C) (Menzel *et al*., 2015). When the gelatinised starch of T-2012 was oven-cooked again at a higher temperature (around 120 °C), its retrogradation enthalpy increased to 8.0 J/g. Moreover, the peak and endset temperatures of retrogradation and the temperature range increased dramatically (Paper IV). The very large fraction of long outer starch chains and large building blocks of T-2012 could favour the formation of more perfect crystals compared with the wild-type potato starch.

5. Conclusions

Starch is a glucose polysaccharide consisting of amylose (essentially long linear molecule) and amylopectin (highly branched molecule). Amylopectin is constructed from A-, B-, and C-chains (carrying the reducing end) connected through branching points. Knowledge of starch internal structure (the structure of chain segments from the outmost branches to the reducing end) is important in understanding the biosynthesis of starch and the starch structure-property relationship. However, there are challenges in measuring the levels of different chain-length and building block categories, especially when newly developed starches do not fall into any of the categories defined previously.

In this thesis, a method for determining the internal structure of starch without prior amylopectin isolation was developed and optimised. Whole starches of potato and barley were degraded into β-LDs, α-LDs and building blocks with this simplified method. The internal B-chain distribution and building block composition were characterised effectively, providing a deeper understanding of the relationship between molecular structure and functional properties.

Potato whole starches had a markedly higher proportion of long internal chains from both amylose and amylopectin fractions compared with barley whole starches, while potato whole starches were lacking the dominant proportion of B_{fp}-chains that is found in barley whole starches. The pattern of building block composition of potato whole starch and barley whole starch was generally similar, with slightly different proportions of the intermediate (G4) building blocks. The general descending order Group 2>Group 1>wild type>Group 7>Group 8) was determined for the composition of intermediate and large building blocks and proportion of B_{fp}-chains. These structure parameters appeared to be

related to genetic background and could be used to predict some physicochemical properties of starches from different plant sources and genetic backgrounds.

The high-amylose potato line T-2012 was found to have higher levels of resistant starch and dietary fibre than the parental variety after cooking, and may therefore have beneficial effects on human health. The resistant starch content was influenced both by the amylose content and by the amylopectin structure. T-2012 starch had a very large fraction of long outer chains and intermediate-sized inner amylopectin chains, and more intermediate and large building blocks, compared with the wild-type potato. The unique amylopectin structure of T-2012 starch promoted the formation of a unique recrystallised amylopectin with properties similar to those of amylose. After recrystallisation, this amylopectin did not split as easily as ordinary potato starch and was resistant to enzyme digestion. The information obtained in this thesis on the unique amylopectin structure in T-2012 can be useful in designing functional starch and healthier food in future.

Links between structural features and thermal properties (gelatinisation and retrogradation) were also uncovered in this thesis. Various parameters of the internal structure of potato starch were found to be significantly correlated with the gelatinisation temperature, enthalpy of gelatinisation and retrogradation of potato starch. Dense structure of the building blocks led to higher gelatinisation temperatures and enthalpy. Retrogradation was found to be favoured by presence of more large building blocks with many short internal chains.

6. Future research

Following on from this thesis, further work needs to be performed in order to customise starch with predicted properties, through:

- Further studies on the effect of genetic modification on starch structure and the relationship between molecular structure and functional properties of starch

- Investigations on the nutritional properties of the "amylose lines" of potato developed via DNA-free CRISPR-Cas9-mediated mutagenesis.

- Further studies on amylose starch with no detectable branching.

- Development of a method to zoom in on amylose structure using HPSEC.

- Comparative investigations of amylose structure from potato lines with different amylose contents.

- Wider application of the simplified analytical method to diverse starch samples from different plant sources, to characterise the internal molecular structure of whole starch.

- Studies on the correlation between amylose structure and functional properties of starch.

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Popular science summary

A good understanding of the relationship between molecular structure and functional properties of potato starch can help plant breeders produce tailor-made starches with desired properties at the genetic level. These starches can be used in various food applications, with beneficial effects on human health and wellbeing.

Potato tubers are an important part of the human diet in the Nordic countries and in many other countries worldwide. The starch contained in potato tubers also has many valuable applications in food and non-food industries, depending on the qualities of the starch. Starch can have different properties depending on the ratio of amylose to amylopectin, granular size distribution and the molecular structure of the individual starch components.

Native starch has some drawbacks when it is used in manufactured foods. In order to overcome these drawbacks, many physical and/or chemical modifications of native starch are applied in food processing. However, modification of starch in this way requires much money, time and energy and is labour- and chemicals-intensive. Efforts are underway to make starch production and downstream processing more sustainable. One option could be to use modern breeding technologies to develop new starch qualities in crops such as potato and cereals.

This thesis evaluated a new potato line that contains a high content of amylose. The results showed that cooked tubers of this high-amylose potato line have a three-fold higher level of resistant starch than cooked tubers of the parental potato. Resistant starch is part of dietary fibre, which has well-known health benefits for humans such as contributing to low glycaemic index, weight loss and probiotic effects. The resistant starch content in the high-amylose potato tubers was found to be increased further by one extra

day of cold storage after cooking, due to the unique amylopectin structure of the starch.

Native potato starch usually contains about 25% amylose and this amylose content cannot be changed through conventional plant crossings. In general, potato starch composition can only be adjusted through molecular genetic techniques. The high-amylose potato line examined in this thesis was developed by down-regulating two starch branching enzymes, using RNA interference or the genome editing technique CRISPR-Cas9. Apart from increasing the amylose content, the amylopectin structure of potato starch was also altered by this molecular genetic modification.

To study the structure of amylopectin in the new potato line, an optimised method for determining the internal structure of starch was developed. Results obtained using this method showed that the high-amylose potato starch had a very large fraction of intermediate-sized outer and inner amylopectin chains, and more intermediate and large building blocks, than the native potato starch. The amylopectin in the new potato line was found to have properties similar to amylose. After cooking and cold storage, it did not split as easily as ordinary potato starch and it was resistant to digestion. Using this structural knowledge of the unique amylopectin in the new potato line, functional starches and healthier foods can be designed in future.

The results also showed that various parameters relating to the internal structure of potato starch were significantly correlated with starch thermal properties (gelatinisation and retrogradation).

In summary, increased understanding of the effect of genetic modification on molecular structure and functional properties of starch is of great importance. So as to custom starch at the genetic level with desired functional properties for multiple applications, without need for further chemical or physical modifications of starch. This would be a green alternative, economically friendly and sustainable approach to develop novel desirable starches in the near future.

Populärvetenskaplig sammanfattning

Genom att ytterligare förstå förhållandet mellan molekylstruktur och funktionella egenskaper hos potatisstärkelse kan vi hjälpa växtförädlare att skräddarsy stärkelse med önskade egenskaper på genetisk nivå för olika livsmedelsapplikationer som kan ha en positiv inverkan på människors hälsa och välbefinnande.

Potatisknölar bidrar till en stor del av den mänskliga kosten i Norden och i resten av världen. Stärkelsen som produceras av denna gröda har också många värdefulla tillämpningar inom livsmedels- och livsmedelsindustrin. Stärkelsens egenskaper är mycket viktiga i både livsmedels- och icke-livsmedelsapplikationer, men stärkelsen kan ha olika egenskaper beroende på förhållandet mellan amylos och amylopektin, granulstorleksfördelning och molekylstrukturen hos de enskilda stärkelsekomponenterna.

Nativ stärkelse har dock vissa nackdelar när den används i en livsmedelsapplikation. För att övervinna nackdelarna görs fysiska och/eller kemiska modifieringar av nativ stärkelse. Emellertid är de kemiska och fysiska modifieringarna av stärkelse både penga-, tid- och energikrävande, liksom arbetskraft och kemikalieintensiva. Fokus läggs på att göra stärkelseproduktion och nedströmsprocess mer hållbar, och en del av en lösning kan vara att använda modern förädlingssteknik för att utveckla nya stärkelsekvaliteter i en gröda.

I doktorandprojektet utvärderade vi en ny potatis som har högt innehåll av amylos. Vi fann att denna högamylospotatis ger en tre gånger högre nivå av resistent stärkelse i de kokta knölarna jämfört med modersorten. Resistent stärkelse är en kostfiber, som har kända hälsofördelar för människokroppar, som att bidra till ett lågt glykemiskt index och viktminskning samt har en probiotisk effekt. Halten resistent stärkelse ökade ytterligare efter en extra

dags kylförvaring efter kokning, som ett resultat av den unika amylopektinstrukturen.

Nativ potatisstärkelse innehåller vanligtvis cirka 25 % amylos och amylosinnehållet kan inte ändras genom konventionella korsningar. Potatisstärkelsekompositionen kan endast påverkas genom molekylärgenetiska tekniker. De potatisar med hög amylos som används i studien har utvecklats genom nedreglering av två stärkelseförgreningsenzymer med RNA-interferens eller genomredigeringsmetoded CRISPR-Cas9. Förutom att öka amylosinnehållet har amylopektinstrukturen också förändrats.

För att studera amylopektinstrukturen har en optimerad metod för att bestämma stärkelsens interna struktur utvecklats inom doktorandprojektet. Genom att tillämpa metoden undersöktes potatisstärkelsen med hög amylos vilket visade på en mycket stora fraktion av mellanstora yttre och inre amylopektinkedjor och mellanstora såväl som stora byggstenar jämfört med den ursprungliga potatisstärkelsen. Detta unika amylopektin har egenskaper som liknar amylos. Efter tillagning och kylförvaring delas den inte lika lätt som vanlig potatisstärkelse och blir motståndskraftig mot matsmältningen. Kunskapen om den unika amylopektinstrukturen är viktig för att utforma funktionell stärkelse och hälsosammare mat i framtiden.

Våra resultat visade också att olika parametrar för potatisstärkelsens inre struktur signifikant korrelerade med stärkelseegenskaperna (gelatinisering och retrogradering.

Sammanfattningsvis är ökad förståelse för effekten av genetisk modifiering på stärkelsens molekylära struktur och funktionella egenskaper av stor betydelse. Förståelsen behövs för att kunna designa stärkelse med önskade funktionella egenskaper för flera applikationer, utan behov av ytterligare kemiska eller fysiska modifieringar. Detta skulle vara ett grönt alternativ; ett ekonomiskt- och hållbart tillvägagångssätt, för att utveckla nya önskvärda stärkelser inom en snar framtid.

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A simplified method was developed for determining the internal molecular structure of whole starch without prior amylopectin isolation. Applying the method, varied internal structural parameters of potato starch were obtained from different genetic backgrounds. Various internal structure parameters were found to affect the thermal properties of potato starch. A dense structure of building blocks led to higher gelatinisation temperatures and enthalpy. Retrogradation was found to be favoured by more large building blocks and many short internal chains.

Xue Zhao, the author of this thesis, conducted her PhD studies in Food Science at the Department of Molecular Sciences, SLU, Uppsala. She received her MSc and BSc degrees in Animal Science from SLU, Sweden and Northwest A & F University, China, respectively.

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