Studies on Starch Structure and the Differential Properties of Starch Branching Enzymes

Lena Andersson
Department of Food Science
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2001

Acta Universitatis Agriculturae Sueciae
Agraria 287

ISSN 1401-6249
ISBN 91-576-5834-X

Tryck: SLU Service/Repro, Uppsala 2001

Abstract

Andersson, L. 2001. *Studies on starch structure and the differential properties of starch branching enzymes*. Doctoral thesis.
ISSN 1401-6249, ISBN 91-576-5834-X.

Starch is a staple food in human and animal diets, but also a raw material widely used for industrial purposes. By genetical modification of starch-synthesising enzymes in crop plants, starch yields could be increased and novel starches with particular qualities could be produced for industrial use. However, the process of starch biosynthesis and its regulation is still not completely understood. One of the major groups of enzymes in starch biosynthesis is the starch branching enzymes (SBEs), which introduce the branch points into amylopectin. This thesis describes some properties of two different families of SBEs from potato and barley. In addition, starch structure from both developing and mature barley has been characterised.

Initially, a linear substrate for *in vitro* studies of SBEI and SBEII from potato was prepared. The linear dextrins were produced by enzymatic hydrolysis of retrograded starch and then further fractionated through ethanol precipitation. The *in vitro* branching properties of SBEI and SBEII from potato were examined using iodine-staining, NMR spectroscopy, chromatographic and enzymatic methods. The two enzyme isoforms showed different substrate specificities and the branched products exhibited different chain length distribution profiles, but a similar degree of branching.

In developing barley, starch structure and the expression of the gene encoding for barley SBEI were examined. The amylose content increased during development and followed a time course similar to that of the expression of the SBEI gene. The amylopectin chain length distribution was examined in the later stages of development, but no changes in the amylopectin structure were observed.

In mature waxy barley, the amylose content and amylopectin structure in different parts of the grain were analysed. It was shown that the amylose content was significantly higher in the peripheral parts of the endosperm than in the centre, whereas the amylopectin chain length distribution was similar in all parts of the endosperm.

Keywords: barley, potato, amylose, amylopectin, *Hordeum vulgare* L., *Solanum tuberosum* L., starch branching enzyme, starch biosynthesis.

Author´s address: Lena Andersson, Dept of Food Science, SLU, P.O Box 7051,
SE-750 07 Uppsala, Sweden.

Contents

Appendices

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Andersson, L., Rydberg, U., Larsson, H., Andersson, R. and Åman, P. Preparation and characterisation of linear dextrins and their use as substrates in *in vitro* studies of starch branching enzymes. Accepted for publication in *Carbohydrate Polymers*.

II. Rydberg, U., Andersson, L., Andersson, R., Åman, P. and Larsson, H. Comparison of starch branching enzyme I and II from potato. *Submitted for publication*.

III. Andersson, L., Andersson, R., Andersson, R.E., Rydberg, U., Larsson, H. and Åman, P. Characterisation of the *in vitro* products of potato starch branching enzymes I and II. *Submitted for publication*.

IV. Sathish, P., Sun, C., Ahlandsberg, S., Andersson, L., Åman, P. and Jansson, C. Characterization of a cDNA encoding starch branching enzyme I in barley. *Submitted for publication*.

V. Andersson, L., Fredriksson, H., Oscarsson Bergh, M., Andersson, R. and Åman, P. 1999. Characterisation of starch from inner and peripheral parts of normal and waxy barley kernels. *Journal of Cereal Science*, 30, 165-171.

Introduction

The importance of starch

Starch is not only a staple food in human and animal diets, it is also widely used as a raw material in the food industry, as well as in paper, textile and other industries. It can be used either in its native form or after chemical or physical modifications. The usefulness of a native or modified starch is based on its functional properties, which may include: adhesiveness, cold-swelling properties, specific viscosity, freeze-thaw stability, processing tolerance, gel texture and film-forming properties. In the food sector, starch is used in various products including frozen foods, dairy products, soups, sauces and baking products. Modified starch has also been used as a fat replacer (Aime *et al.*, 2001) and its film-forming abilities make it useful in coatings, for enhanced texture and crispness, and for the inclusion of fats and flavours (Krochta and de Mulder-Johnston, 1997; Shinsato *et al.*, 1999). Some of the most important non-food uses of starch include the production of paper and board, textiles and adhesives. In the paper industry, starch is used to enhance paper strength and printing properties. Due to its swelling capacity, pregelatinised starches can be used in the pharmaceutical industry for the preparation of tablets and capsules that quickly dissolve in water (http://www.excipients.com: Accessed 5 July, 2001).

New applications require new starches

The awareness of environmental issues has led to an increased interest in renewable and degradable materials. The use of starch in plastics and packaging, products that were formerly made out of petroleum-based raw materials, is gaining more interest (Slattery *et al.*, 2000). One example of such a product is the foamed maize starch used as loose-fill packaging material, with properties similar to polystyrene (http://www.eco-foam.com: Accessed 5 July, 2001). This, together with an increase in processed and pre-prepared food products, has created a demand for the development of starches with certain qualities required for these applications (Bligh, 1999). These starches can be found by searching for and studying other plant varieties or species than those used in starch production today. Another way of developing novel starches, or to increase starch yields, could be to genetically modify plants by changing the enzyme machinery that is responsible for starch biosynthesis (Bligh, 1999; Heyer *et al.*, 1999; Slattery *et al.*, 2000). The development of new starches might also reduce the need for chemical modifications and thus reduce the amount of chemicals used within the starch industry.

Starch production – facts and figures

The main starch sources on a global basis are maize, wheat, potato and tapioca (Gordon, 1999). Maize is the most important starch source and as much as 12% of the world production of maize is used for starch production. The European Union

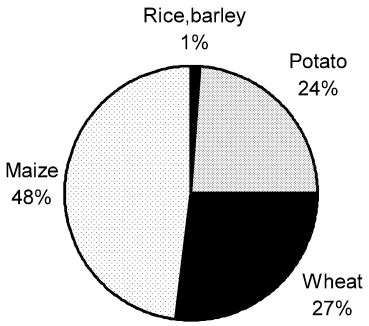

Figure 1. Starches produced within the European Union in 1998 (De Baere, 1999).

is self-sufficient with respect to starch and nearly a fifth of the starch produced within the EU is exported (De Baere, 1999). Maize starch accounts for about half of the starch produced within the EU. Potato and wheat starch makes up only a few percent of the total world production, though in Europe these starches are of great importance (Fig. 1).

Potato is one of the major starch sources in the EU and in Eastern Europe. Germany is the largest producer of potato starch within the EU, followed by the Netherlands, France, Denmark, Sweden, Finland and Austria (De Baere, 1999). In Sweden, approx. 304 000 tonnes of potato were processed into starch last year (http://www.lyckeby.com/Lyckeby/Svenska/index2.htm: Accessed 5 July, 2001). This gave around 65 000 tonnes of potato starch, of which 80-85% was chemically and/or physically modified while the remaining part was sold in its native form (Jakobsson, 2001). A smaller quantity of wheat starch is also produced in Sweden and is used mainly as a raw material in the production of starch syrup.

Starch structure and characteristics

Storage and transitory starch

Starch is found in both photosynthetic and non-photosynthetic tissues. In green leaves, it is found in the chloroplasts and often termed transitory starch due to its rapid, diurnal turnover, where the starch is produced and accumulated during photosynthesis and then degraded into sucrose during darkness. Storage starch is found in amyloplasts, which are specialised plastids present in plant storage organs such as seeds, tubers and roots. Storage starch is deposited over longer periods of time and mobilised *e.g.* during the germination of seeds or the sprouting of tubers. Storage starch is composed of both amylose and amylopectin in various ratios depending on plant species, while amylopectin is the dominant component in transitory starch (Hovenkamp-Hermelink *et al.*, 1988; Tomlinson *et al.*, 1997). This thesis focuses mainly on the characteristics of storage starch.

Table 1. Some characteristics of amylose and amylopectin[a]

Property	Amylose	Amylopectin
Average M_w [b]	~10^5	10^7-10^9
Average chain length	100-550	18-25
Degree of branching (%)	0.2-0.7	4.0-5.5
β-Amylolysis limit (%)	70-95	55-60
λ_{max} of iodine-complex (nm)	640-660	530-570

[a] From Hizukuri, 1996.
[b] From Buléon *et al.*, 1998.

Amylose and amylopectin

Amylose and amylopectin are the two principal components of starch. Amylose is defined as a mainly linear molecule of α-(1→4)-linked glucose residues with a small fraction of α-(1→6)-linkages. It makes up a minor fraction of the starch granule where it generally accounts for 20-30% of the total starch. There are also a number of natural *waxy* mutants of *e.g.* barley, maize, wheat and rice, with starch containing little or no amylose (Morrison *et al.*, 1984). High-amylose genotypes, with up to 70% amylose, have been found for *e.g.* maize and barley (Morrison and Laignelet, 1983; Salomonsson and Sundberg, 1994). Some characteristics of amylose and amylopectin are summarised in Table 1.

Amylopectin is the major constituent of starch and consists of large, highly branched molecules. It is composed of linear α-(1→4)-linked glucose chains connected by α-(1→6)-linkages. The outer and inner chains of amylopectin were classified into A-, B- and C-chains by Peat *et al.* (1952). A-chains are the linear chains attached to another chain, by its potential reducing end, through an α-(1→6)-linkage. B-chains and C-chains both carry one or more A- and/or B-chain.

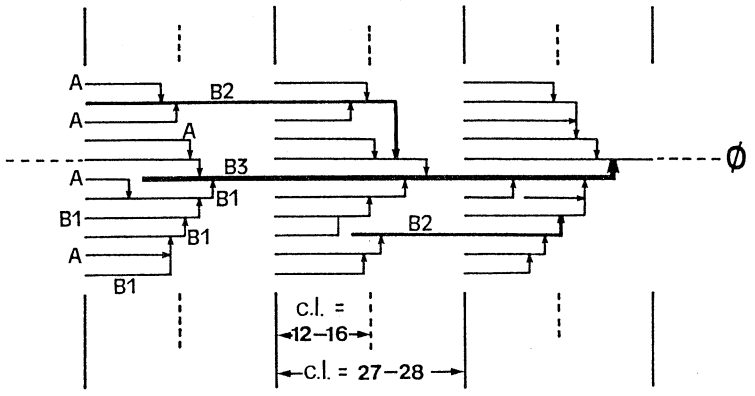

Figure 2. A-, B- and C-chain arrangement in amylopectin as proposed by Hizukuri (1986). ∅ represents the reducing end of the C-chain. Reproduced by permission of the publisher.

There is only one C-chain per molecule and it contains the only reducing end of the molecule. The ratio of A- to B-chains is a parameter used when characterising the structures of different amylopectins (Manners, 1989; Hizukuri, 1996). The chain length distribution of amylopectin is another important characteristic that may vary between plant species. In debranched amylopectin, Hizukuri (1986) observed a polymodal distribution of chains, with five populations (A, B1, B2, B3 and B4). The shortest chains, fraction A, had a chain length maximum at 11-16, whereas the average chain lengths in the B1, B2 and B3 fractions were 20-24, 42-48, and 69-75, respectively. Their relative lengths were almost 1:2:3, which indicates that they were involved in the formation of one, two or three amylopectin clusters (see below) as illustrated in Figure 2. The B4 fraction was suggested to be part of more than three clusters.

Minor starch components

The physical properties of starch are affected by amylose/amylopectin ratios and chain length distributions of amylopectins (Jane *et al*., 1999), but also by other components of the starch granules such as lipids and phosphorylated glucose residues (Morrison *et al*., 1984; Jane *et al*., 1996). The lipids associated with isolated starch granules include surface as well as internal lipids (Morrison, 1995). Lipids extracted from starch granules may also include non-starch lipids that have associated with the granules during starch extraction. Starch lipids are present in various amounts in different species, but are generally associated with cereal starches, which have a lipid content of around 1% (Morrison *et al*., 1984).

Barley, wheat and rye starches contain mainly lysophospholipids (LPL), whereas lipids in other cereal starches also contain 30-60% of free fatty acids (Morrison, 1993a). The content of LPL and free fatty acids in cereals is positively correlated with the amylose content. Amylose chains are able to form amylose-lipid complexes, but evidence for the presence of naturally occurring amylose-lipid complexes in native starch granules is inconclusive (Hoover, 1998). In 1993, however, Morrison *et al*. reported that both lipid-complexed and lipid-free amylose were present in native barley starch.

Phosphorus is not only present as phospholipids but can also be found as phosphate monoesters covalently linked to the glucose residues. In contrast to cereal starches, root and tuber starches generally contain significant amounts of phosphate groups (Lim *et al*., 1994). These are primarily linked either to the C3- or C6-position of the glucosyl units (Hizukuri *et al*., 1970; Tabata and Hizukuri, 1971) and are found exclusively in amylopectin (Hizukuri, 1996). Starch granules also contain small amounts of proteins that usually account for less than 0.5% of the granule weight (Ellis *et al*., 1998).

Starch granule organisation

Starch granules from different sources vary in both shape and size. Potato granules are oval or spherical in shape and have a diameter of up to 75 μm while amaranth has granules that are 0.5-2 μm in diameter (Jane *et al*, 1994). Barley, wheat and rye starches have bimodal granule size distributions with large lens-

shaped A-granules and small spherical B-granules (MacGregor and Fincher, 1993; Ellis *et al*., 1998). However, all starch granules are essentially organised in the same way (Fig. 3). The first level of organisation is the arrangement of the side chains in amylopectin. These side chains are arranged as clusters, a model that was originally proposed 30 years ago by Nikuni (1969) and French (1972). Hizukuri (1986) later suggested that the polymodal distribution of amylopectin chains could be explained by the linear chains being part of one, two or more clusters as described above. The amylopectin clusters are part of crystalline and amorphous lamellae. The crystalline lamellae are believed to consist of amylopectin chains forming double helices, while the branch points are located in the amorphous lamellae. In all species examined, the thickness of a crystalline and an amorphous lamella is 9-10 nm as determined by small angle X-ray scattering (Jenkins *et al*., 1993).

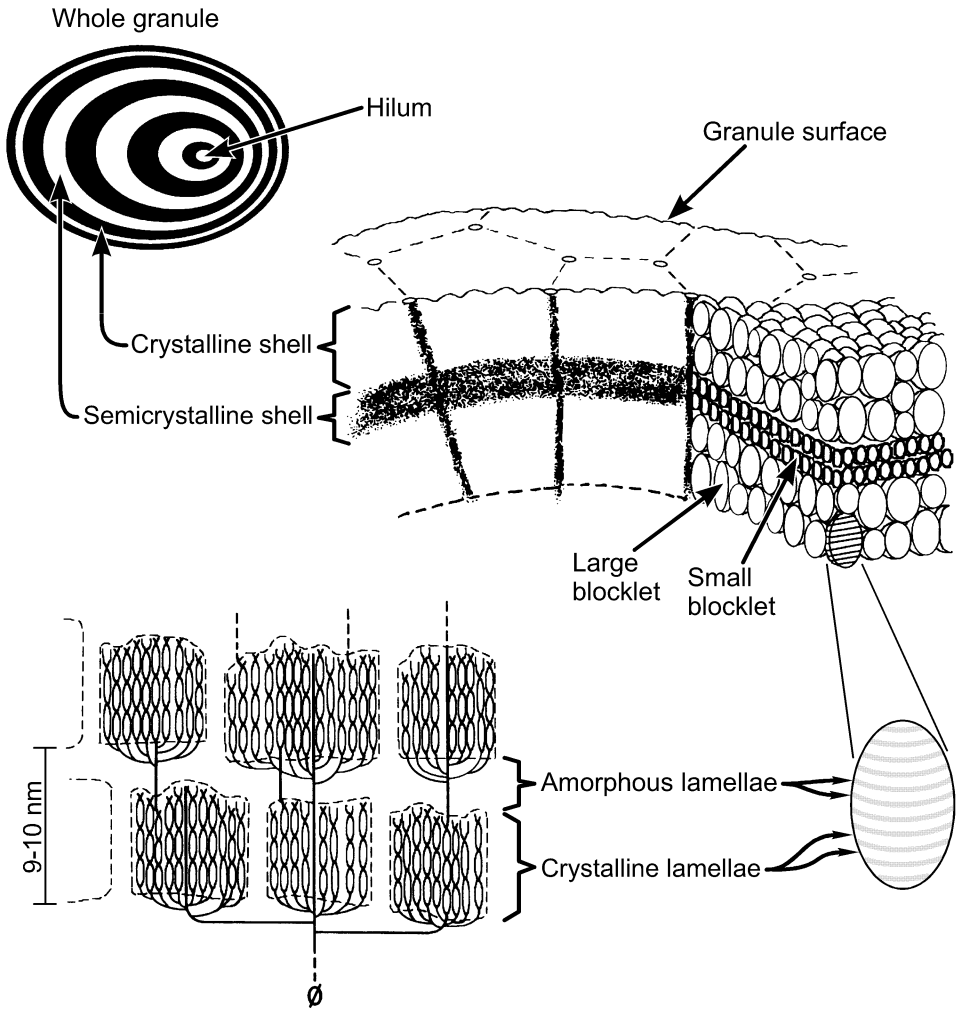

Figure 3. Overview of starch granule organisation (modified from Gallant *et al*., 1997). Reproduced by permission of the publisher.

Crystalline and amorphous lamellae are organised into elongated, spherical structures termed blocklets, which is proposed to be the next level of granule organisation (Gallant *et al*., 1997). These blocklet structures, described already in 1936 (Badenhuizen), were examined by scanning electron microscopy (Gallant *et al*., 1992) and their existence was further supported from recent studies using atomic force microscopy (Baldwin *et al*., 1998; Baker *et al.*, 2001). The blocklet structures are in turn proposed to be organised into growth rings, which are layers of semi-crystalline and crystalline shells visible by light microscopy. The starch polymers are arranged radially with their molecular axes aligned perpendicular to the growth rings and to the granule surface (Yamaguchi *et al*., 1979; Baker *et al*., 2001).

The crystallinity of starch

Three types of x-ray diffraction patterns, termed A, B and C, have been observed for native starch granules from various botanical sources (Zobel, 1988). Starches with A-type x-ray diffraction patterns include most cereal starches (*e.g*. normal maize, wheat, and oat) while potato and other tuber starches exhibit the B-type pattern. Some high-amylose starches, *e.g*. high-amylose maize, also display B-type patterns. The C-type diffraction pattern has been shown to be a mixture of A- and B-type patterns and is characteristic of pea and other legume starches. The A- and B-patterns are characteristic of two different types of crystallites, which differ in the mode of packing the amylopectin double helices, and in the water content (Imberty and Pérez, 1988; Imberty *et al*., 1988). A-type crystallites are more densely packed than B-type crystallites, the unit cell structure of which has a cavity holding water molecules. The amylopectin double helices in the unit cell structures of A- and B-crystallites are essentially identical and are packed as left-handed, parallel-stranded double helices. It is believed that amylopectin chain length influences the crystallinity. It has been shown that amylopectins from A-type starches have shorter average chain lengths than those from B-type starches (Hizukuri, 1985). Other factors such as the presence of lipids or the growing conditions (temperature, moisture) may also affect the crystallinity (Hizukuri, 1996; Buléon *et al*., 1998).

Starch biosynthesis

As described earlier, starch polymers are composed of only one type of sugar residue (glucosyl units) and two types of linkages: α-(1→4)- and α-(1→6)-linkages. Despite the simple building blocks, starch is stored in highly organised granule structures and the biosynthesis of starch involves a number of enzymes and enzyme isoforms, which are differently expressed in different tissues and in developing storage organs. The biosynthesis of starch and its regulation have been extensively studied through a number of years and have been reviewed by many authors (for recent reviews, see Smith *et al*., 1997; Sivak and Preiss, 1998; Ball *et*

al., 1998; Kossmann and Lloyd, 2000; Myers *et al*., 2000; Denyer *et al*., 2001). Many of the enzymes and their corresponding genes have been isolated from various plant species. In spite of this, the precise role of each starch biosynthetic enzyme has yet not been established.

Figure 4 illustrates a simplified version of a proposed pathway for sucrose breakdown and starch synthesis in starch storage organs. Sucrose entering the plant cells can be broken down by sucrose synthase, generating UDP glucose and fructose. UDP glucose was first suggested to be the substrate in starch synthesis (Leloir *et al*, 1961), although, ADP glucose is now generally accepted to be the preferred substrate (reviewed in Sivak and Preiss, 1998). Three major groups of enzymes are involved in starch biosynthesis: ADP glucose pyrophosphorylases (EC 2.7.7.27), starch synthases (EC 2.4.1.21) and starch branching enzymes (EC 2.4.1.18) all of which are briefly described below.

ADP glucose pyrophosphorylase

In the first step of starch biosynthesis, ADP glucose pyrophosphorylase (AGPase)

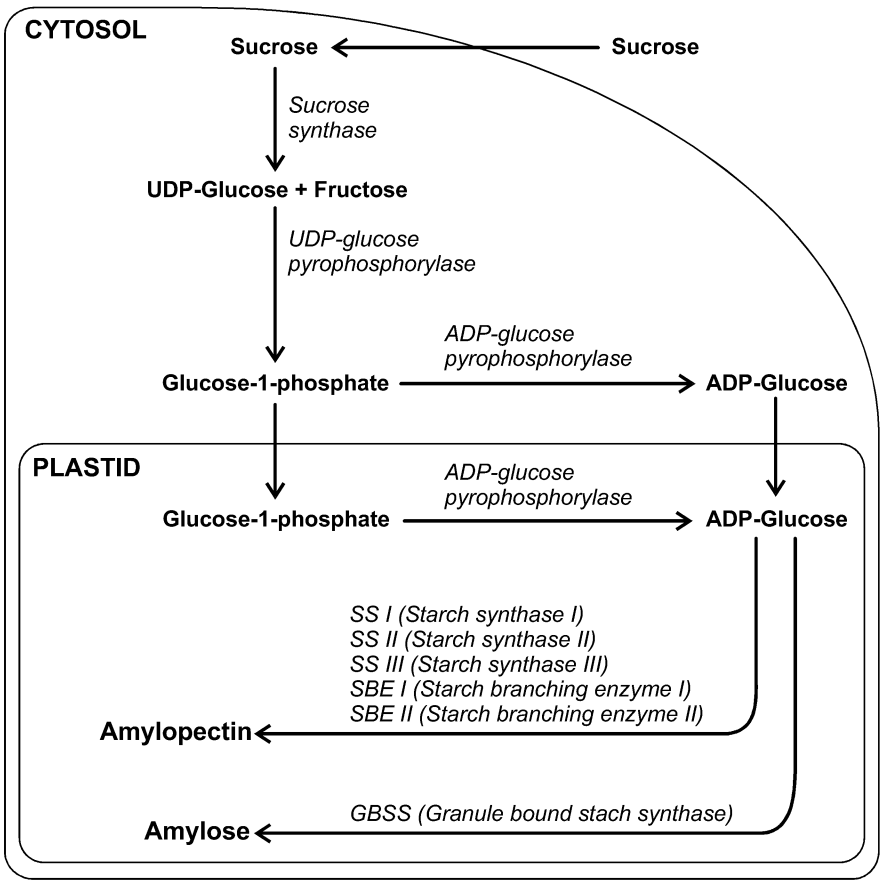

Figure 4. A proposed pathway of sucrose breakdown and starch synthesis in storage organs.

synthesizes ADP-glucose from glucose-1-phosphate. ADP-glucose is the substrate for starch synthases. AGPase activity has often been considered to be a rate-limiting step in starch biosynthesis because of its sensitivity to allosteric regulation (Reviewed in Sivak and Preiss, 1998; Buléon et al., 1998; Kossmann and Lloyd, 2000). AGPase present in photosynthetic tissues is generally activated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (P_i) (Preiss, 1982; Kleczkowski, 1999). Conflicting results, however, have been obtained for AGPases in storage organs, where *e.g.* barley (Kleczkowski *et al.*, 1993) and wheat endosperm AGPases have shown a rather low or no sensitivity to 3-PGA/P_i-regulation (reviewed in Kosegarten and Mengel, 1998). In maize and barley, both cytoplasmic and plastidial forms of the enzyme has been found (Kossmann and Lloyd, 2000).

Starch synthase

The starch synthases can be divided into two groups: granule-bound starch synthases (GBSSs), which are found tightly bound to starch granules and starch synthases (SSs) that are either found in the soluble phase or as granule-bound enzymes (Edwards *et al.*, 1996). All starch synthases transfer glucosyl units from ADP-glucose to the nonreducing ends of growing α-(1→4)-glucans. It was previously shown that the granule-bound form of starch synthase was responsible for the synthesis of amylose (Nelson and Rines, 1962). Naturally occurring amylose free (*waxy*) mutants with defects in GBSS have been isolated for a number of species including waxy maize (Nelson and Rines, 1962), waxy barley (Rohde *et al.*, 1988), and amylose-free potato (Hovenkamp-Hermelink *et al.*, 1987). Multiple isoforms of SS have been identified, and based on their amino-acid sequences they can be divided into three distinct classes: SSI, SSII and SSIII (Smith, 1999). They are generally accepted to be involved in the synthesis of amylopectin, although, relatively little is known about the relative contributions of the different SS isoforms.

Starch branching enzyme

Starch branching enzymes (SBEs) are involved in the synthesis of amylopectin where they create the branch points by hydrolysis of an α-(1→4)-linkage and subsequent formation of an α-(1→6)-glucosidic bond between the cleaved chain and a C6 hydroxyl group of a α-(1→4) glucan. Amino acid sequence alignment and structure prediction studies suggest that the branching enzymes contain the catalytic $(\beta/\alpha)_8$-barrel domains found in many other amylolytic enzymes (Jespersen *et al.*, 1993).

At least two isoforms of SBE have been identified in various plant species, such as maize, pea, rice, potato, wheat and barley (Boyer and Preiss, 1978; Smith, 1988, Mizuno *et al.*, 1992; Larsson *et al.*, 1996; Morell *et al.*, 1997; Sun *et al.*, 1997). The SBE isoforms can be divided into two distinct families, A and B, based on sequence homology (Burton *et al.*, 1995). There are two types of nomenclature used for the branching enzyme families; SBE A and B, or I and II. The members of family A include *e.g.* potato SBEII, maize SBEII, pea SBEI, rice

SBEIII and barley SBEII, whereas potato SBEI, maize SBEI, pea SBEII, rice SBEI and barley SBEI belong to the B family.

SBEs from the different classes differ in their enzymatic properties. In *in vitro* studies, maize SBEI and SBEII differed in their substrate affinities and in the chain lengths of the branched products created by each enzyme (Takeda *et al*., 1993). The branched products made from an amylose-like substrate by the individual SBE isoforms were analysed and the results showed that maize SBEI transferred longer chains than SBEII. When amyloses with different average chain lengths (cl) were used, both maize SBEI and II were more active on longer chains (cl 405). Maize SBEI still had a high activity on an amylose with an average cl of 197, whereas the activity of SBEII decreased substantially with a decrease in chain length. A comparison of the activities on amylose and amylopectin substrates showed that maize SBEI had the highest activity in branching amylose (Guan and Preiss, 1993). In contrast, maize SBEII isoforms catalysed branching of amylopectin at a higher rate than maize SBEI.

Studies of mutants or transgenic plants, where the activities of SBEs are reduced using antisense techniques, may also provide some insights into the roles of the SBE isoforms. Mutants lacking SBEII, known as *amylose extender* (*ae*) mutants in maize and rice and *rugosus* (*r*) mutants in pea are characterised by a reduced starch content, a high proportion of amylose, and amylopectin with a high average chain length (Baba and Arai, 1984; Takeda and Hizukuri, 1987; Lloyd *et al*., 1996; Jane *et al*., 1999). No mutant lacking SBEI is known, but transgenic potato plants deficient in SBEI and/or SBEII have recently been produced (Safford *et al*., 1998; Jobling *et al*., 1999; Schwall *et al*., 2000). When SBEI was down-regulated, no significant change in amylose content or amylopectin chain length profile was observed. A reduction in potato SBEII increased the apparent amylose content, seemingly due to an increase in the proportion of long chains in amylopectin. Only the simultaneous antisense-inhibition of both SBEI and SBEII resulted in a notable increase in amylose content. All starches, however, showed elevated levels of phosphorus. In a different study, the genes encoding maize SBEI and II were expressed in glycogen branching enzyme deficient *Escherichia coli* (Guan *et al*., 1995). The resulting polysaccharides produced together with glycogen synthase were different from amylopectin, stressing the importance of the whole enzyme machinery, including interactions with starch synthases, in determining starch structure.

Other enzymes

Already in 1958 (Erlander) it was proposed that starch debranching enzymes were active during starch biosynthesis. Recent studies of certain *sugary* mutants of maize (Pan and Nelson, 1984), rice (Nakamura, 1996) and the *sta7* mutant of *Chlamydomonas reinhardtii* (Mouille *et al*., 1996) deficient in the activity of a debranching enzyme, showed that these mutants produced phytoglycogen together with, or instead of starch. A model was proposed which involve the debranching enzymes in trimming an extensively branched preamylopectin molecule to produce a more ordered amylopectin (Ball *et al*., 1996). Another model has been

proposed where soluble glucans produced as a by-product by starch synthases and SBEs are degraded by glucan-degrading enzymes, including debranching enzymes (Zeeman *et al*., 1998). The absence of debranching enzymes would then result in phytoglycogen accumulation.

Another enzyme suggested to be involved in starch metabolism is the disproportionating enzyme (D-enzyme). It is an α-1,4-glucanotransferase that catalyses the transfer of maltooligosaccharides from one α-(1→4)-glucan chain to another, or to glucose (Takaha *et al*., 1993). Its role in starch metabolism is not clear, but it has been proposed to be involved in starch degradation (Takaha *et al*., 1998; Critchley *et al*., 2001). Studies of a mutant of *C. reinhardtii* led to the proposal that the D-enzyme might be involved in the regulation of branch length and the number of branches in amylopectin synthesis, together with debranching enzymes (Myers *et al*., 2000).

A protein called R1 has recently been found in potato and other plant species (Lorberth *et al*., 1998; Ritte *et al*., 2000). Its precise function has not yet been elucidated but a role in phosphorylation and/or degradation of starch has been suggested (reviewed in Kossmann and Lloyd, 2000). When R1 was down-regulated in transgenic potato plants, there was an increased accumulation of starch and a substantial decrease in phosphate content compared to normal plants (Lorberth *et al*., 1998).

Present investigations

Objectives

The overall purpose of this project was to study the properties of the branching enzymes involved in starch biosynthesis in potato and barley. This was performed through *in vitro* studies of potato starch branching enzymes and by examining starch structure in developing and mature barley endosperm, since differences in starch structure could be related to the differential expression of starch biosynthetic enzymes.

Specifically, the aims were to:

• Develop suitable substrates and methods to be used in *in vitro* studies of starch branching enzymes from potato (*Solanum tuberosum* L.).

• Compare the branching activities of starch branching enzymes I and II from potato using various enzyme substrates and different methods to study the enzyme products.

• Study the amylose content and amylopectin structure in connection with an analysis of the expression pattern of starch branching enzyme I in developing barley endosperm (*Hordeum vulgare* L.).

• Characterise starch isolated from inner and outer parts of waxy and normal barley grain.

Studies of starch branching enzymes I and II from potato

Starch branching enzymes from potato

As early as 1944, starch branching enzyme activity was found in potato by Haworth *et al*., who discovered an enzyme system that catalysed the conversion of glucose-1-phosphate into an amylopectin-like polysaccharide. Multiple forms of the branching enzyme, formerly called Q-enzyme, were later purified from potato tubers (Borovsky *et al*., 1975; Vos-Scheperkeuter *et al*., 1989; Blennow and Johansson, 1991; Praznik *et al*., 1992; Khoshnoodi *et al*., 1996). Some of the purified forms of the protein, identified as potato SBEI, varied in their molecular masses but have been suggested to be products of the same gene (Khoshnoodi *et al*., 1996). It was not until recently that an SBEII isoform was found in potato by Larsson *et al*. (1996). SBEI is thought to be the dominating isoform in potato tubers, whereas SBEII is the predominant form found in leaves (Larsson *et al*., 1998; Jobling *et al*., 1999). Still, down-regulation of SBEI activity in transgenic potato plants has a limited impact on the structure of the tuber starch (Safford *et al*., 1998). Small differences, however, were observed in the physical properties of the starch.

Preparation of substrates for SBE in vitro *assays*

There is not much information available about the conformation and structure of the native substrates for SBEs. Amylopectin and amylose substrates have been used in *in vitro* enzyme studies but these polysaccharides are not always that well defined. Commercially available amylose may contain substantial amounts of branched, amylopectin-like material, while amylopectin has a highly complex structure and is itself a final product in starch biosynthesis. In order to produce a linear substrate with a defined molecular weight range, we hydrolysed a retrograded high-amylose maize starch (Paper I). Retrograded starch can be partially hydrolysed by acid or by digestion with α-amylase (Jane and Robyt, 1984), leaving an enzyme-resistant dextrin fraction of linear amylose-type chains (Russell *et al*., 1989; Cairns *et al*., 1996). The linear dextrins prepared by α-amylase digestion of retrograded starch in Paper I gave a gel permeation chromatography (GPC) elution profile with a peak maximum around dp 50-60 (Fig. 5). This was in line with average chain lengths previously reported for enzyme-resistant residues produced from retrograded starch or amylose (Jane and Robyt, 1984; Russell *et al*., 1989; Siljeström *et al*., 1989). Degradation by β-amylase gave a β-amylolysis limit of 92%, suggesting that the glucose chains were essentially linear.

Analysis of the linear dextrins by high performance anion exchange chromatography (HPAEC) gave a chain length distribution where most chains contained nine or more glucose units. This could be the minimum length of the chains forming double helices in the crystalline enzyme-resistant residue, as

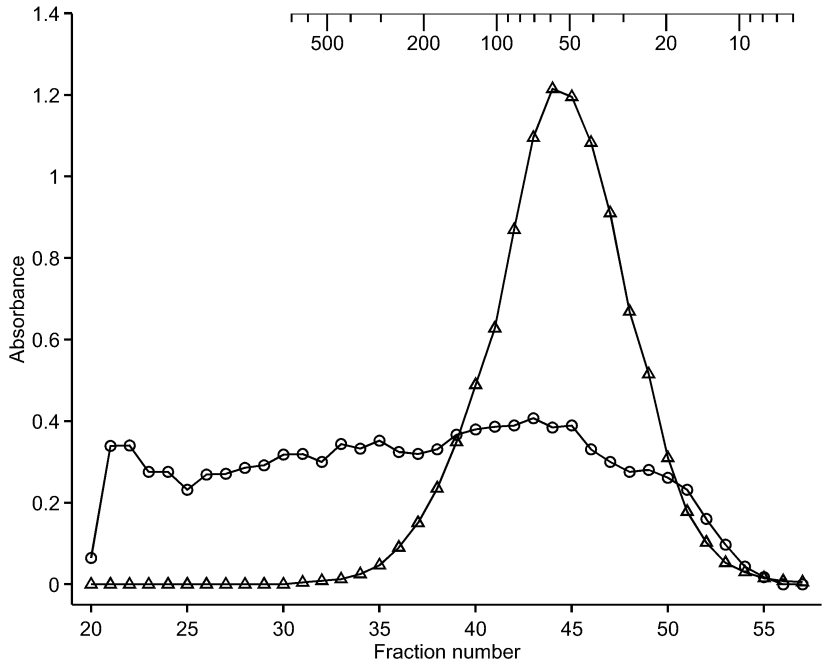

Figure 5. GPC elution profile of retrograded starch prior to (-o-) and after (-Δ-) hydrolysis by α-amylase and amyloglucosidase. Dp values obtained after column calibration with pullulans are shown on the upper axis.

suggested in a previous study of enzyme-resistant starch (Gidley *et al*., 1995). The linear dextrins obtained from two different samples of retrograded high-amylose maize starches, "Novelose" and "Cerestar", were characterised. They had slightly different chain length distribution patterns (Fig. 2 in Paper I), but were otherwise similar. The linear dextrins produced from "Cerestar" starch were used as a substrate for SBEI in Paper I and for both SBE isoforms in Paper II.

Fractionation of linear dextrins

Further fractionation of the linear dextrins described above was desirable in order to produce fractions with different molecular weight ranges that could be used as substrates for the branching enzymes. Attempts to remove low-molecular weight material by dialysis failed, supposedly due to interactions between low- and high-molecular weight material. However, starch polymers can be fractionated by the gradual addition of alcohols, such as ethanol, methanol or isopropanol (Everett and Foster, 1959; Young, 1984). Initially, a fraction with a high average molecular weight was generated (Paper III). By ethanol precipitation from a solution of linear dextrins dissolved in DMSO (80%), a selective fractionation was obtained. Ethanol was added drop-wise during continuous stirring of the sample until a final concentration of 45% ethanol was reached. The precipitate, containing the high molecular weight material, was collected by centrifugation and characterised by

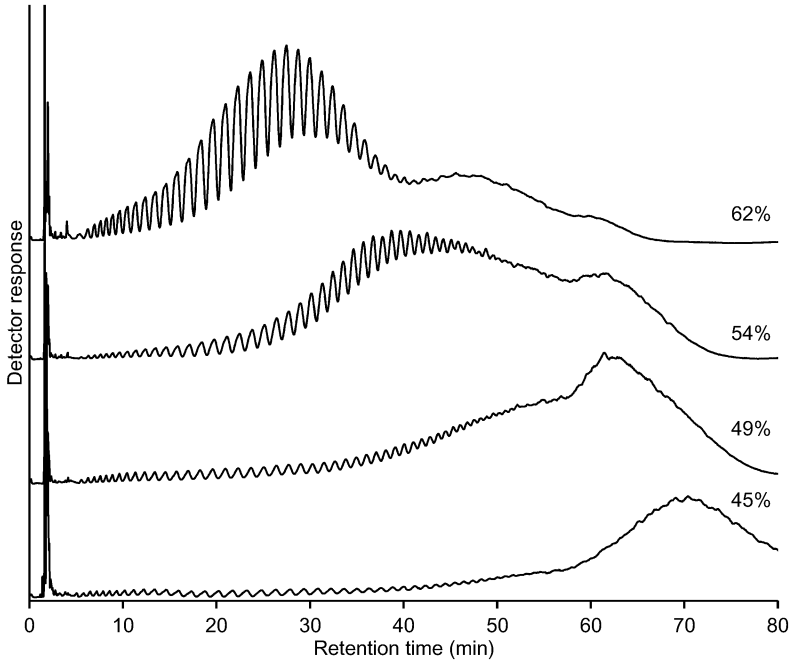

Figure 6. HPAEC chromatograms of dextrin fractions obtained after a sequential ethanol precipitation of linear dextrins dissolved in 80% DMSO. Ethanol concentrations are indicated.

HPAEC which showed that only minor amounts of shorter glucose chains (dp<40) were present. These dextrins were used as an enzyme substrate in Paper III.

The supernatant, containing material of a lower molecular weight than in the precipitate, could be used for further fractionation by adding more ethanol in the same way as described above. Dextrin fractions precipitated at different ethanol concentrations from one single sample solution are shown in Figure 6 (unpublished data). Furthermore, a repeated fractionation, *e.g.* of a 49% ethanol fraction as shown in Figure 7, resulted in a total removal of all shorter chains but gave a yield of only 15%, which is less than half of the yield obtained after the first precipitation. The procedure involving only one precipitation step was therefore chosen for the preparation of the enzyme substrate.

Characterisation of the in vitro *products of SBEI and SBEII*

Both SBEI and SBEII from potato have recently been expressed in *Escherichia coli* (Khoshnoodi, 1997; Larsson, 1999). In Papers I, II and III, the *in vitro* activities of the purified potato SBE isoforms expressed in *E. coli* were examined. The activities on amylose and amylopectin substrates were compared using the starch/iodine assay (Paper II). The progressing branching process was characterised by a shift in λ_{max} and a decrease in the absorbance measured at the wave lengths typical for amylose and amylopectin, 655 and 520 nm, respectively.

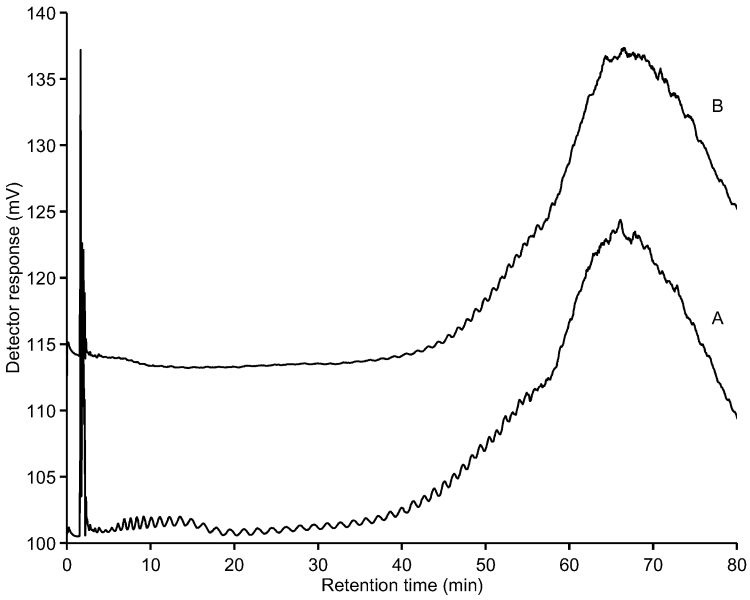

Figure 7. HPAEC chromatograms of 49%-ethanol fractions of linear dextrins collected after the first precipitation (A) and after a repeated precipitation (B).

As shown by the relative absorbances (Table 2), SBEI was more active than SBEII on an amylose substrate and SBEII was slightly more active than SBEI on amylopectin. With the amylopectin substrate, however, the shift in λ_{max} did not follow the decrease in absorbance. The difference in substrate preference is consistent with similar studies of maize SBEs (Guan and Preiss, 1993), and studies of wheat SBEs with an amylose substrate (Morell *et al.*, 1997).

The linear substrate and the branching products were analysed by GPC, which showed that the molecular size of the branched product was not considerably different from that of the substrate (Fig. 4 in Paper I, Fig. 4A in Paper II). That branch points actually had been formed was shown when the debranched samples

Table 2. Properties of the branching products of SBEI and SBEII after 180 min of incubation with amylose and amylopectin substrates (Paper II)

Substrate + enzyme isoform	λ_{max} (nm)	Relative absorbance*
Amylose	616	100%
+ SBEI	543	27%
+ SBEII	574	46%
Amylopectin	551	100%
+ SBEI	522	74%
+ SBEII	538	64%

* The absorbance of the iodine complex was measured at 655 nm (amylose) and 520 nm (amylopectin). The absorbance of the control sample was set to 100%.

were analysed, causing a clear shift towards lower molecular weights. These results could be interpreted as intra-molecular branching taking place, which would give branched products with molecular weights similar to that of the original linear substrate. On the other hand, inter-molecular branching could not be excluded from these results and further studies are necessary to establish the presence of intra-molecular branching.

The *in vitro* studies of potato SBEI and SBEII performed in Paper II also showed that both enzyme isoforms were stimulated when inorganic phosphate was added to the incubation buffer. Whether the enzyme itself was affected by the phosphate or if the substrate was also influenced by the addition of P_i, leading to increased enzyme affinity, could not be established. Phosphate stimulation has previously been reported for wheat SBEs, where phosphorylated compounds as well as P_i increased the branching activities (Morell *et al.*, 1997).

In Paper III, the shortest chains of the enzyme substrate were removed, thereby reducing the risk of short substrate chains affecting the chain length profiles of the branching products. HPAEC analysis showed that the branched products made by SBEI contained a large number of chains of dp 6 and 11-12, and a population around dp 29-30. The most abundant chains in the SBEII products were shorter, with peaks at dp 6-7 and 9-11 (Fig. 8). Similar chain length distributions were also reported in Paper I (SBEI) and Paper II (SBEI and II). Thus, SBEI preferably transferred long chains, whereas SBEII transferred shorter chains.

When samples were incubated with SBE for various times, the relative composition of chains of dp 9-35 was found to be essentially constant throughout the branching reaction (Fig. 2 in Paper III). This showed that the change in structure undergone by the substrate during the branching process did not significantly affect the patterns of chains produced by each enzyme. A rapid decrease in the number of long chains was shown for SBEI, whereas SBEII worked more slowly on this substrate (Fig. 9). Additionally, the final SBEII product contained more long chains than the final product of SBEI. Whether this depends on SBEII being less efficient than SBEI in using longer chains or if there are some structural differences in the branching products making the long chains in the SBEII product unavailable to further branching could not be established from these results.

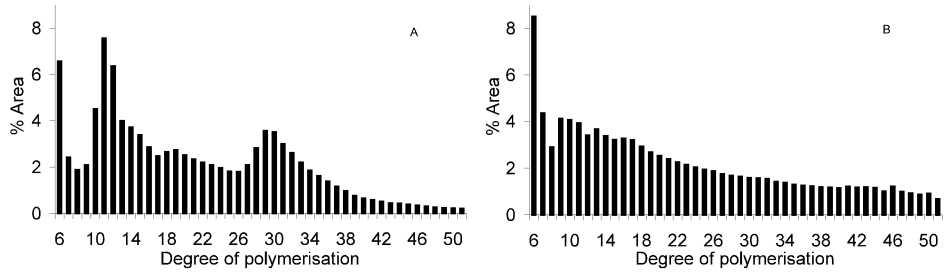

Figure 8. HPAEC chain length distribution profiles of the debranched products of potato SBEI (A) and SBEII (B).

By ^{1}H NMR spectroscopy, the degree of branching of the final products was determined to 3.7% for both enzyme products (Paper III), which means that a branching density similar to that of amylopectin could be obtained with each of the enzymes. β-Amylolysis values for the final branching products were determined to 66-74%. This means that a great part of the branched molecule was available for enzymatic hydrolysis and indicates that branch points were generally not formed very close to non-reducing ends. This was also confirmed when the structural differences between the branching products and their β-limit dextrins were visualised by ^{1}H-^{1}H 2D-NMR spectroscopy (COSY). The 2D-NMR spectra showed that branch points were not present on the second or third glucose residue from the non-reducing ends of the branched products.

The differences in chain length patterns and branching rates displayed by the two potato SBE isoforms are consistent with those reported for maize SBEs (Takeda *et al*., 1993) and support the theory that the two isoforms have different roles in amylopectin synthesis. Still, it has been difficult to draw any conclusions about the specific roles of each enzyme isoform from *in vitro* as well as from previous *in vivo* studies due to the following reasons. *In vivo*, the SBEs most likely interact with other enzymes such as starch synthases and possibly other enzymes during amylopectin synthesis. Furthermore, it seems likely that some

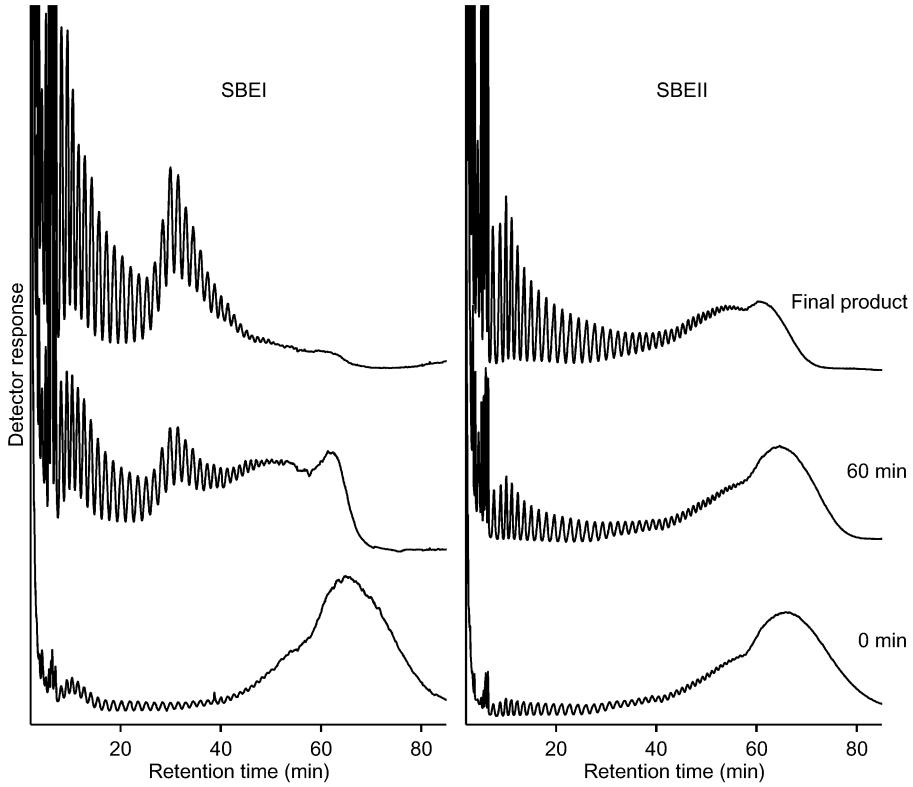

Figure 9. HPAEC chromatograms of debranched products of potato SBEI and SBEII obtained after different incubation times.

enzyme isoforms can compensate for a reduction in the activity of another enzyme (Safford *et al*., 1998) which indicates that their *in vivo* activities are not necessarily clearly different from each other. Additionally, some properties of the native substrates are still unknown, which means that *in vitro* experiments may be very different in terms of concentrations and substrate structures compared to the *in vivo* conditions.

Studies on starch structure in mature and developing barley endosperm.

Barley starch

Barley was the second most common cereal grown in Sweden last year (http:// apps.faostat.org: Accessed 5 July 2001). The major component of the barley grain is starch, which accounts for 50-67% of the dry kernel weight (Åman *et al*., 1985; Morrison *et al*., 1986; Oscarsson *et al*., 1997). Normal barley starch generally contains around 25-30% of amylose. High-amylose as well as waxy mutants are also available, typically containing 40-44% and 2-8% of amylose respectively (Morrison *et al*., 1986). The granule size distribution is bimodal with large, A-type, granules that are 10-25 μm in diameter and small, B-type, granules with diameters less than 6 μm. The A-type granules make up around 10-20% of the total number of granules, but represent a high proportion (85-95%) of the total granule volume (Morrison *et al*., 1986). Analyses of amylose content in small and large granules show that large granules contain more amylose than small granules (MacGregor and Morgan, 1984; Kang *et al*., 1985; Tang *et al*., 2001a), though, conflicting results have been reported (Vasanthan and Bhatty, 1996).

Characterisation of barley starch in developing barley endosperm

In barley, the genes encoding SBEIIa and SBEIIb have previously been isolated (Sun *et al*., 1998). In developing endosperm, it has been shown that both SBEII genes are expressed at an early stage of development with a peak around 12 days after pollination (d.a.p.). SBEIIb is exclusively expressed in the endosperm, whereas SBEIIa is found in both leaf and endosperm. In connection with the characterisation of the SBEI gene and a study of its expression pattern in developing barley kernels, the structure of starch isolated from developing endosperm was examined (Paper IV). RNA gel-blot analysis showed that the SBEI gene was mainly found in endosperm where it was expressed at a later stage than the genes coding for SBEII. In accordance with the transcriptional assay, immunoblot analysis of protein extracts using an SBEI-specific antibody showed that SBEI could be detected after 15 d.a.p. and that the enzyme levels peaked at around 24 d.a.p. (Fig. 5 in Paper IV).

Amylose contents were determined by GPC in samples isolated from barley endosperm harvested at 6-27 d.a.p. (Fig. 10). An increase in the amylose/amylopectin ratio was observed, beginning at 15 d.a.p. The amylose contents obtained at 24-27 d.a.p. corresponded well to that of the mature barley cultivar Golf (Oscarsson *et al*., 1997; Paper V). Interestingly, the increase in amylose content coincided with the accumulation of SBEI.

Amylopectin chain length distributions were determined by HPAEC in samples harvested 12-27 d.a.p. The chain length profiles were characteristic of barley amylopectin with a peak maximum at dp 11-12 and a slight shoulder around dp 18-20 (Fig. 11). The peak profiles of all samples were similar and the small differences noticed between individual samples could not be correlated with

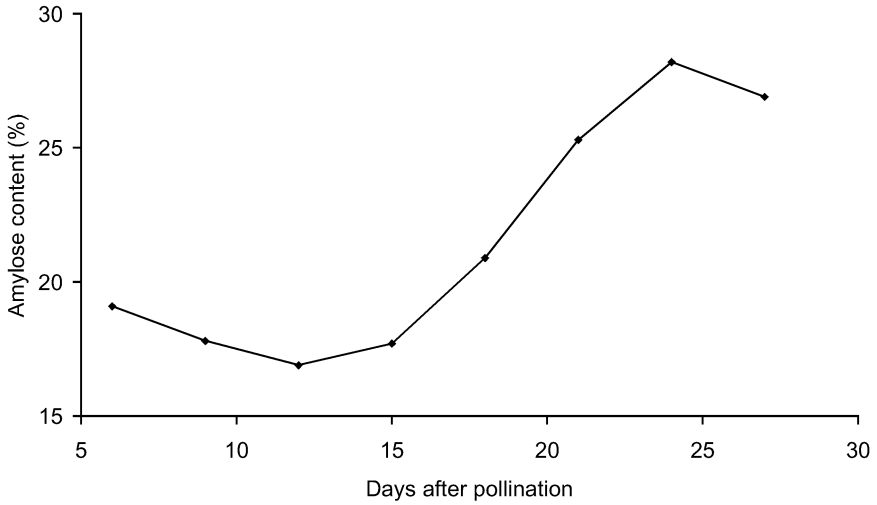

Figure 10. Amylose content in starch isolated from barley endosperm during seed development.

maturity. This suggests that the distribution of the short amylopectin chains (dp 6-30) examined in this study did not change significantly during barley seed development. Previous studies of developing seeds have shown similar results with increasing amylose contents and unaffected amylopectin chain length profiles in barley (Kang *et al*., 1985), rice (Asaoka *et al*., 1985), and pea starch (Biliaderis, 1982). Even though the expression of SBEI and the amylose content seemed to follow a similar time course, the enzyme expression does not necessarily have to correlate with enzyme activity. Furthermore, a previous study

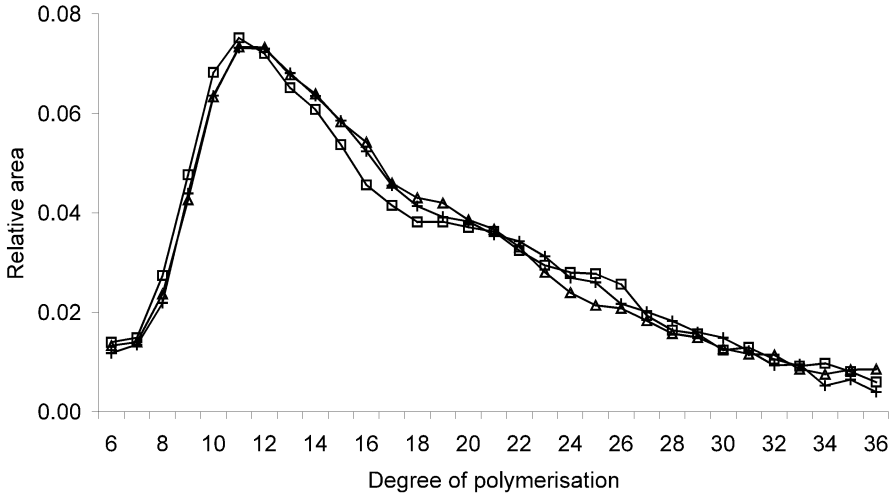

Figure 11. HPAEC chain length distribution profiles of samples harvested 12 (-□-), 21(-+-) and 27 (-Δ-) days after pollination.

in potato has shown that the down-regulation of SBEI has no detectable effect on amylopectin chain length distribution or on amylose content (Safford *et al*., 1998). Other factors, such as the expression and properties of other starch-synthesising enzymes or the availability of ADP-glucose, probably have a greater impact in determining the relative rates of amylose and amylopectin synthesis (Denyer *et al*., 2001).

Characterisation of starch from inner and outer parts of the barley kernel

In a previous study where grains of the waxy barley cultivar SW 7142-92 were examined by microscopy, the amylose seemed unevenly distributed within the kernels (Oscarsson *et al*., 1997). Starch granules in the subaleurone layer of these kernels stained black with iodine while granules in the inner parts of the kernel were mainly brown. Starch isolated from three pearling fractions representing outer, middle and inner parts of these waxy barley kernels was analysed with respect to amylose content and amylopectin structure (Paper V). A comparison was made with the corresponding starch samples isolated from a normal barley variety (Golf). Apparent and total amylose contents, which are analysed before and after the removal of lipids, were measured using a colorimetric method with iodine. In waxy barley, the amylose content decreased from the surface to the centre of the kernel, with total amylose contents of 8.6%, 5.9% and 2.2% in outer, middle and inner fractions respectively (Table 3). Normal barley starch had similar amylose contents in all fractions (24.0-25.2%). The lipid-complexed amylose was calculated as the difference between total and apparent amylose. The amount of lipid-complexed amylose correlates positively with the amylose content (Morrison, 1993b) and both waxy and normal starch showed slightly higher contents of lipid-complexed amylose in the outer fractions. However, lipid-complexed amylose made up a higher proportion of the total amylose in waxy barley than in normal barley. When the amylose content was analysed by GPC, similar results were obtained. The normal barley starch fractions had amylose contents of 28-29%. The innermost fraction of waxy barley had an amylose

Table 3. Content of total and lipid-complexed amylose in starch isolated from different parts of waxy and normal barley kernels. Expressed as percentage of starch (dry weight)

Sample	Starch fraction	Total amylose	Lipid-complexed amylose
Waxy starch	Outer	8.6	4.2
	Middle	5.9	3.7
	Inner	2.2	1.7
Normal starch	Outer	24.0	7.9
	Middle	25.2	5.8
	Inner	24.7	5.4

content of 5%, whereas the middle and outer fraction both had amylose contents of 12%. The high amylose value obtained for the middle fraction was attributable to some undefined material eluting between the void and the amylopectin peaks, and thereby being included in the amylose fraction. The higher amylose content in the surface layer of waxy barley grain has very recently been confirmed in a different barley cultivar (Tang *et al*., 2001b), while similar amylose levels were found in all parts of normal barley grain (Tang *et a*l., 2001a).

The chain length distributions of amylopectins isolated from the different starch fractions were analysed by HPAEC and size exclusion chromatography. A peak maximum at dp 11-12 and a shoulder around dp 19 were observed in the HPAEC chromatograms, which were similar to elution profiles reported by Silverio *et al*. (2000). Both methods gave similar chain length profiles for amylopectin isolated from the inner and peripheral parts of the kernels (Table III in Paper V).

These results indicate that the expression of starch biosynthetic enzymes and the regulation of starch synthesis vary not only in different genotypes or during different stages of development but also within different parts of the endosperm. How these results correspond with the specific expression of individual starch biosynthetic enzymes has yet to be revealed.

Conclusions

- Linear dextrins with a relatively narrow molecular weight distribution were prepared. These linear dextrins were fractionated by ethanol precipitation and a high molecular weight fraction was collected. The linear dextrins and the dextrin fraction could be made on a relatively large scale and were found to be suitable as substrates in *in vitro* studies of SBEI and SBEII from potato.

- The *in vitro* enzyme studies showed that SBEI and SBEII from potato differed in their substrate preferences. The enzyme products made by each SBE isoform displayed clearly different chain length patterns, which were visible already after very short incubation times. However, both enzyme products displayed a similar degree of branching.

- In developing barley endosperm, the amylose content analysed between 6 to 27 days after pollination (d.a.p.) increased from around 15 d.a.p. The expression of the gene encoding for barley SBEI followed a similar time course. Amylopectin chain length distribution did not change during endosperm development.

- In mature waxy barley, there was a higher amylose content in starch isolated from the outer part of the endosperm than in starch from the inner part, whereas the amylopectin structure did not differ between fractions. In the normal barley variety, however, the amylose content was similar in all fractions. This suggested that starch biosynthetic enzymes were differentially expressed in different parts of the waxy barley endosperm.

References

Aime, D.B., Arntfield, S.D., Malcolmson, L.J. and Ryland, D. 2001. Textural analysis of fat reduced vanilla ice cream products. *Food Research International*, 34, 237-246.

Asaoka, M., Okuno, K., Sugimoto, Y. and Fuwa, H. 1985. Developmental changes in the structure of endosperm starch of rice (*Oryza sativa* L.). *Agricultural and Biological Chemistry*, 49, 1973-1978.

De Baere, H. 1999. Starch policy in the European Community. *Starch/Stärke*, 51, 189-193.

Baba, T. and Arai, Y. 1984. Structural characterization of amylopectin and intermediate material in amylomaize starch granules. *Agricultural and Biological Chemistry*, 48, 1763-1775.

Baker, A.A., Miles, M.J. and Helbert, W. 2001. Internal structure of the starch granule revealed by AFM. *Carbohydrate Research*, 330, 249-256.

Baldwin, P.M., Adler, J., Davies, M.C. and Melia, C.D. 1998. High resolution imaging of starch granule surfaces by atomic force microscopy. *Journal of Cereal Science*, 27, 255-265.

Ball, S., Guan, H.P., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P. and Preiss, J. 1996. From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. *Cell*, 86, 349-352.

Ball, S.G., van de Wal, M.H.B.J. and Visser, R.G.F. 1998. Progress in understanding the biosynthesis of amylose. *Trends in Plant Science*, 3, 462-467.

Biliaderis, C.G. 1982. Characteristics of starch in developing pea seeds. *Phytochemistry*, 21, 37-39.

Blennow, A. and Johansson, G. 1991. Isolation of a Q-enzyme with M_r 103 000 from potato tubers. *Phytochemistry*, 30, 437-444.

Bligh, F.J. 1999. Genetic manipulation of starch biosynthesis: Progress and potential. *Biotechnology and Genetic Engineering Reviews*, 16, 177-201.

Borovsky, D., Smith, E.E. and Whelan, W.J. 1975. Purification and properties of potato 1,4-α-D-glucan : 1,4-α-D-glucan 6-α-(1,4-α-glucano)-transferase. *European Journal of Biochemistry*, 59, 615-625.

Boyer, C.D. and Preiss, J. 1978. Multiple forms of (1→4)-α-D-glucan, (1→4)-α-D-glucan-6-glycosyl transferase from developing *Zea mays* L. kernels. *Carbohydrate Research*, 61, 321-334.

Buléon, A., Colonna, P., Planchot, V. and Ball, S. 1998. Starch granules: structure and biosynthesis. *International Journal of Biological Macromolecules*, 23, 85-112.

Burton, R.A., Bewley, J.D., Smith, A.M., Bhattacharyya, M.K., Tatge, H., Ring, S., Bull, V., Hamilton, W.D.O. and Martin, C. 1995. Starch branching enzymes belonging to distinct enzyme families are differently expressed during pea embryo development. *Plant Journal*, 7, 3-15.

Cairns, P., Morris, V.J, Botham, R.L. and Ring, S.G. 1996. Physicochemical studies on resistant starch *in vitro* and *in vivo*. *Journal of Cereal Science*, 23, 265-275.

Critchley, J.H., Zeeman, S.C., Takaha, T., Smith, A.M. and Smith, S.M. 2001. A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in *Arabidopsis*. *Plant Journal*, 26, 89-100.

Denyer, K., Johnson, P., Zeeman, S. and Smith, A.M. 2001. The control of amylose synthesis. *Journal of Plant Physiology*, 158, 479-487.

Edwards, A., Marshall, J., Denyer, K., Sidebottom, C., Visser, R.G., Martin, C. and Smith, A.M. 1996. Evidence that a 77-kilodalton protein from the starch of pea embryos is an isoform of starch synthase that is both soluble and granule-bound. *Plant Physiology*, 112, 89-97.

Ellis, R.P., Cochrane, M.P., Dale, M.F.B., Duffus, C.M., Lynn, A., Morrison, I.M., Prentice, R.D.M., Swanston, J.S. and Tiller, S.A. 1998. Starch production and industrial use. *Journal of the Science of Food and Agriculture*, 77, 289-311.

Erlander, S. 1958. Proposed mechanism for the synthesis of starch by glycogen. *Enzymologia*, 19, 273-283.

Everett, W.W. and Foster, J.F. 1959. The subfractionation of amylose and characterization of the subfractions by light scattering. *Journal of the American Chemical Society*, 81, 3459-3464.

FAO Statistical Data. *Http://apps.faostat.org:* Accessed 5 July 2001.

French, D. 1972. Fine structure of starch and its relationship to the organization of starch granules. *Denpun Kagaku*, 19, 8-25.

Gallant, D.J., Bouchet, B., Buléon, A. and Peréz, S. 1992. Physical characteristics of starch granules and susceptibility to enzymatic degradation. *European Journal of Clinical Nutrition*, 46, S3-S16.

Gallant, D.J., Bouchet, B. and Baldwin, P.M. 1997. Microscopy of starch: Evidence of a new level of granule organization. *Carbohydrate Polymers*, 32, 177-191.

Gidley, M.J., Cooke, D., Darke, A.H., Hoffmann, R.A., Russell, A.L. and Greenwell, P. 1995. Molecular order and structure in enzyme-resistant retrograded starch. *Carbohydrate Polymers*, 28, 23-31.

Gordon, I. 1999. Starches from differing sources – supply, demand, price formation. *Starch/Stärke*, 51, 193-196.

Guan, H.P. and Preiss, J. 1993. Differentiation of the properties of the branching isozymes from maize (*Zea mays*). *Plant Physiology*, 102, 1269-1273.

Guan, H., Kuriki, T., Sivak, M. and Preiss, J. 1995. Maize branching enzyme catalyzes synthesis of glycogen-like polysaccharide in glgB-deficient *E. coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 964-967.

Haworth, W.N., Peat, S. and Bourne, E.J. 1944. Synthesis of amylopectin. *Nature*, 154, 236.

Heyer, A.G., Lloyd, J.R. and Kossmann, J. 1999. Production of modified polymeric carbohydrates. *Current Opinion in Biotechnology*, 10, 169-174.

Hizukuri, S. 1985. Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. *Carbohydrate Research*, 141, 295-306.

Hizukuri, S. 1986. Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research*, 147, 342-347.

Hizukuri, S. 1996. Starch: Analytical Aspects. In *Carbohydrates in Food*. Edited by A.-C. Eliasson. Food Science and Technology series. Marcel Dekker Inc., New York, USA, pp 347-429.

Hizukuri, S., Tabata, S. and Nikuni, Z. 1970. Studies on starch phosphate. Part 1. Estimation of glucose 6-phosphate residues in starch and the presence of other bound phosphates. *Starch/ Stärke*, 22, 338-343.

Hoover, R. 1998. Starch-lipid interactions. In *Polysaccharide Association Structures in Food*. Edited by R.H Walter. Marcel Dekker Inc., New York, pp 227-256.

Hovenkamp-Hermelink, J.H.M., Jacobsen, E., Ponstein, A.S., Visser, R.G.F., Vos-Scheperkeuter, G.H., Bijmolt, E.W., de Vries, J.N., Witholt, B. and Feenstra, W.J. 1987. Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). *Theoretical and Applied Genetics*, 75, 217-221.

Hovenkamp-Hermelink, J.H.M., de Vries, J.N., Adamse, P., Jacobsen, E., Witholt, B. and Feenstra, W.J. 1988. Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue of the potato. *Potato Research*, 31, 241-246.

Imberty, A. and Pérez, S. 1988. A revisit to the three-dimensional structure of B-type starch. *Biopolymers*, 27, 1205-1221.

Imberty, A., Chanzy, H., Perez, S., Buléon, A. and Tran, V. 1988. The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology*, 201, 365-378.

Jakobsson, B. 2001. Lyckeby Stärkelsen. Personal communication.
Jane, J-L. and Robyt, J.F. 1984. Structure studies of amylose V-complexes and retrograded amylose by action of alpha amylases, and a new method for preparing amylodextrins. *Carbohydrate Research*, 132, 105-118.
Jane, J., Kasemsuwan, T., Leas, S., Zobel, H. and Robyt, J.F. 1994. Anthology of starch granule morphology by scanning electron microscopy. *Starch/Stärke*, 46, 121-129.
Jane, J., Kasemsuwan, T. and Chen, J.F. 1996. Phosphorus in rice and other starches. *Cereal Foods World*, 41, 827-832.
Jane, J., Chen, Y.Y., Lee, L.F., McPherson, A.E., Wong, K.S., Radosavljevic, M. and Kasemsuwan, T. 1999. Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry*, 76, 629-637.
Jenkins, P.J., Cameron, R.E. and Donald, A.M. 1993. A universal feature in the structure of starch granules from different botanical sources. *Starch/Stärke*, 45, 417-420.
Jespersen, H.M., MacGregor, E.A., Henrissat, B., Sierks, M.R. and Svensson, B. 1993. Starch- and glycogen-debranching and branching enzymes: Prediction of structural features of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. *Journal of Protein Chemistry*, 12 (6), 791-805.
Jobling, S.A., Schwall, G.P., Westcott, R.J., Sidebottom, C.M., Debet, M., Gidley, M.J., Jeffcoat, R. and Safford, R. 1999. A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: Cloning and characterisation of multiple forms of SBE A. *Plant Journal*, 18, 163-171.
Kang, M.Y., Sugimoto, Y., Sakamoto, S. and Fuwa, H. 1985. Developmental changes in the amylose content of endosperm starch of barley (*Hordeum vulgare*) during the grain filling period after anthesis. *Agricultural and Biological Chemistry*, 49, 3463-3466.
Khoshnoodi, J. 1997. Starch branching enzymes in *Solanum tuberosum* and *Arabidopsis thaliana*. Doctoral thesis, *Swedish University of Agricultural Sciences*, Uppsala, Sweden.
Khoshnoodi, J., Blennow, A., Ek, B., Rask, L. and Larsson, H. 1996. The multiple forms of starch-branching enzyme I in *Solanum tuberosum*. *European Journal of Biochemistry*, 242, 148-155.
Kleczkowski, L.A. 1999. A phosphoglycerate to inorganic phosphate ratio is the major factor in controlling starch levels in chloroplasts via ADP-glucose pyrophosphorylase regulation. *FEBS Letters*, 448, 153-156.
Kleczkowski, L.A., Villand, P., Lüthi, E., Olsen, O.-A. and Preiss, J. 1993. Insensitivity of barley endosperm ADPglucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation. *Plant Physiology*, 101, 179-186.
Kosegarten, H. and Mengel, K. 1998. Starch deposition in storage organs and the importance of nutrients and external factors. *Zeitschrift für Pflanzenernährung und Bodenkunde*, 161, 273-287.
Kossmann, J. and Lloyd, J. 2000. Understanding and influencing starch biochemistry. *Critical Reviews in Plant Science*, 19, 171-226.
Krochta, J.M. and De Mulder-Johnston, C. 1997. Edible and biodegradable polymer films: Challenges and opportunities. *Food Technology*, 51, 61-74.
Larsson, C.-T. 1999. Studies of starch branching enzyme A from potato (*Solanum tuberosum*). Doctoral thesis, *Swedish University of Agricultural Sciences*, Uppsala, Sweden.
Larsson, C.-T., Hofvander, P., Khoshnoodi, J., Ek, B., Rask, L. and Larsson, H. 1996. Three isoforms of starch synthase and two isoforms of branching enzyme are present in potato tuber starch. *Plant Science*, 117, 9-16.
Larsson, C.-T., Khoshnoodi, J., Ek, B., Rask, L. and Larsson, H. 1998. Molecular cloning and characterisation of starch-branching enzyme II from potato. *Plant Molecular Biology*, 37, 505-511.

Leloir, L.F., de Fekete, M.A.R. and Cardini, C.E. 1961. Starch and oligosaccharide synthesis from uridine diphosphate glucose. *Journal of Biological Chemistry*, 236, 636-641.

Lim, S.-T., Kasemsuwan, T. and Jane, J. 1994. Characterization of phosphorus in starch by ^{31}P-nuclear magnetic resonance spectroscopy. *Cereal Chemistry*, 71, 488-493.

Lloyd, J.R., Hedley, C.L., Bull, V.J. and Ring, S.G. 1996. Determination of the effect of *r* and *rb* mutations on the structure of amylose and amylopectin in pea (*Pisum sativum* L.). *Carbohydrate Polymers*, 29, 45-49.

Lorberth, R., Ritte, G., Willmitzer, L. and Kossmann, J. 1998. Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Nature Biotechnology*, 16, 473-477.

Lyckeby Stärkelsen, Kristianstad, Sweden. *Http://www.lyckeby.com/Lyckeby/Svenska/index2.htm*: Accessed 5 July, 2001.

MacGregor, A.W. and Morgan, J.E. 1984. Structure of amylopectins isolated from large and small starch granules of normal and waxy barley. *Cereal Chemistry* 61, 222-228.

MacGregor, A.W. and Fincher, G.B. 1993. Carbohydrates of the barley grain. In *Barley: Chemistry and Technology*. Edited by A.W. MacGregor and R.S. Bhatty. American Association of Cereal Chemists Inc., St Paul, pp 73-130.

Manners, D.J. 1989. Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers*, 11, 87-112.

Mizuno, K., Kimura, K., Arai, Y., Kawasaki, T., Shimada, H. and Baba, T. 1992. Starch branching enzymes from immature rice seeds. *Journal of Biochemistry*, 112, 643-651.

Morell, M.K., Blennow, A., Kosar-Hashemi, B. and Samuel, M.S. 1997. Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. *Plant Physiology*, 113, 201-208.

Morrison, W.R. 1993a. Cereal starch granule development and composition. In *Seed Storage Compounds*. Edited by P.R. Shewry, and K. Stobart. Clarendon Press, Oxford, pp 175-190.

Morrison, W.R. 1993b. Barley lipids. In *Barley: Chemistry and Technology*. Edited by A.W. MacGregor and R.S. Bhatty. American Association of Cereal Chemists, Inc., St. Paul, pp. 199-236.

Morrison, W.R. 1995. Starch lipids and how they relate to starch granule structure and functionality. *Cereal Foods World*, 40, 437-446.

Morrison, W.R. and Laignelet, B. 1983. An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. *Journal of Cereal Science*, 1, 9-20.

Morrison, W.R., Milligan, T.P. and Azudin, M.N. 1984. A relationship between the amylose and lipid contents of starches from diploid cereals. *Journal of Cereal Science*, 2, 257-271.

Morrison, W. R., Scott, D. C. and Karkalas, J. 1986. Variation in the composition and physical properties of barley starches. *Starch/ Stärke*, 38, 374-379.

Morrison, W.R., Tester, R.F., Snape, C.E., Law, R. and Gidley, M.J. 1993. Swelling and gelatinisation of cereal starches. IV. Some effects of lipid-complexed amylose and free amylose in waxy and normal barley starches. *Cereal Chemistry*, 70, 385-391.

Mouille, G., Maddelein, M.-L., Libessart, N., Talage, P., Decq, A., Delrue, B. and Ball, S. 1996. Preamylopectin processing: A mandatory step for starch biosynthesis in plants. *Plant Cell*, 8, 1353-1366.

Myers, A.M., Morell, M.K., James, M.G. and Ball, S.G. 2000. Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiology*, 122, 989-997.

Nakamura,Y. 1996. Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Science*, 121, 1-18.

National starch and Chemical Company, Bridgewater, USA. *Http://www.eco-foam.com*: Accessed 5 July, 2001.

National starch and Chemical Company, Bridgewater, USA. *Http://www.excipients.com*: Accessed 5 July, 2001.
Nelson, O.E. and Rines, H.W. 1962. The enzymatic deficiency in the waxy mutant of maize. *Biochemical and Biophysical Research Communications*, 9, 297-300.
Nikuni, J. 1969. Starch and cooking. *Chori Kagaku*, 2, 6-14.
Oscarsson, M., Parkkonen, K., Autio, K. and Åman, P. 1997. Composition and microstructure of waxy, normal and high amylose barley samples. *Journal of Cereal Science*, 26, 259-264.
Pan, D. and Nelson, O.E. 1984. A debranching enzyme deficiency in endosperms of the *sugary-1* mutants of maize. *Plant Physiology*, 74, 324-328.
Peat, S., Whelan, W.J. and Thomas, G.J. 1952. Evidence of multiple branching in waxy maize starch. *Journal of the Chemical Society*, 4546-4548.
Praznik, W., Rammesmayer, G., Spies, T. and Huber, A. 1992. Characterisation of the (1→4)-α-D-glucan-branching 6-glycosyltransferase by *in vitro* synthesis of branched starch polysaccharides. *Carbohydrate Research*., 227, 171-182.
Preiss, J. 1982. Regulation of the biosynthesis and degradation of starch. *Annual Review of Plant Physiology*, 54, 431-454.
Ritte, G., Eckermann, N., Haebel, S., Lorberth, R. and Steup, M. 2000. Compartmentation of the starch-related R1 protein in higher plants. *Starch/Stärke*, 52, 179-185.
Rohde, W., Becker, D. and Salamini, F. 1998. Structural analysis of the waxy locus from *Hordeum vulgare*. *Nucleic Acids Research*, 16, 7185-7186.
Russell, P.L., Berry, C.S. and Greenwell, P. 1989. Characterisation of resistant starch from wheat and maize. *Journal of Cereal Science*, 9, 1-15.
Safford, R., Jobling, S.A., Sidebottom, C.M., Westcott, R.J., Cooke, D., Tober, K.J., Strongitharm, B.H., Russell, A.L. and Gidley, M.J. 1998. Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch. *Carbohydrate Polymers*, 35, 155-168.
Salomonsson, A.-C. and Sundberg, B. 1994. Amylose content and chain profile of amylopectin from normal, high amylose and waxy barleys. *Starch/Stärke*, 46, 325-328.
Schwall, G.P., Safford, R., Westcott, R.J., Jeffcoat, R., Tayal, A., Shi, Y-C., Gidley, M.J. and Jobling, S.A. 2000. Production of very-high-amylose potato starch by inhibition of SBE A and B. *Nature Biotechnology*, 18, 551-554.
Shinsato, E., Hippleheuser, A.L., and Van Beirendonck, K. 1999. Products for batter and coating systems. *The World of Ingredients*, Jan-Feb, 38-42.
Siljeström, M., Eliasson, A.-C. and Björck, I. 1989. Characterisation of resistant starch from autoclaved wheat starch. *Starch/Stärke,* 41, 147-151.
Silverio, J., Fredriksson, H., Andersson, R., Eliasson, A.-C. and Åman, P. 2000. The effect of temperature cycling on the amylopectin retrogradation of starches with different amylopectin unit-chain length distribution. *Carbohydrate Polymers*, 42, 175-184.
Sivak, M.N. and Preiss, J. 1998. In *Starch: Basic Science to Biotechnology*. Adv Food Nutr Res, 41, Academic Press, San Diego, pp 33-161.
Slattery, C.J., Kawakli, I.H. and Okita, T.W. 2000. Engineering starch for increased quantity and quality. *Trends in Plant Science*, 5, 291-298.
Smith, A.M. 1988. Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.). *Planta*, 175, 270-279.
Smith, A.M. 1999. Making starch. *Current Opinion in Plant Biology*, 2, 223-229.
Smith, A.M., Denyer, K. and Martin, C. 1997. The synthesis of the starch granule. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48, 67-87.
Sun, C., Sathish, P., Ahlandsberg, S., Deiber, A. and Jansson, C. 1997. Identification of four starch-branching enzymes in barley endosperm: Partial purification of forms I, IIa and IIb. *New Phytology*, 137, 215-222.

Sun, C., Sathish, P., Ahlandsberg, S. and Jansson, C. 1998. The two genes encoding starch branching enzymes IIa and IIb are differentially expressed in barley. *Plant Physiology*, 118, 37-49.

Tabata, S. and Hizukuri, S. 1971. Studies on starch phosphate. Part 2. Isolation of glucose 3-phosphate and maltose phosphate by acid hydrolysis of potato starch. *Starch/Stärke*, 23, 267-272.

Takaha, T., Yanase, M., Okada, S. and Smith, S.M. 1993. Disproportionating enzyme (4-α-glucanotransferase; EC 2.4.1.25) of potato. Purification, molecular cloning, and potential role in starch metabolism. *Journal of Biological Chemistry*, 268, 1391-1396.

Takaha, T., Critchley, J., Okada, S. and Smith, S.M. 1998. Normal starch content and composition in tubers of antisense potato plants lacking D-enzyme (4-α-glucanotransferase). *Planta*, 205, 445-451.

Takeda, Y. and Hizukuri, S. 1987. Structures of rice amylopectins with low and high affinities for iodine. *Carbohydrate Research*, 168, 79-88.

Takeda, Y., Maruta, N. and Hizukuri, S. 1992. Structures of amylose subfractions with different molecular sizes. *Carbohydrate Research*, 226, 279-285.

Takeda, Y., Guan, H-P. and Preiss, J. 1993. Branching of amylose by the branching isoenzymes of maize endosperm. *Carbohydrate research*, 240, 253-263.

Tang, H., Ando, H., Watanabe, K., Takeda, Y. and Mitsunaga, T. 2001a. Physicochemical properties and structure of large, medium and small granule starches in fractions of normal barley endosperm. *Carbohydrate Research*, 330, 241-248.

Tang, H., Ando, H., Watanabe, K., Takeda, Y. and Mitsunaga, T. 2001b. Fine structures of amylose and amylopectin from large, medium, and small waxy barley starch granules. *Cereal Chemistry*, 78, 111-115.

Tomlinson, K.L., Lloyd, J.R. and Smith, A.M. 1997. Importance of isoforms of starch-branching enzyme in determining the structure of starch in pea leaves. *Plant Journal*, 11, 31-43.

Vasanthan, T. and Bhatty, R.S. 1996. Physicochemical properties of small- and large-granule starches of waxy, regular, and high-amylose barleys. *Cereal Chemistry*, 73, 199-207.

Vos-Scheperkeuter, G.H., de Wit, J.G., Ponstein, A.S., Feenstra, W.J. and Witholt, B. 1989. Immunological comparison of the starch branching enzymes from potato tubers and maize kernels. *Plant Physiology*, 90, 75-84.

Yamaguchi, M., Kainuma, K. and French, D. 1979. Electron microscopy observations of waxy maize starch. *Journal of Ultrastructure Research*, 69, 249-261.

Young, A.H. 1984. Fractionation of starch. In *Starch: Chemistry and Technology*. 2nd Edition. Edited by R.L. Whistler, J.N. Bemiller and E.F. Paschall. Academic Press Inc., Orlando, pp 249-283.

Zeeman, S.C., Umemoto, T., Lue, W.-L., Au-Yeung, P., Martin, C., Smith, A.M. and Chen, J. 1998. A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell*, 10, 1699-1711.

Zobel, H.F. 1988. Starch crystal transformations and their industrial importance. *Starch/Stärke*, 40, 1-7.

Åman, P., Hesselman, K. and Tilly, A-C. 1985. The variation in chemical composition of Swedish barleys. *Journal of Cereal Science*, 3, 73-77.

Tack

En mängd personer har på olika sätt varit involverade i mitt liv under min doktorandtid, och några av dessa personer skulle jag särskilt vilja tacka.

Ett stort tack till:

- Mina handledare, Per och Roger, för att ni alltid har tid, för er entusiasm, och för ert stöd och uppmuntran under hela min doktorandtid.

- Mina närmaste samarbetspartners: Ulrika, för gott samarbete i vått och torrt, för trevliga lunchmöten och diskussioner om både det ena och det andra; Håkan, för gott samarbete, för din entusiasm och för att du alltid är full av nya idéer och uppslag.

- Mina medförfattare: Rolf, för den korta men spännande introduktionen till NMR-teknikens värld; Sathish, Sun, Staffan och Christer samt ovan nämnda personer för gott samarbete.

- Alla nuvarande och före detta kollegor på institutionen för livsmedelsvetenskap där jag tillbringat en stor del av min arbets- och "fritid" de senaste åren. Ett särskilt tack till alla trevliga rumskamrater (9 st, tror jag) som jag har haft under dessa år. Till Ann-Christine och Einar för att ni så gärna hjälpt till att lösa diverse praktiska problem. Till Gertrud, för att du så gärna delat med dig av köttavdelningens forskningsmaterial (mina katter avgudar dig!). Alastair, för att du har hjälpt till då mina kunskaper i det engelska språket satts på prov.

- Andrea, Kristin, Christine, Emma, Helena och Maria som under sina 3-veckorsprojekt lyckades åstadkomma så mycket i stärkelselabbet.

- Mina vänner på och utanför jobbet som förgyllt min arbets- och fritid: Susanne, Jin, samt ett stort tack till det gamla mat.-nat.-gänget för mångårig vänskap, roliga upptåg och fester.

- Till sist vill jag tacka min familj. Mamma Kerstin, för att du finns och för att du alltid ställer upp för mig. Min Kristoffer, för din omtanke, uppmuntran och stöd. Mina katter, Björn och Johnny, för att ni ser till att jag inte sover för länge på morgnarna.

- Dessutom, ett litet tack till Stiftelsen för strategisk forskning som finansierat detta projekt.