Characterisation of starches isolated from *Arracacha xanthorriza*, *Canna edulis* and *Oxalis tuberosa* and extracted from potato leaf

Stalin Santacruz
Department of Food Science
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2004

Acta Universitatis Agriculturae Sueciae
Agraria 486

ISSN 1401-6249
ISBN 91-576-6763-2

Tryck: SLU Service/Repro, Uppsala 2004

Abstract

Santacruz, S. 2004. Characterisation of starches isolated from *Arracacha xanthorriza*, *Canna edulis* and *Oxalis tuberosa* and extracted from potato leaf. Doctoral thesis, ISSN 1401-6249, ISBN 91-576-6763-2

Starches from *Arracacha xanthorriza*, *Canna edulis* and *Oxalis tuberosa* grown in the Andean region were characterised. All three starches revealed a B-type X-ray diffraction pattern. The amylose content determined by gel permeation chromatography was 4% for *A. xanthorriza*, 18% for *O. tuberosa*, and 24% for *C. edulis*. The complexation ability of the three starches with a surfactant was investigated, and the amylose content was found to be positively correlated with the enthalpy of the complex. The dynamic rheological behaviour of gels made with the three starches was studied in strain-sweep mode. *A. xanthorriza* starch formed gels which had a stable elastic modulus when stored for three days at 4 ºC. A decrease in pH from 6.5 to 4.0 resulted in a reduction of the elastic modulus for all three starches. The starch content in potato leaves collected at different times on a day and night in July varied between 2% and 13%, with a minimum in early morning and a maximum in late afternoon. Much lower starch content was found in leaves collected in August, when, the morning samples again had the lowest content. Isolated leaf starch granules had either an oval or a round shape with a size smaller than 5 μm. Dimethylsulphoxide-extracted and debranched starches contained small amounts of amylose and a material with intermediate chain length. Unit chains with less than 6 glucose residues were detected in isolated amylopectin. The afternoon samples showed a similar chain length distribution as reported for potato tuber starch, where the populations around DP 19 and DP>35 became significant during the day, whereas the short chains remained basically unchanged. Results from iodine staining indicated that amylose from the afternoon samples had a high proportion of shorter chains. Both leaf and tuber starches seemed to be composed only of amylose and amylopectin, with constant chain length distribution over their molecular weight ranges. The results indicate that the material with molecular weights between the pure amylopectin and amylose is a mixture of the two and not an intermediate material. Purified fragments of isolated leaf starch were analysed with ^{31}P-NMR and no signals corresponding to phosphate monoesters linked to glucose at the C_3 and/or C_6 positions were detected. The results show that starch in potato leaves does not contain any detectable amounts of phosphate monoesters.

Keywords: *Arracacha xanthorriza*, *Canna edulis*, *Oxalis tuberosa*, iodine-staining, leaf starch, characterisation, starch complex, starch-extraction, starch phosphate monoesters

Author's address: Stalin Santacruz, Department of Food Science, P.O. Box 7051, Swedish University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden.
e-mail address: Stalin.Santacruz@lmv.slu.se

Sammanfattning

Arracacha xanthoriza, *Canna edulis* och *Oxalis tuberosa* är tre stärkelserika växter som odlas i Anderna. Isolerad stärkelse från de tre växterna visade ett röntgendiffraktionsmönster av B-typ. Särkelse från *A. xanthorriza* innehöll endast 2 % amylos medan innehållet av amylos i de andra två stärkelserna var mer normalt. Stärkelsernas komplexbildning med polära lipider visade sig korrelera med amylosinnehållet. Dynamisk reologi visade att stärkelse från *C. edulis* bildade starkare geler än de andra två stärkelsetyperna. Stärkelse från *A. xanthorriza* hade en stabil elastisk modul under tre dagars lagring vid kylskåpstemperatur. En sänkning av pH från 6.5 till 4.0 under lagringen gav svagare geler. Stärkelsehalten varierade mellan 2 och 13% i potatisblad som samlats in under ett dygn i juli med aktiv fotosyntes. Halten var högst på eftermiddagen och lägst tidigt på morgonen. I blad som samlats in under ett dygn i agusti var halten låg. Stärkelsegranuler som isolerats från bladen i lågt utbyte hade en oval eller rund form och en storlek som var mindre än 5 µm. Mer än 80% av stärkelsen i bladen kunde extraheras med dimetylsulfoxid. Denna stärkelse hade ett lågt innehåll av amylos. Amylopektin med en hög andel långa enhetskedjor (15-20 glukosenheter och mer än 35 glukosenheter) bildades under dagen och utnyttjades under natten. Mycket korta enhetskedjor (mindre än 6 glukosenheter) kunde påvisas i amylopektinet. Gelfiltrering med jodfärgningsdetektion visade att amylopektinet från bladstärkelse hade en vid molekylviktsfördelning jämfört med amylopektin från potatisknölar. Amylosen i prover som samlats in under eftermiddagen hade en högre andel korta kedjor än i prover från morgonen. Både blad- och knölstärkelse från potatis tycks bara bestå av amylos och amylopektin med konstant kedjelängdsfördelning över hela sina molekylvikstområden. Resultaten tyder på att det material som har en molekylvikt mellan ren amylos och amylopektin är en blandning av de två och inte ett material med en annorlunda struktur. Renade fragment av extraherad bladstärkelse analyserades med fosfor-NMR. Inga signaler från fosfater som är esterbundena till stärkelse kunde påvisas. Resultaten tyder på att bladstärkelse från potatis, till skillnad från knölstärkelse, inte innehåller några påvisbara mängder av stärkelsebundet fosfat.

Resumen

El almidón es un material que tiene gran variedad de aplicaciones que van desde la industria alimenticia a la producción de papel y adhesivos. Solo una pequeña parte del almidón es usado en su estado original, la mayor parte es modificado mediante el uso de agentes químicos. La tendencia actual es la de consumir productos naturales, por lo tanto es interesante el estudio de nuevas fuentes de almidón que reemplacen los modificados químicamente. Es por ello que tres almidones provenientes de zanahoria blanca (*Arracacha xanthorriza*), achira (*Canna edulis*) y oca (*Oxalis tuberosa*), fueron estudiados. El almidón tiene dos components, amilosa y amilopectina. La cantidad de amilosa y amilopectina hacen que el arroz tenga diferente textura después de la cocción. Arroces con contenido bajo de amilosa son pegajosos y son preferidos en países asiáticos. En la mayor parte de América Latina, se prefieren arroces con alto contenido de amilosa que los hacen sueltos y firmes. El almidón de zanahoria blanca tiene un bajo contenido de amilosa comparado con achira y oca. La cocción del almidón da como resultado soluciones espesas (viscosas) que al ser enfriadas producen geles con diferente grado de firmeza. El almidón de achira produce geles más firmes que los almidones de oca y zanahoria blanca. Cuando los geles de almidón son almacenados en refrigeración, como en el caso de pudines, se producen cambios en la firmeza de los geles. Los geles de zanahoria blanca almacenados a refrigeración no reflejaron ningún cambio en su firmeza. Los geles de achira y oca en cambio presentaron un aumento en la firmeza después de un día de almacenamiento en refrigeración.

En la naturaleza, el almidón es producido durante el día en las hojas y transportado durante la noche para ser almacenado, en el caso del maíz, en los granos, en la papa en el tubérculo. Existen muchos factores responsables de la producción del almidón. Es posible modificar uno o más de estos factores para obtener almidones con diferentes aplicaciones en la industria. Sin embargo, el proceso de producción del almidón no está bien entendido y es por ello que en este trabajo se extrajo almidón de hojas de papa para su estudio. Los resultados indican que la producción de almidón es alta cuando los tubérculos de papa están aumentando de tamaño y se reduce cuando los tubérculos alcanzan su tamaño final. La amilopectina del almidón de las hojas es muy similar a la amilopectina del almidón del tubérculo, no así la amilosa que tiene diferente tamaño en las hojas y en el tubérculo.

El almidón contiene minerales en pequeñas cantidades, como por ejemplo fósforo. El fósforo hace posible que el almidón pueda ser utilizado en productos que se almacenan por largo tiempo sin que existan cambios en su textura. Este mineral no fue encontrado en el almidón de las hojas de papa.

El conocimiento obtenido acerca de cómo es producido el almidón en las hojas podría ser aplicado a los tubérculos con el fin de obtener almidones “modificados” en la planta en lugar de realizar modificaciones químicas o físicas.

Story of a man who loved a star

Day after day the plantation of potatoes was getting empty. Everything happened during the night. The owner tried to stay awake to catch the thieves, but the moment he closed his eyes, the thieves took the opportunity to steal the potatoes. However one night he pretended to fall sleep and when this happened, he could see a group of very shiny beings. He ran after them and caught one of them, a woman who was a star. She begged him for her freedom; however he did not listen to her and kept her in his house.

Once when he was away on the plantation she ran away to the sky. The farmer asked his friend, a condor, to take him up to the sky to look for her. There, he recognized his star, and asked her to come and stay with him.

Every night she went to shine up in the sky and every morning she came to be with him. They lived happily for years, and every time the man got older, she took him to the Lake of Time, where he became young again. However one morning the star did not come back. The farmer went together with the condor to look for her. They looked all over the sky to no avail. Finally the man came back to Earth, where he died of sorrow.

Some said that she did not love him any longer and that is why she left him. Others say that she did not want see him die. Still others said that the Sun and the Moon had forbidden that relationship and that the star listened to the Gods, but after some years she regretted this and went to look for the farmer. It was too late.

The "shooting stars" we see in the sky are not many, there is only one. It is the star who once felt the danger and the happiness of love but became afraid of it and ran away. Since then her body knows that she was born to be two and not one, and she is now trying to find her way back….

-- Eduardo Galeano –

A papá, mamá y mis hermanos

Contents

Appendix

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-V)

I. Santacruz, S., Koch, K., Svensson, E., Ruales, J. & Eliasson, A.-C. 2002. Three underutilised sources of starch from the Andean region in Ecuador. Part I. Physico-chemical characterization. *Carbohydrate Polymers 49*, 63-70.

II. Santacruz, S., Ruales, J. & Eliasson, A.-C. 2003. Three underutilised sources of starch from the Andean region in Ecuador. Part II. Rheological characterization. *Carbohydrate Polymers 51*, 85-92.

III. Santacruz, S., Koch, K., Andersson, R. & Åman, P. 2004. Characterisation of potato leaf starch. *Journal of Agricultural and Food Chemistry 52*, 1985-1989.

IV. Santacruz, S., Andersson, R. & Åman, P. 2004. Characterisation of potato leaf starch with iodine-staining. (*Submitted*)

V. Santacruz, S., Andersson, R. & Åman, P. 2004. On the presence of starch bound phosphate in potato leaf starch. *Carbohydrate Polymers. In press*.

Reprints and accepted papers were published with the permission of the publishers.

Authors' contribution to the papers

Planning, analysis and writing of Paper I and II were carried out by S. Santacruz under the supervision of A.-C. Eliasson and J. Ruales, with the exception of the analyses of amylopectin chain length distribution and amylose content which were carried out by K. Koch, and the X-ray diffraction, which was carried out by E. Svensson.

Planning, method development, analysis and writing of Papers III-V were carried out by S. Santacruz under the supervision of K. Koch, R. Andersson and P. Åman, with the exception of the collection and analyses of starch content in leaf samples, which were performed by K. Koch.

List of abbreviations

ADP	Adenosine diphosphate
D-enzyme	Disproportionating enzyme
DBE	Debranching enzyme
DMSO	Dimethylsulphoxide
DP	Degree of Polymerisation
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetra-acetic acid
G′	Elastic modulus
G″	Viscous modulus
GBSS	Granule bound starch synthase
GPC	Gel Permeation Chromatography
GWD	α-glucan water dikinase
HPAEC-PAD	High performance anion exchange chromatography with pulse amperometric detection
HPSEC	High performance size exclusion chromatography
^{31}P-NMR	Phosphorus Nuclear Magnetic Resonance
SDS	Sodium Dodecyl Sulphate
SBE	Starch branching enzyme
SS	Starch synthase
δ	Phase angle

Introduction

Background

Starch is the predominant carbohydrate reserve in many plants. It provides the main source of energy in the human diet and animal feed. Starch is a very useful raw material with a wide field of applications from gelling systems of foods to manufacturing of paper and adhesives (Swinkels, 1985; Ellis *et al*., 1998). It is currently used in the production of biodegradable packing materials (Shogren, Fanta & Doane, 1993; Riaz, 1999). Expansion of dietary and industrial uses of starch is creating an increased demand for new starches in the market place. This can be achieved by searching for unconventional sources of starch or by chemical or genetic modification of starch (Visser *et al*., 1997; Schwall *et al*., 2000; Slattery, Kavakli & Okita, 2000). In the present study the physico-chemical and rheological properties of three unconventional Andean starches isolated from *Arracacha xanthorriza*, *Canna edulis*, and *Oxalis tuberosa* were investigated.

One of the most abundant and universally distributed forms of storage polysaccharide is leaf starch, known also as transient starch (Buleón *et al*., 1998). Leaf starch is synthesised during the course of a single photoperiod rather than over days or weeks, as in storage organs. It is accumulated during the day in the green plant leaf cells and mobilised during the night to achieve a more or less constant supply of sucrose to the non-photosynthetic tissues. Relatively little is known about the starch accumulation-degradation process and the structure of leaf starch in different botanical sources (Smith *et al*., 2003). The possibility of designing starches for industrial purposes by using genetic manipulation of starch synthesizing enzymes in crop plants has led to an increased interest in the mechanisms by which starch granules are synthesised (Slattery, Kavakli & Okita, 2000; Kok-Jacon *et al.*, 2003). With the advances in genetic engineering technologies, it is now possible to modify starch biosynthesis *in planta*. However, starch biosynthesis is a very complicated and incompletely characterised process which requires further research (Davis *et al*., 2003).

To improve the understanding of the accumulation-degradation of leaf starch, potato leaves with different starch accumulation rates (morning and afternoon samples) were collected from plants at two different times during the growing season. Starch was extracted from potato leaves and its amylose and amylopectin were characterised and compared with those from potato tuber.

Starch structure and characteristics

Starch granule organisation is very complicated and depends strongly on the botanical origin. Starch exists naturally in the form of discrete granules within plant cells. The starch granule is mainly composed of amylose and amylopectin. Natural amylopectin crystallises during biosynthesis as A-, B- or C-type polymorph (Hizukuri, 1985; Zobel, 1988). The A polymorph is generally found in cereal starches, while the B polymorph is mostly found in tuber and root starches (Imberty *et al*., 1988). The C-type polymorph is a mixture of A- and B-type

starches and is found in legumes and some tropical tuber starches (Gidley & Bulpin, 1987).

The amylose content of starch can be determined by various methods that tend to produce differing results. The original colorimetric procedure (McCready & Hassid, 1943) has been modified by many workers in order to make it more accurate (Morrison & Laignelet, 1983; McGrane, Cornell & Rix, 1998). Gel permeation chromatography (GPC) is being widely used to estimate the amylose content (Torneport, Salomonsson & Theander, 1990; Fredriksson *et al*., 1998). The ability of the amylose to complex with lipids and the thermal transition of this complex offer a possibility for using differential scanning calorimetry (DSC) for amylose determinations (Kugimiya & Donovan, 1981). The content of amylose and amylopectin varies for the different starches. Normal, non-mutant starch contains 15-35% amylose and 65-85% amylopectin (Eliasson & Gudmundsson, 1996; Ball, van de Wal & Visser, 1998). Amylopectin is a highly branched molecule with a percentage of branching varying from 4.0 to 5.5 (Table 1). It is composed of linear α-(1,4)-linked glucose chains connected by α-(1,6)-linkages. Compared to amylopectin, amylose is a smaller molecule with an average molecular weight of 10^5-10^6 g mol^{-1}. Amylose has longer chains and a limited number of branch linkages (percentage of branching 0.2-1.0). The long chains of amylose have a high capacity to bind iodine in solution and this imparts a blue colour to amylose-containing starches when these are stained with iodine. Amylopectin has a much lower iodine-binding capacity and stains red-brown with iodine solution. Some starches contain dextrins that cannot be classified as either amylose or amylopectin. These dextrins are called 'intermediate material' and are usually found in starches with high amylose content (Banks, Greenwood & Muir, 1974; Baba & Arai, 1984; Bertoft, Qin & Manelius, 1993). They have characteristics that are between those of amylose and amylopectin, e.g. molecular weight, β-amylolysis limit and iodine binding (Klucinec & Thompson, 1998). The

Table 1. *Some characteristics of amylose and amylopectin.*

Property	Amylose	Amylopectin
General structure	Essentially unbranched	Branched
Number average unit chain length	100-550	18-25
Degree of polymerisation	700-6000	10^3-10^6
Weight average molecular weight (g mol^{-1})	10^5-10^6	10^7-10^9
A-chains/B-chains	--	1.0-1.5
λ_{max} (nm)	640-660 (blue)	530-550 (red-brown)
β-Amylolysis limit (%)	70-95	55-60
Branch (%)	0.2-1.0	4.0-5.5
Exterior chain length	--	13-16
Interior chain length	--	10-15

(Manners & Sturgeon, 1982; Hizukuri, 1993, 1996; Ball, van de Wal & Visser, 1998; Buleón *et al*., 1998; Yoo & Jane, 2002)

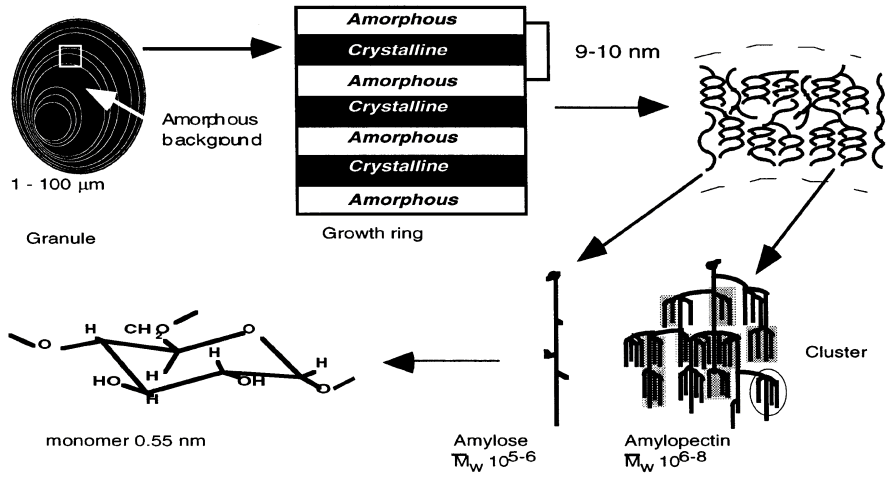

Fig. 1. Overview of the various levels of polymer organisation within the starch granule. Reprinted from Buleón *et al*. (1998) with permission from Elsevier.

intermediate material has a branching pattern more closely related to amylopectin than to amylose, but with a higher proportion of long chains and clusters of short chains (Bertoft, Qin & Manelius, 1993; Klucinec & Thompson, 1998).

Starch granule organisation

It is accepted that the amylopectin glucan chains are radially arranged in the granules and ordered in alternating crystalline and amorphous lamellae with a periodicity of 9 to 10 nm (Fig. 1). These arrays are the basis of the semi-crystalline nature of starch. In the crystalline lamellae the chains are associated in double helices and are packed together in an array to form clusters, while it is probable that the α-(1,6) branch points are within the amorphous lamellae. The alternating lamellae form concentric, semi-crystalline zones within the granule. These zones alternate, with a periodicity of a few hundreds of nanometres, with amorphous zones in which amylopectin molecules are in a less organised state and in which much of the amylose component of the granule is thought to occur (Ball, van de Wal & Visser, 1998; Thompson, 2000; Myers *et al*., 2000; Donald *et al*., 2001; Ridout *et al*., 2002). One repeat on this scale is known as a growth ring (Fig. 1). The organisation of amylopectin within the granule has been proposed to be into blocklets (Gallant, Bouchet & Baldwin, 1997), whereas studies by X-ray scatter and micro diffraction, and ^{13}C cross-polarisation magic angle spinning NMR, reveal that amylopectin may have the structure of a side-chain liquid crystalline polymer. If this is the case, it is expected that the organisation of the newly synthesised amylopectin chains that form a semi-crystalline matrix of the granule is primarily a physical rather than a biological process (Waigh *et al*., 2000).

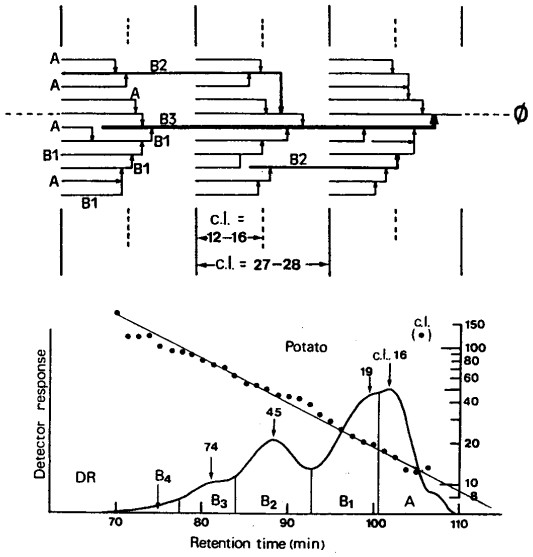

Fig. 2. Model of the cluster structure of amylopectin with chains from fractions A-B3 (upper) together with distribution profile from HPSEC of debranched potato amylopectin (lower). Reprinted from Hizukuri (1986) with permission from Elsevier.

Hizukuri (1986) suggested the arrangement of amylopectin chains into clusters, which are comprised of different classes of chains, often classified into A-, B- and C- chains (Fig. 2). A- chains are non-branched and are glucosidically linked to the C_6 on a B- chain. B- chains are linked in the same way but also carry one or more A- or B- chains, and the C- chains carry the reducing end group. The ratio of A- to B- chains is a parameter used when characterising the structures of different amylopectins (Table 1; Manners, 1989; Hizukuri, 1996). Debranched amylopectin can be analysed by chromatographic methods, e.g. high performance size exclusion chromatography, HPSEC, or high performance anion exchange chromatography, HPAEC (Koch, Andersson & Åman, 1998). High resolution with individual peaks for α-(1,4)-linked linear glucans with a DP 6 to 60 can be obtained by using HPAEC. Results from debranched amylopectin showed a polymodal distribution of chains (Hizukuri, 1986) with five populations (A, B1, B2, B3 and B4). The A fraction was proposed to contain the A- chains, while the B1, B2, B3 and B4 represent the B- chains that stretch across 1, 2, 3 and 4 clusters, respectively. The location of amylose within the amylopectin matrix of the granule is not entirely known. It is believed that amylose resides largely in the amorphous regions of the granule (Jenkins, Cameron & Donald, 1993; Gallant, Bouchet & Baldwin, 1997; Smith, 2001). However some amylose may also be present within the crystalline areas of the starch granules (Jenkins & Donald, 1995). Partial gelatinisation of potato starch granules showed that amylose has a higher concentration in the periphery of the granule and a smaller molecular size than amylose at the core (Jane & Shen, 1993). However studies of starch from potato tubers with low amylose content showed that amylose is located almost exclusively in the core of the granules (Kuipers, Jacobsen & Visser, 1994).

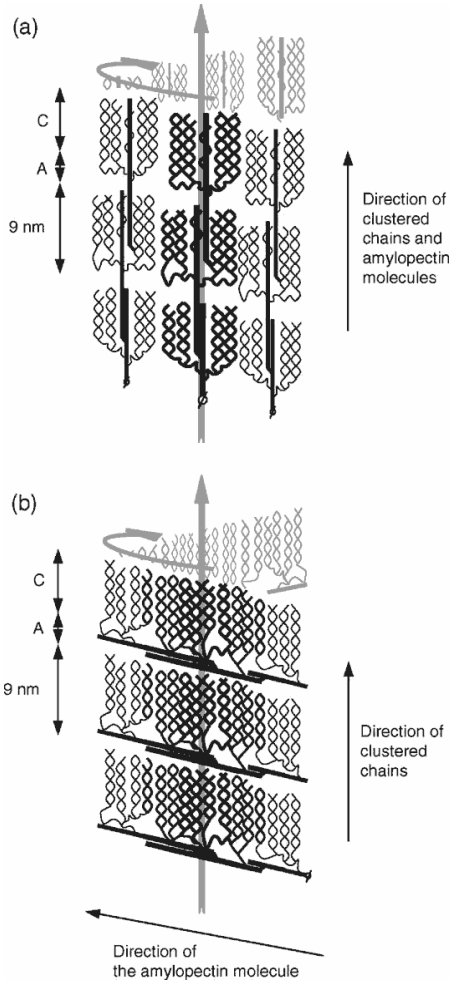

Fig. 3. The super-helix model of amylopectin proposed by Oostergetel & Bruggen (1993) and Waigh *et al.* (1999). (a) Based on the cluster model of Hizukuri (1986), (b) Based on a two-directional backbone model as proposed by Bertoft (2004). Reprinted from Bertoft (2004) with permission from Elsevier.

Oostergetel & van Bruggen (1993) and Waigh *et al*. (1999) proposed a super-helix model for amylopectin. The super-helix based on the cluster model of Hizukuri (1986) is a cooperative structure built up of several individual amylopectin molecules lined up close together, forming a left-handed crystalline lamella with an inner radius of 4 nm and an outer radius of 9 nm (Fig. 3a). The turns of the super-helix are built up of lamellar motifs. The amorphous lamellae between those motifs form a backbone for the double helices. Bertoft (2004) proposed a two-directional backbone model (Fig. 3b) that can be adapted to the super-helix. The new model differs from the cooperative alternative in that the backbone structure

is built of a single amylopectin molecule. The direction of the clustered chains is similar to the super-helix axis, but the direction of the amylopectin molecule follows the turns of the super-helix.

Minor components

Minor components of starch include proteins, lipids and minerals (Hizukuri, 1996; Buleón *et al*., 1998). Starch proteins can be classified as surface proteins which can be extracted in aqueous solutions, or as integral proteins, which are extractable only when a starch solution is heated to temperatures near the gelatinisation temperature (Morrison & Karkalas, 1990). Integral starch protein levels range from 0.06% in potato to 0.9% in fava bean (Eliasson & Gudmundsson, 1996).

Lipid levels are lower in tuber than in cereal starches. In tuber starches, lipids are only found on the granule surface, while starches from cereal endosperm have surface and integral lipids (Morrison, 1981, 1988; Davis *et al*., 2003). It is well established that polar lipids, e.g. monoglycerides and fatty acids, form a helical inclusion complex with the amylose molecule (Zobel, French & Hinkle, 1967; Biliaderis, Page & Maurice, 1986; Rutschman & Solms, 1990). The complex is thermally reversible and melts at 100-120 ºC. The complex formation depends on the length of the carbon chains of the lipid/surfactants, the degree of saturation of the hydrocarbon chain of the lipid, the presence of a hydrophilic polar group and the type of aggregate of polar lipids in wet systems (Krog & Nybo-Jensen, 1970; Eliasson & Krog, 1985). Lipids and surfactants are required to have a minimum of four to eight carbons in the chain, with an optimum between 12 and 18 carbons for efficient complex formation. The complex can be studied using DSC, which gives information on the melting temperatures and enthalpy of the melting complex. Since complex formation is an exothermic process that probably takes place during the gelatinisation, the enthalpy of the gelatinisation measured by DSC could be lower than it really is (Eliasson, 1986; Villwock *et al*., 1999).

Starch contains several different minerals in small amounts, but the most important mineral is phosphorus (Buleón *et al*., 1998). Organic phosphorus in starch exists in two major forms, starch phosphate monoesters and phospholipids. Most cereal starches contain phosphorus that is mainly in the form of phospholipids, whereas root and tuber starches contain phosphorus in the form of starch phosphate monoesters. These phosphate monoesters are covalently bound to starch (Lim, Kasemsuwan & Jane, 1994; Kasemsuwan & Jane, 1994) and starch phosphate monoesters in native potato starch are mainly found in the amylopectin (Jane, Kasemsuwan & Chen, 1996). ^{31}P-NMR spectroscopy has been applied for determination of the positions of the phosphate groups on potato amylopectin (Muhrbeck & Tellier, 1991; Frigård, 2002). The distribution of the phosphate monoester content on the C_6 of the glucose unit of potato starch has been reported to be 60-70%, on C_3 30-40% and trace on C_2 (Hizukuri, Tabata & Nikuni 1970; Lim & Seib, 1993; Blennow *et al*., 1998). Potato amylopectin contains one phosphate monoester group per 200-320 glucosyl residues (Takeda & Hizukuri, 1982; Muhrbeck & Tellier, 1991). Blennow *et al*. (2000a) have shown that the amorphous parts of the starch may contain between 68 and 92% of the total starch

phosphate content, depending on the botanical source. The phosphate groups are mainly located in the longer unit chains, 30-100 glucose units (Takeda & Hizukuri, 1982). A unit chain may contain one or more phosphate groups but no phosphate groups have been reported to be located on the non-reducing end or closer than nine glucosyl units from an α-1,6 branch point (Takeda & Hizukuri, 1981, 1982). However, Frigård (2002) found that phosphate groups are often located close to the branch point. Such differences may be attributed to different analytical techniques. Jane & Shen (1993) showed that phosphorous in potato starch is present densely in the granule core together with amylopectin. The high phosphate monoester content of potato starch confers enhanced paste clarity, water binding capacity, swelling power, high peak consistency, significant shear thinning and slow rate and extent of retrogradation (Kasemsuwan & Jane 1994; Lim, Kasemsuwan & Jane, 1994; Jane, Kasemsuwan, & Chen, 1996). The function of starch phosphate in the starch metabolic system is not clear but it has been shown that starch phosphorylation occurs concomitantly with starch biosynthesis (Nielsen *et al*., 1994).

Starch structure affects the phosphorylation process. First, phosphorylation of amylopectin but not amylose takes place, due to the fact that the enzyme responsible for the phosphorylation process does not accept amylose as a substrate. Second, phosphorylated chains in native starch are, on average, longer than unsubstituted chains, which fits well with the fact that elongated and branched glucans are much better substrates than branched short chain substrates (Blennow *et al*., 2002).

Starch biosynthesis

Elongation of the α-1,4-linked glucan

Glucose is transferred from ADP-glucose to the non-reducing end of a growing α-1,4-linked glucan, thus generating an extra glucose with simultaneous release of ADP. The enzymes responsible for the process is called starch synthases, SS (Fig. 4). To date, four classes of SS have been identified in higher plants. One class is exclusively granule-bound (GBSSI-types I and II), while the three other classes of starch synthases (I, II and III) may be located partially or entirely in the soluble phase (Edwards *et al*., 1996; Myers *et al*., 2000). GBSSI has been shown to be responsible for amylose biosynthesis in plant storage organs (Nelson & Rines, 1962), although a number of studies have shown that it incorporates glucose both in amylose and amylopectin. Studies of SS have demonstrated the key role of these enzymes in the determination of the structure of amylopectin (Davis *et al*., 2003).

Formation of the α-1,6 branch

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

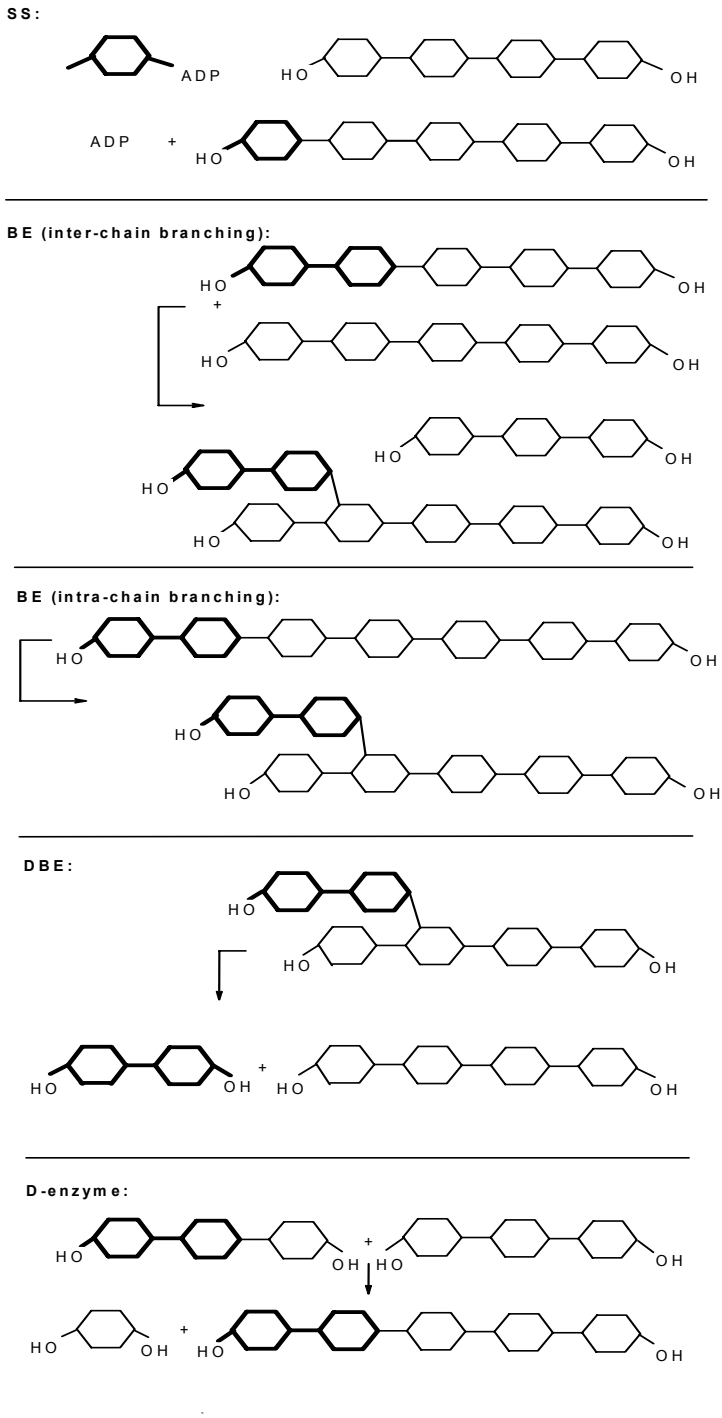

Fig. 4. Diagrammatic representation of the chemical reactions catalysed by enzymes involved in amylopectin biosynthesis. Reprinted from Myers *et al*. (2000) with kind permission from the American Society of Plant Biologists.

subsequent formation of an α-1,6-glucosidic bond between the cleaved chain and a C_6 hydroxyl group of a α-1,4-glucan (Fig. 4). The incorporation of glucans into starch may be inter- and intra-chain (Borovsky, Smith & Whelan, 1976; Rydberg *et al*., 2001; Andersson *et al.*, 2002). In all plants, there appears to be at least one isoform of branching enzyme belonging to two distinct families of enzyme. Type I SBE, which has been identified in maize, wheat, pea, potato, rice and cassava, prefers longer amylose-like chains and transfers longer chains. Type II SBE prefers amylopectin as an in vitro substrate and transfers short chains (Guan & Preiss, 1993; Rydberg *et al.*, 2001). SBE II has been identified in maize, wheat, barley, potato, pea and rice (Buleón *et al*., 1998; Davis *et al*., 2003). In addition, another SBE, named SBE Ic, was found in plant species exhibiting a bimodal starch granule size distribution, which suggests that it may have a role in the determination of starch granule size and morphology (Båga *et al*., 2000; Peng *et al*., 2000).

Other enzymes

Debranching enzymes (DBE) hydrolyse the α-1,6 glucan branches in amylopectin. Two classes of debranching enzymes, pullulanase and isoamylase (Doehlert & Knutson, 1991; Zhu *et al*., 1998), have been identified in plants. DBE is mainly associated with starch degradation, but it also seems to be necessary for normal starch biosynthesis since several mutants accumulate phytoglycogen in its absence (Ball *et al*., 1996; Zeeman *et al*., 1998a). Three isoforms of isoamylase have been identified in potato tubers (Hussain *et al*., 2003), Stisa1, Stisa2 and Stisa3. Stisa1 and Stisa2 are associated as a multimeric enzyme and are responsible for the removal of branched, soluble glucan during synthesis. Stisa3 has different substrate specificity and may play a more significant role in starch mobilisation than in starch synthesis.
The protein α-glucan water dikinase (GWD) is responsible for the phosphorylation at the C_3 and C_6 positions of glucose residues (Lorbert *et al*., 1998). GWD homologues recognised by potato GWD antibodies have been found in sweet potato, yam, seeds of maize and barley, and banana fruit (Ritte *et al*., 2000). This suggests that GWD has a general function in the plant. However some of these plants, e.g. maize, pea and barley, synthesise storage starch with low or not detectable phosphate content. Hence the presence of GWD does not necessarily result in significant starch phosphorylation (Blennow *et al*., 2002).

Several other enzymes have been implicated as being involved in starch biosynthesis, such as phosphorylases and the disproportionating enzyme (D-enzyme). D-enzyme catalyses the transfer of α-1,4-linked oligosaccharides from the end of one glucan chain to the end of another (Fig. 4). First, it may create longer glucans from short malto-oligosaccharides produced by other enzymes during starch degradation. By creating longer glucans from short malto-oligosaccharides, D-enzyme would increase the efficiency of glucan degradation by phosphorylase and β-amylase (Takaha *et al*., 1993; Smith *et al*., 2003; Wattebled *et al*., 2003). Second, D-enzyme might be involved in starch synthesis. In the pre-amylopectin model of synthesis of amylopectin it may act by recovering

malto-oligosaccharides produced during the maturation of amylopectin molecules and linking them back (Myers *et al.*, 2000; Wattebled *et al*., 2003).

Starch phosphorylases catalyse a reversible reaction where glucose 1-phosphate is liberated from (or incorporated into) a glucan chain at the non-reducing end. As this is a reversible reaction, it is possible for these enzymes to contribute both to the synthesis and degradation of starch (Kossman & Lloyd, 2000). However the degradative direction should be favoured (Preiss & Levi, 1980; Steup, 1988). Studies on potato tubers (Duwening *et al*., 1997) showed that starch phosphorylases may be involved in the elongation of specific glucans in the amylopectin molecule. However, another possible mechanism that has been suggested is that it may degrade starch during synthesis and produce low molecular weight primer oligosaccharides for starch synthase enzymes (Smith & Denyer, 1992; Zeeman *et al*., 1998a).

Models of starch synthesis

The pre-amylopectin trimming model (Fig. 5) proposes a direct involvement of DBE in amylopectin synthesis (Ball *et al*., 1996). It is envisaged that a sequence of synthetic events at the surface of the granule creates a cluster within an amylopectin molecule as follows: (1) short chains are elongated by starch synthase; (2) when chains reach a sufficient length to become substrates for SBE, a highly branched pre-amylopectin is formed; (3) selective trimming of this structure by DBE creates a bed of short chains from which the next round of synthesis can occur. Investigations performed in mutants of *Chlamydomonas* and *Arabidopsis* (Mouille *et al*., 1996; Zeeman *et al*., 1998a), which lack DBE, showed that the trimming model occurs but the process is more complex. According to the trimming model a lack of DBE would result in presence of phytoglycogen only (Myers *et al*., 2000). However phytoglycogen and normal amylopectin were present in these types of mutants (Ball *et al*., 1996).

The soluble glucan recycling model (Fig. 5) proposes that DBE is only indirectly involved in starch synthesis (Zeeman *et al*., 1998a). Amylopectin synthesis requires only starch synthase and starch-branching enzyme. Malto-oligosaccharides present in the soluble fraction of the amyloplast may be elongated by starch synthase and then branched by SBE. Any glucan thus synthesised will be degraded by a suite of enzymes including DBE, preventing the accumulation of such products. In a plastid in which DBE activity is reduced or eliminated, this degradative mechanism would be incomplete. Soluble branched glucans formed by starch synthase and SBE from malto-oligosaccharides tend to accumulate as phytoglycogen. The synthesis of these soluble glucans restricts the availability of enzyme and substrate for starch synthesis, reducing the rate of starch accumulation (Zeeman *et al*., 1998a). Both models are difficult to test and at present the question of whether DBE is directly involved in amylopectin synthesis remains open (Smith, 1999, 2001).

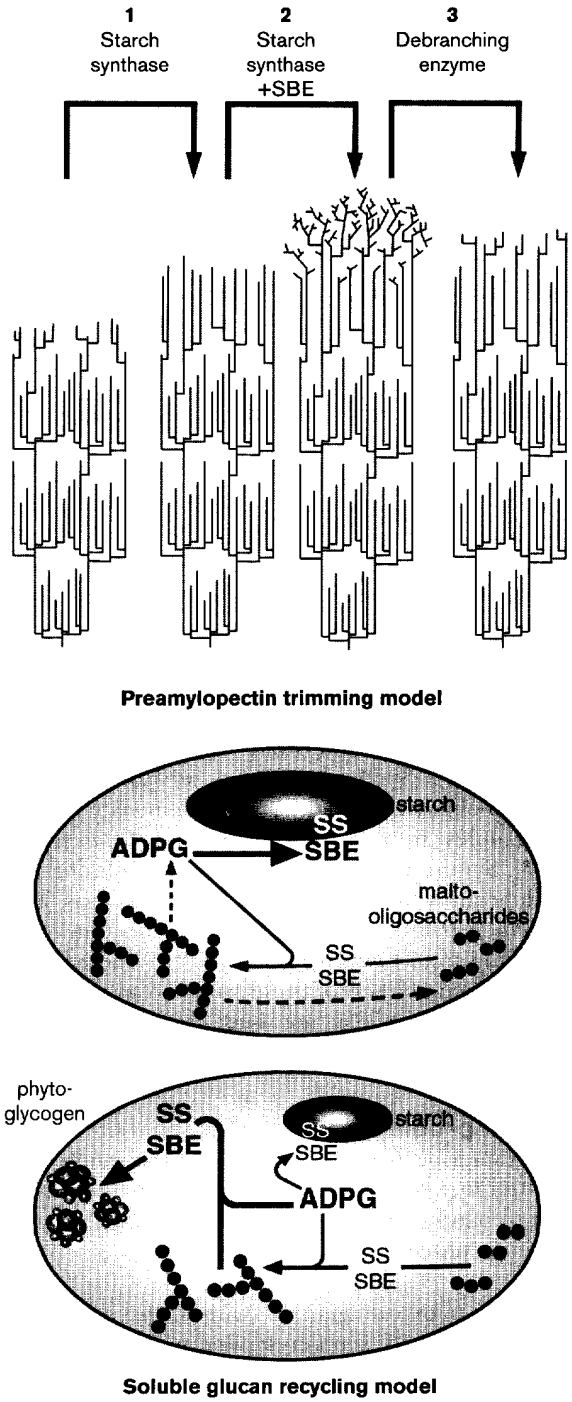

Fig. 5. Pre-amylopectin trimming model and soluble glucan recycling model of the synthesis of amylopectin. Reprinted from Smith (1999) with kind permission from Elsevier.

Three starch crops from the Andean region in Ecuador

Arracacha xanthorriza

A. xanthorriza grows between 1000 and 3100 metres above sea level, especially in the more humid valleys from Colombia to Bolivia (CONDESAN-CIP, 1997; Hermann, 1997). It is frequently grown with maize and beans, or underneath coffee plants in Colombia. It takes up to 10 months to reach maturity. *A. xanthorriza* incorporates some characteristics from both carrot and celery, being of the same family. The economically valuable part of the plant is a starchy storage root, with a starch content of 24% of root dry matter (Pereira, 1995). The starch extraction yields in cottage industries are as low as 24-50% of the starch (Hurtado *et al*., 1997). In general, root yields are below 20 tonnes per hectare, whereas potato yield in Sweden is around 40 tonnes per hectare. The young stems can be used in salads or as a cooked vegetable, and the leaves are often fed to livestock. There are two major drawbacks to *A. xanthorriza* use. Firstly, the roots have a short shelf-life, since they must reach consumers within a week of harvest. Secondly, the plants are highly susceptible to viruses. *A. xanthorriza* has been cultivated in southern Brazil for some 100 years, but over the past 40 years it has developed into a major horticultural operation providing income to thousands of farming families. The crop is currently planted on 12000 hectares and is one of the country's most highly prized vegetables. Farmers like *A. xanthorriza* because it needs only a fraction of the inputs required for potato, and because it produces a high return on investment. *A. xanthorriza* is a rare case of a legal, high-value cash crop that poor farmers can grow. Food companies process *A. xanthorriza* roots into a number of products such as baby food, chips, instant soups and pre-cooked roots.

Canna edulis

C edulis is grown from sea level up to 2000 metres a.s.l. *C. edulis* looks like a large-leaved lily and grows 2 m tall or more. It is commonly used as a living fence. *C. edulis* is a market vegetable from Mexico to Argentina, but only in Perú, Ecuador and Colombia is it a substantial crop. The average yields are between 22 and 50 tonnes per hectare (National Research Council, 1989; Hermann *et al.*, 1996). Its fleshy rhizomes, sometimes as long as an adult's forearm, can be cooked and eaten as a starch-rich food. However, the long cooking time that is required to soften the rhizome tissue limits direct consumption. Owing to its exceptionally large starch granules, which settle quickly out of a suspension of grated rhizome tissue, the starch can be extracted economically with home-made equipment, and cottage industries have been developed in some Andean locations. The starch is then sold for use in foods, *e.g.* manufacturing of pastries in Ecuador and biscuits in Colombia. In Vietnam, where its starch is used in making high-quality noodles, about 10000 hectares of *C. edulis* are grown, mostly in hilly areas (National Research Council, 1989; CONDESAN-CIP, 1997).

Oxalis tuberosa

A perennial herb that grows from Venezuela to Argentina, *O. tuberosa* grows between 2800 and 4000 metres a.s.l. Evidence from historical accounts indicates

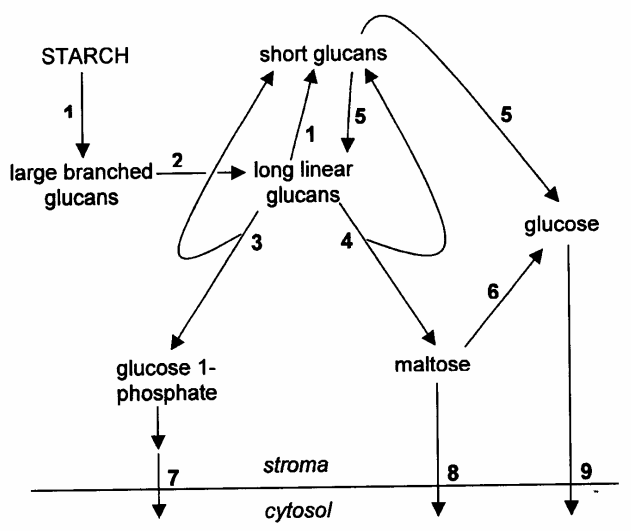

Fig. 6. Possible pathways of starch mobilization in a plastid. Reactions catalysed by enzymes known to occur in starch-containing plastids. 1) α-amylase, 2) debranching enzymes, 3) starch phosphorylase, 4) β-amylase, 5) D-enzyme, 6) α-glucosidase, 7) triose-phosphate translocator, 8) hypothetical maltose translocator and 9) glucose translocator. Reprinted from Smith *et al*. (2003) with kind permission from the publisher.

that *O. tuberosa* was a major Andean staple food in pre-colonial times, second in importance to potato. During the past 30 years it has become popular in New Zealand (National Research Council, 1989; CONDESAN-CIP, 1997) where it is known as New Zealand yam. Because of its high yield (approximately 60 tonnes per hectare) and good taste, it is frequently used in rural Andean cuisine, where it is eaten boiled in soups and stews. It is known for its resistance to frost damage. Its cultivation is severely constrained by a beetle that often destroys the entire crop.

Leaf starch

Leaf starch (transitory starch) and reserve starch can be distinguished from one another on the basis of physical and chemical characteristics: size, shape, and composition. Leaf starch granules tend to be smaller (Grange, Hammond & Andrews, 1989), and reserve granules have species-specific shapes. Leaf starch is composed mainly of the branched amylopectin, whereas reserve starch contains significant amounts of amylose chains in addition to amylopectin (Matheson, 1996; Slattery, Kavakli & Okita, 2000; Cairns, Begley & Sims, 2002; Zeeman *et al*., 2002).

Starch mobilisation in leaves

Several inherent problems are associated with identifying starch degradation pathways. First, plants contain many different enzymes that can degrade starch or

products derived from starch. Second, many of the enzymes exist as multiple isoforms, which may have different properties or roles in the plant (Steup, 1988; Trethewey & Smith, 2000). Furthermore, many of the well-studied presumptive starch degradative enzymes (e.g. α- and β- amylase and phosphorylase) are located primarily outside of the plastid (Beck & Ziegler, 1989). Since leaf starch is synthesised and accumulates only inside plastids, the role of the extra-chloroplastic forms of these enzymes in starch degradation is unknown (Caspar *et al.*, 1991). This means that there are several possible pathways for starch degradation in most cells (Kossmann & Lloyd, 2000; Smith *et al*., 2003). Possible routes for degradation using enzymes known to occur in many plastids are illustrated in Fig. 6.

Gelatinisation

Gelatinisation is a process that occurs when starch granules are heated in the presence of water. Gelatinisation is not a single reaction but a process with a number of irreversible reactions including swelling of the starch granules, leakage of amylose from the starch granules and loss of crystallinity (Colonna, Leloup & Buleón, 1992). The terms used to describe changes that take place during heating are shown in Fig. 7a and the physico-chemical processes that cause the changes are shown in Fig. 7b. Gelatinisation takes place over a temperature interval and is influenced by factors such as the starch source, water content, (Donovan, 1979; Biliaderis *et al*., 1986; Eliasson & Gudmundsson, 1996), presence of damaged starch, starch isolation procedure, annealing and environmental conditions during growth (Shi, Seib & Bernardin, 1994; Morrison, 1995). Depending on the property being measured, gelatinisation can be detected by different techniques. One of them is Differential Scanning Calorimetry (DSC), which quantifies the temperatures and enthalpy of gelatinisation (Biliaderis, 1983; Tester, 1997). Other techniques are:

Melting of starch crystallites detected by X-ray diffraction
Loss of anisotropic order detected by birefringence under polarised light
Hydration detected by Proton Magnetic Resonance

Gel formation and retrogradation

In the majority of cases, food that contains starch is processed by heating in the presence of water, allowing the product to develop some characteristics such as viscosity, firmness, elasticity, etc. However, those characteristics do not last for long time since there are some physico-chemical changes in the structure of the starch during storage of the food (Fig. 7b; Biliaderis, 1991). Some of the changes are related to the retrogradation process. Retrogradation is the term used to describe the molecular interactions, mainly hydrogen bonding, that occur between glucan molecules in the gelatinised starch during cooling (Davis *et al*., 2003). Retrogradation is dependent on time and temperature and affects quality, acceptability and shelf-life of starch-containing foods (Orford *et al*., 1987; Biliaderis, 1991). It is important to distinguish between the short-term development of gel structure via amylose crystallisation and long-term reordering

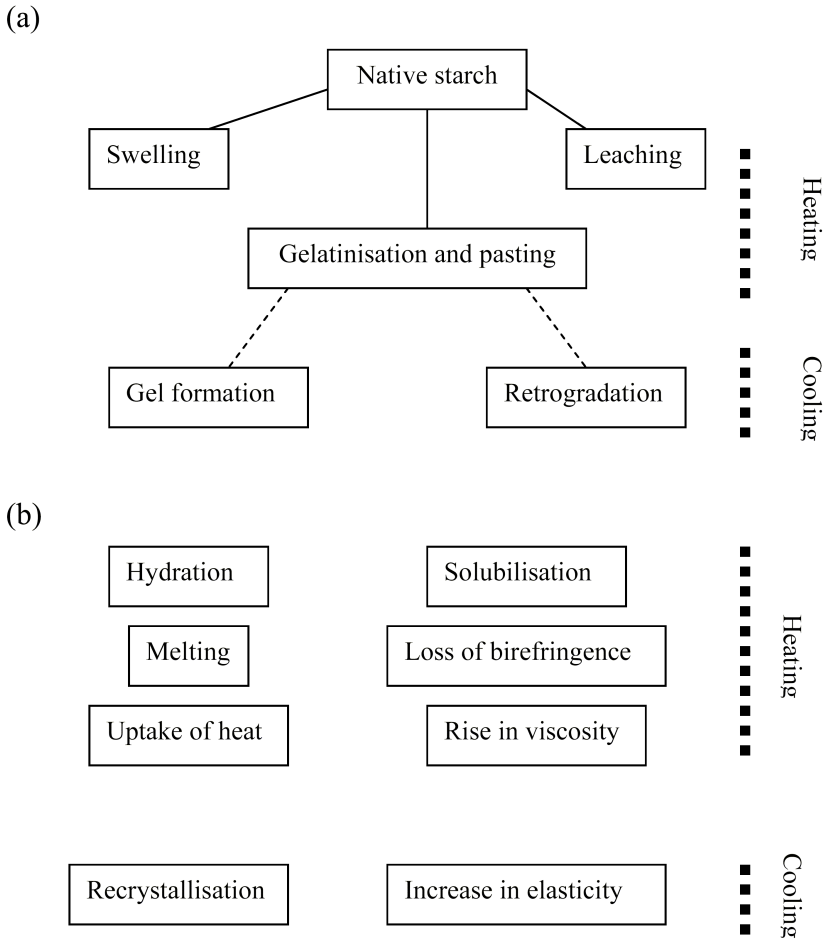

Fig. 7. (a) Terms to describe changes induced by heating and cooling (b) Physico-chemical changes that take place during heating and cooling. Reprinted from Svegmark (1992) with kind permission from the author.

of amylopectin, which is a much slower process (Miles *et al.*, 1985; Ring *et al.*, 1987; Silverio *et al.*, 1996).

Because of its industrial significance, many methods for the study of starch retrogradation have been developed, such as rheology (Ring, 1985; Eliasson & Gudmundsson, 1996), ^{13}C solid-state NMR (Kalichevsky *et al.*, 1992) and DSC (Kalichevsky, Orford & Ring, 1990; Eliasson & Gudmundsson, 1996). Dynamic rheological testing has become a common method for studying the viscoelastic behaviour of food, by determination of the elastic and viscous character of a sample. The equation describing the material behaviour is as follows:

$\tan(\delta) = G''/G'$

In this equation G′ (elastic modulus) indicates the elastic nature (solid character), G″ (viscous modulus) the viscous nature (liquid behaviour) of a substance and δ (phase angle) the degree of elasticity (Steventon *et al*., 1990). If a material is an ideal elastic material the stress and strain are in phase and δ=0. Hence G″ is equal to 0 because there is no viscous dissipation of energy. If a material behaves as an ideal viscous substance (Newtonian material) the stress and strain are 90 degrees out of phase and δ=π/2. Hence G′ is zero because the material does not store energy. Viscoelastic materials show both viscous and elastic behaviours. Examples of viscoelastic materials are polymer melts, bread dough and natural or artificial gels. Observations of polymer systems give the following numerical ranges for tangent of phase angle (tan δ): high for dilute solutions, 0.2 to 0.3 for amorphous polymers, and low (near 0.01) for glassy crystalline polymers and gels (Steventon *et al*., 1990).

Objectives of the present investigations

- Characterisation and studies of the lipid complexation ability of three unconventional starches from the Andean region in Ecuador.
- Investigation of rheological properties of gels from those three unconventional starches, influences of storage conditions and pH.
- Development of a method for extraction of leaf starch in good yield.
- Investigation of changes in potato leaf starch structure during the accumulation-degradation process.
- Study of the presence of phosphate groups in potato leaf starch.

Results

Characterisation of starches from *A. xanthorriza*, *C. edulis* and *O. tuberosa*

Physico-chemical characterisation (Paper I)

The sources of starch production vary all over the world depending on local traditions and climatic conditions, but it is more or less only starch and starch derivatives of maize, wheat, potato, cassava and rice that are of commercial interest (Swinkels, 1985; Ellis *et al*., 1998). There is always a concern with using different kinds of modified starches for food production, and consumer demand for non-modified foods is constantly increasing (Ellis *et al*., 1998). Another aspect is that many countries lack their own domestic production of starch, even though they have conceivable conditions for it (National Research Council, 1989). The structure and physico-chemical properties of many tuber and root starches have not been studied extensively (Hoover, 2001). *A. xanthorriza, C. edulis* and *O. tuberosa* are starch-rich crops grown in the Andean region of Ecuador. Their starches are not well studied but reports in the literature include information on the gelatinisation properties, starch granule size and the chain length distribution of amylopectin (Snyder 1984; Hizukuri 1985; Cortella & Pochettino 1995; Pérez, Breene & Bahnassey, 1998). However, more knowledge of the functional properties of these starches is required before they can be more commonly used (CONDESAN-CIP, 1997; Hoover, 2001). In Paper I some structural and physical properties of three Andean starches, isolated from *A. xanthorriza*, *C. edulis* and *O. tuberosa*, were investigated.

The starches exhibited the same B-type X-ray crystallinity pattern (Paper I) that is typical for almost all root and tuber starches (Zobel, 1988). The starch granule size distribution was between 35 and 101 µm for *C. edulis*, 20-55 µm for *O. tuberosa* and 7-23 µm for *A. xanthorriza*. The granules of *C. edulis* were large and can be compared with those of potato starch with sizes between 10 and 100 µm. Similar

results were later obtained by Thitipraphunkul *et al*. (2003). Starch granules from *C. edulis* and *O. tuberosa* were oval-shaped whereas those from *A. xanthorriza* starch displayed irregular shapes. Amylose content for *C. edulis*, *O. tuberosa* and *A. xanthorriza* starches was 24%, 18% and 4%, respectively. For *C. edulis* and *O. tuberosa* starch, this is in the range of a 'normal' starch with a typical value of around 25% amylose, while *A. xanthorriza* starch is a typical high-amylopectin starch. The amylose content of *C. edulis* starch from different cultivars varies between 19 and 25% (Thitipraphunkul *et al*., 2003). Weight average chain length of amylopectin from the three starches was 22.6 for *A. xanthorriza*, 22.4 for *O. tuberosa* and 21.9 for *C. edulis*. A linear amylose molecule will be completely hydrolysed by β-amylase, yielding a β-limit of 100%, while for amylopectin the hydrolysis will stop when the branching points are reached, yielding a value between 54 and 61% (Hizukuri, 1996). The β-amylolysis of isolated amylopectin had the highest value for *C. edulis* (68%) followed by *O. tuberosa* (65%) and *A. xanthorriza* (57%). Since starches from *A. xanthorriza* and *O. tuberosa* had similar weight average chain length but different β-amylolysis values, this suggests a similar degree of branching of *A. xanthorriza* and *O. tuberosa* but a different pattern of branching. The native starches of *A. xanthorriza*, *C. edulis* and *O. tuberosa* contain very little lipids, and to obtain information about the lipid complexation ability of the starches, their interaction with SDS was investigated. Lipids play an important role in the ageing stability of starch gels by inhibiting the retrogradation (Gudmundsson & Eliasson, 1990; Huang & White, 1993). It was found that the enthalpy of the transition of the starch-SDS complex was positively correlated to the amylose content, being highest for *C. edulis*, 2.7 J g^{-1}, followed by *O. tuberosa* and *A. xanthorriza* with enthalpies of 2.0 J g^{-1} and 0.2 J g^{-1}, respectively. Starch from *A. xanthorriza* with a natural amylose content of only 4% could be an interesting alternative to other high-amylopectin starches.

Rheological properties. Influence of storage conditions and pH (Paper II)

Ready to-eat foods in most cases contain starch which has been heated in the presence of water. This results in gelatinisation of the starch, producing favourable changes in the appearance and texture of the food. The changes in rheological behaviour during heating of a starch suspension are considerable, and rheological data may therefore be very helpful both in evaluating the behaviour of the starch and in the development of new products with controlled rheological properties (Steeneken, 1989). Storage of food containing gelatinised starch often results in undesirable changes in the texture (Kulp & Ponte, 1981; Wong & Lelievre, 1982; White, Abbas & Johnson, 1989; Ferrero, Martino & Zaritzky, 1993). Some of these changes are related to the retrogradation process, i.e. changes from an amorphous to a more ordered or crystalline state (Eliasson & Gudmundsson, 1996). Retrogradation depends on time and temperature as well as the botanical source, concentration, amylopectin/amylose ratio, amylopectin structure, lipids, pH and so on (Longton & LeGrys, 1981; Kalichevsky, Orford & Ring, 1990; Shi & Seib, 1992; Ward, Hoseney & Seib, 1994; Eliasson & Gudmundsson, 1996). For practical applications it is of interest to examine the rheological and gel-forming properties of the three starches from the Andean region.

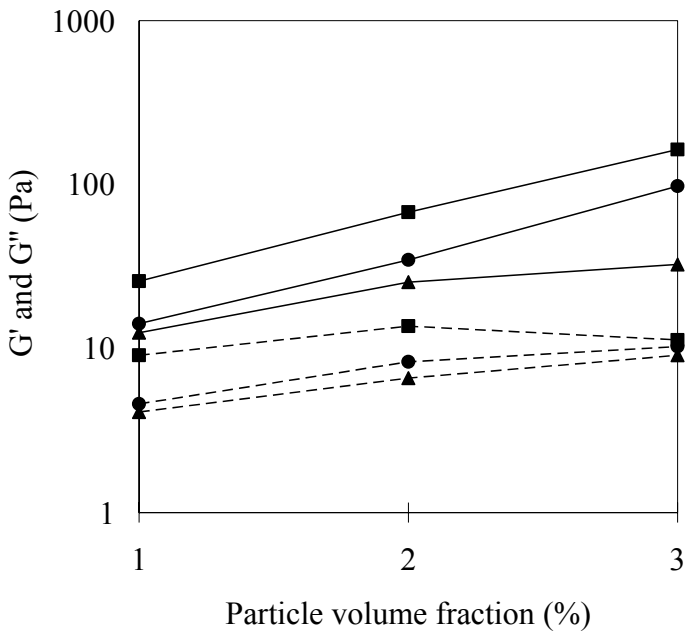

Fig. 8. Elastic (G′, solid lines) and viscous (G″, dotted lines) moduli measured at 2 Hz at room temperature in different starch particle volume fractions. ▲ *A. xanthorriza*, ■ *C. edulis*, ● *O. tuberosa.*

It was shown in Paper II that the starch particle volume fraction had a higher effect on the elastic behaviour (G′) of the three starch gels than on the viscous behaviour (G″) (Fig. 8). A more solid-like behaviour (strong gel) was obtained at a high starch particle volume fraction, which results from the gel formation of the amylose fraction.

Results in Figure 9 show that *A. xanthorriza* starch gels of particle volume fraction 1.0 exhibited only small changes in G′, compared with *C. edulis* and *O. tuberosa*, during three days of storage under refrigeration. Similar results were obtained for particle volume fractions of 2.0 and 3.0. *C. edulis* and *O. tuberosa* gels kept at the same refrigeration conditions showed a high increase in G′ during the first day of storage. Results on the storage of the three starch gels with a particle volume fraction of 1.0 at freezing temperature (-20 °C) are shown in Figure 9. Starch gels stored at freezing conditions were thawed every 24 h before the analyses were performed and stored again until the subsequent measurement was performed. Higher changes in G′ for the three starches were obtained at freezing conditions compared to refrigeration conditions. The freezing-thawing transformed the gel into a more elastic one (Eliasson & Gudmundsson, 1996).

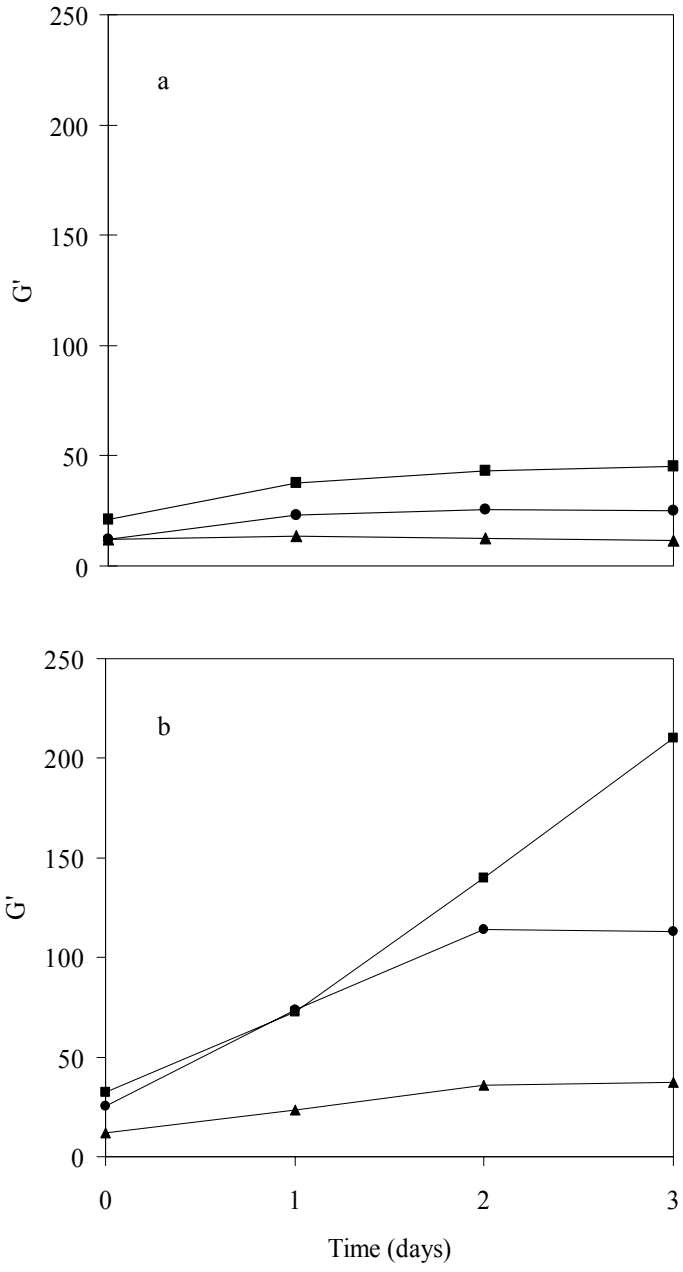

Fig. 9. Changes in the elastic modulus (G′) of ▲ *A. xanthorriza*, ■ *C. edulis* and ● *O. tuberosa* starch gels at starch particle volume fraction of 1.0. (a) Storage at 4°C (b) storage at –20°C. Measurements at 2 Hz.

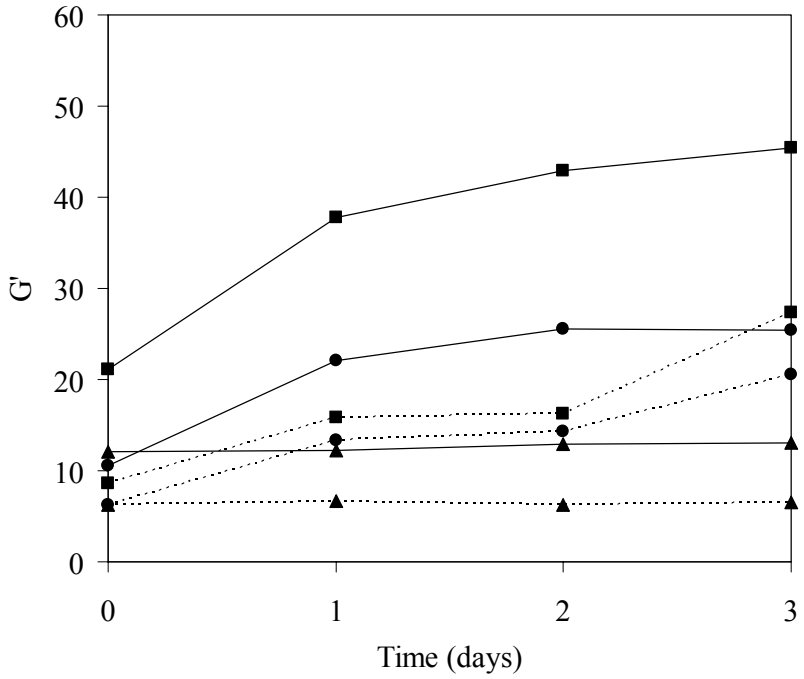

Fig. 10. Changes in the elastic modulus (G′) of ▲ *A. xanthorriza*, ■ *C. edulis* and ● *O. tuberosa* starch gels at starch particle volume fraction 1.0 during storage at 4°C. Dotted lines pH 4 and solid lines pH 6.5. Measurements at 2 Hz.

The high increase in G′ for *C. edulis* and *O. tuberosa* showed a formation of a strong gel structure at both refrigeration and freezing conditions. A decrease in pH from 6.5 to 4.0 produced a loss of structure in the three starch gels, as was shown by the reduction of G′ (Fig. 10). The rapid increase in G′ observed for *C. edulis* and *O. tuberosa* starch gels may be attributed to retrogradation of solubilised amylose. *A. xanthorriza* starch gels exhibited low retrogradation and may be used in the food industry at low storage temperature conditions.

Changes in starch structure during the accumulation-degradation process in potato leaves

Isolation and characterisation of potato leaf starch (Paper III)

Despite leaf starch being one of the most abundant and universally distributed forms of storage polysaccharide, little is known about its synthesis, composition and structure compared to starches from storage organs (Matheson, 1996; Watanabe, Nakamura & Ishii, 1997; Zeeman *et al*., 2002). Studies on the starch granule size of leaves from potato and *Arabidopsis* (Grange, Hammond & Andrews, 1989¸ Zeeman *et al*., 1998b) and the content and characterisation of amylose and amylopectin from potato, pea and cotton leaf starch have been performed (Chang, 1979; Hovenkamp-Hermelink *et al*., 1988; Zeeman *et al*., 1998b; Tomlison, Lloyd & Smith 1997; Tomlison, Craig & Smith, 1998). However, more information regarding the accumulation-degradation process and the structure of leaf starch from different sources is needed. Therefore potato leaves with different starch accumulation rates were collected at two different times (morning and afternoon) during the growing season (July and August) at a site in Uppsala, Sweden. The starch was extracted from the leaves, and amylose and amylopectin were characterised and compared with those from potato tuber starch (Paper III).

Different methods for starch isolation from leaves have been reported (Radwan & Stocking, 1957; Chang, 1979; Zeeman *et al.*, 1998a, 1998b; Blauth *et al*., 2001). However, none of the methods cited report starch yield. Results in Paper III showed that attempts to isolate leaf starch granules led to very low yields (recovery below 3%) and impure starch fractions. The low yield may be due to the fact that some of the analysed starch in the leaves was not present as granules and was therefore washed out during the isolation procedure. Therefore, an extraction technique with DMSO was used instead (Fig. 11). Disintegrated freeze-dried leaves were freed from chlorophyll and other soluble compounds, and enzymes were inactivated by boiling in 90% ethanol. After several washing steps the remaining material was mixed with ethylenediaminetetra-acetic acid (EDTA) and stirred overnight at room temperature. EDTA binds calcium and helps to extract other polysaccharides such as pectin and hemicellulose in the leaf (Åman & Westerlund, 1996). After centrifugation, starch was extracted from the pellet with 90% dimethylsulphoxide (DMSO) during heating in boiling water and in an oven. The DMSO soluble fraction was separated by centrifugation and mixed with ethanol to precipitate the starch. Starch extraction with DMSO showed high yields with an average recovery above 80%. In order to check the presence of other polysaccharides, DMSO-extracted starch was hydrolysed with α-amylase and amyloglucosidase and fractionated by GPC (Fig. 12). Afternoon samples from July showed only one peak from hydrolysed starch, whereas the morning samples showed two regions, one between 200 and 380 min corresponding to polysaccharides other than starch and a region between 400 and 440 min corresponding to hydrolysed starch (Fig. 12). The much lower amount of starch present in the morning sample compared to the afternoon sample may explain this difference.

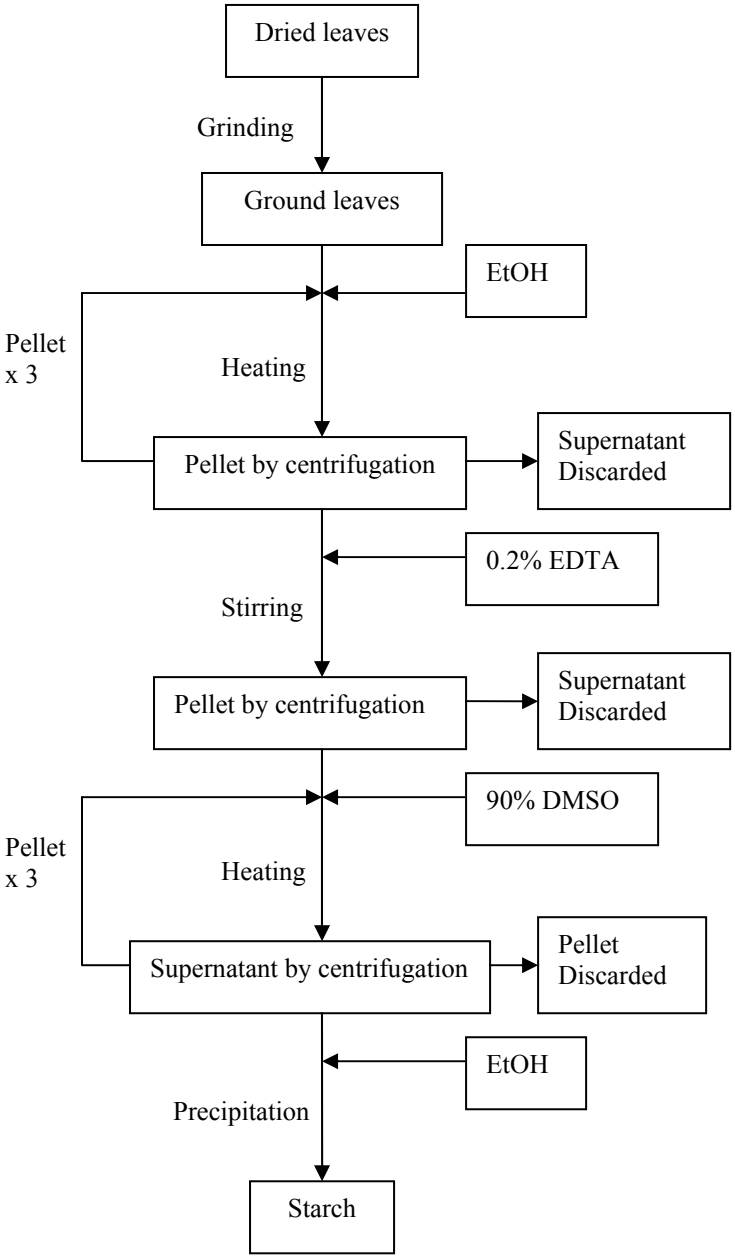

Fig. 11. Flow chart of potato leaf starch extraction with DMSO.

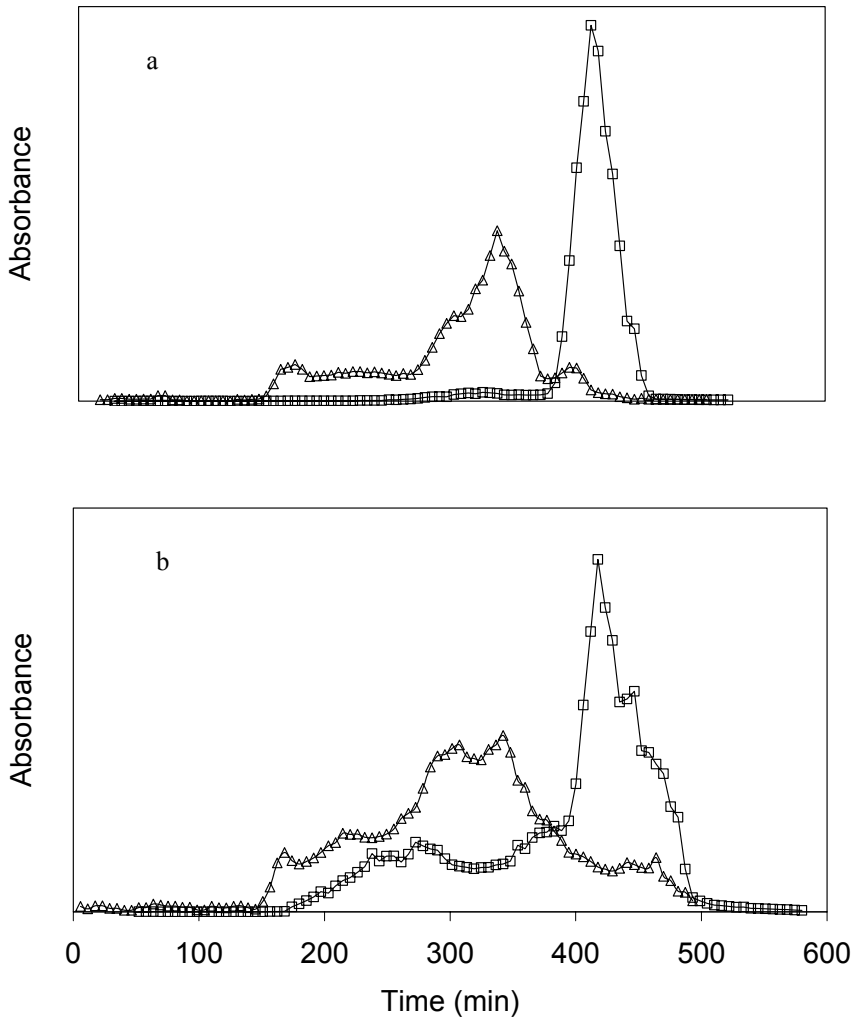

Fig. 12. Elution profile on Sepharose CL-6B of DMSO-extracted potato leaf starch detected by the phenol-sulphuric acid method. (a) July sample taken at 5:00 p.m., (b) July sample taken at 5:00 a.m., Δ debranched with isoamylase, □ hydrolysed with thermostable α-amylase and amyloglucosidase.

A significant amount of starch, analysed as released glucose after hydrolysis with a thermostable α-amylase and amyloglucosidase, was accumulated in potato leaves during the day in July, whereas in August the rate of accumulation was much lower (Fig. 13). The accumulated starch is translocated to the sinks during the night. Similar results have previously been obtained in, for example, soybean leaves (Upmeyer & Koller, 1973; Huber, Rogers & Mowry, 1984) and pea leaves (Tomlison, Lloyd & Smith, 1997).

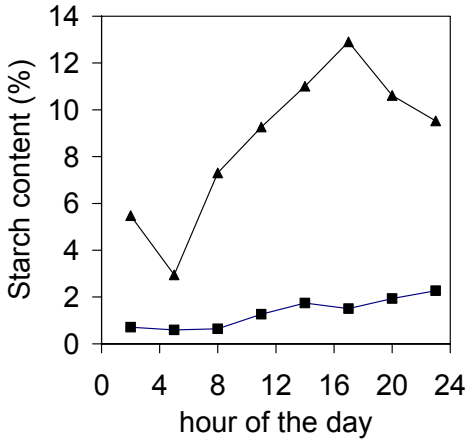

Fig. 13. Starch content in dry potato leaves collected at different times during two days of the growing season ▲ July 12th and ■ August 20th.

DMSO-extracted starches were debranched with isoamylase and analysed by GPC on Sepharose CL-6B (Fig. 14). Samples from July showed a small peak of amylose between 150 and 200 min, a material with a chain length distribution between that found for amylose and amylopectin between 210 and 290 min and debranched amylopectin between 300 and 360 min. Samples from August had similar trends with a higher relative amount of a material between 210 and 290

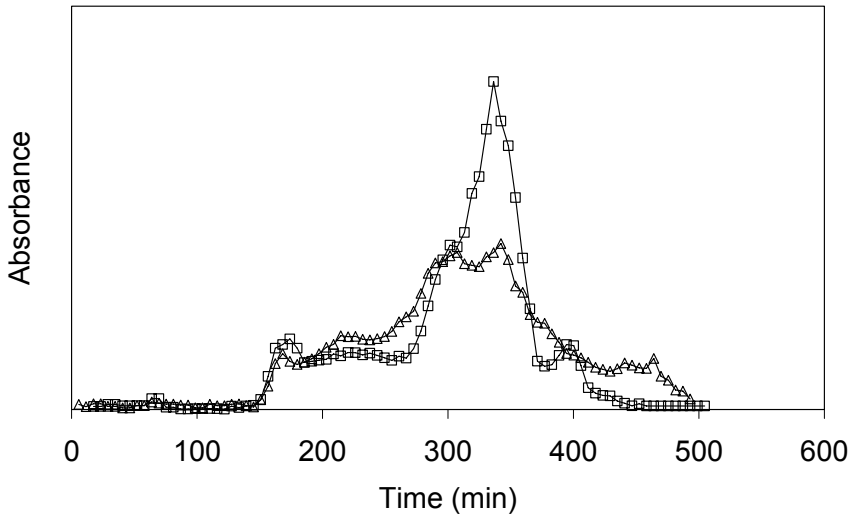

Fig. 14. Elution profile on Sepharose CL-6B of debranched DMSO-extracted potato leaf starch detected by the phenol-sulphuric acid method. Δ July sample taken at 5:00 a.m. and □ July sample taken at 5:00 p.m.

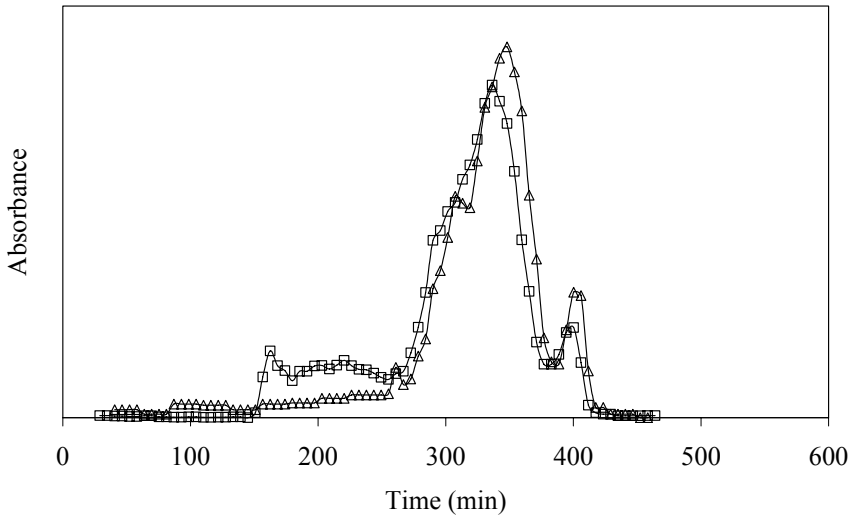

Fig. 15. Elution profile of DMSO-extracted potato leaf starch from July 5:00 p.m. on Sepharose CL-6B detected by the phenol-sulphuric acid method. □ Whole starch debranched with isoamylase; Δ amylopectin isolated on Sepharose CL-2B and debranched with isoamylase.

min in the morning sample. A peak between 380 and 430 min was observed in all four samples.

In order to find out the origin of the peak between 380 and 430 min, amylopectin was isolated on Sepharose CL-2B and debranched with isoamylase before being analysed by GPC on Sepharose CL-6B. The elution profile on Sepharose CL-6B (Fig. 15) showed a peak between 380 and 430 min elution time corresponding to oligomers with less than 7 glucose units. It was present in the profile of both, whole starch and isolated amylopectin. This peak is consequently a true part of the amylopectin structure in potato leaf starch but is not present in potato tuber starch.

HPAEC analyses of debranched potato leaf starch showed the presence of very short unit chains, especially DP 5 (Fig. 16), which are not present in potato tuber starch (Hizukuri & Maehara, 1990; Koch, Andersson & Åman, 1998). In the afternoon samples, with a higher content of leaf starch, the populations around DP 19 and an additional population with DP>35 became significant, whereas the chain length distribution between DP 5 and 15 remained basically unchanged. The afternoon samples showed a similar chain length distribution as reported for potato tuber starch (Koch, Andersson & Åman, 1998). The results indicate that the leaf starch contains a primer of short unit chains (DP 5 to 15) as well as longer unit chains, which are produced during the day and used during the night.

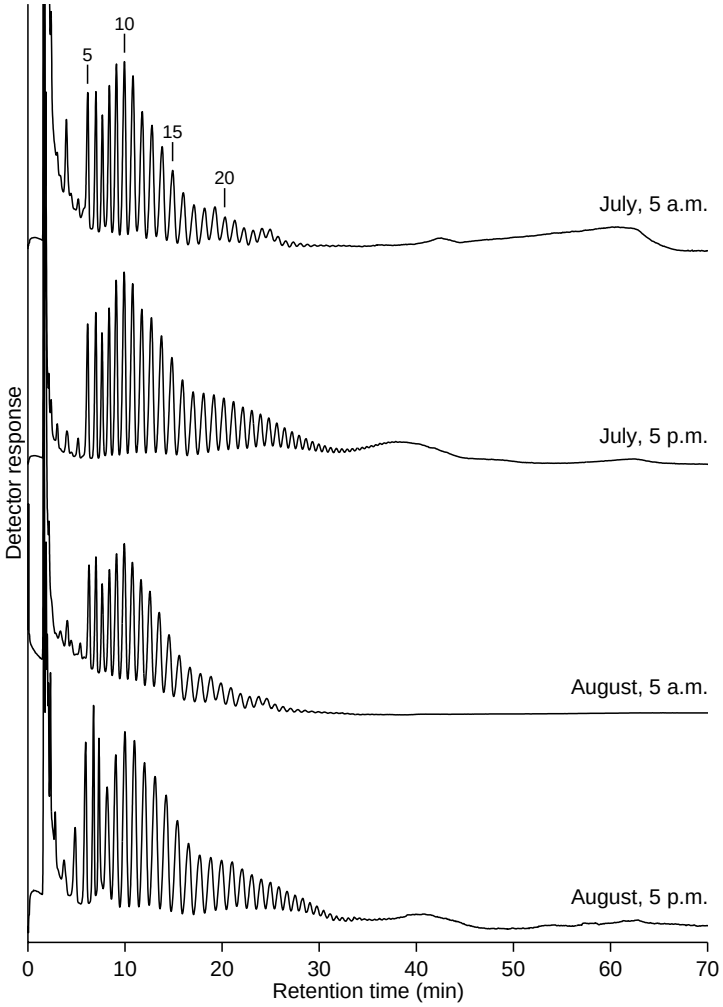

Fig. 16. DMSO-extracted potato leaf starch debranched with isoamylase and analysed by HPAEC-PAD. (a) July sample taken at 5:00 a.m. (b) July sample taken at 5:00 p.m. (c) August sample taken at 5:00 a.m. (d) August sample taken at 5:00 p.m. DP is indicated

Molecular distribution of amylose and amylopectin in potato leaf starch (Paper IV)

The formation of colour by the interaction between starch and iodine is an important property for starch characterisation. Iodine forms a complex with α-1,4 linked glucans by insertion in the hydrophobic cavity of the linear glucan helices. Amylose interacts with iodine, forming a blue-coloured complex with a λ_{max} of between 620 and 650 nm (Banks & Greenwood, 1975). Amylopectin develops a violet/purple coloured complex with a λ_{max} of approximately 550 nm. The λ_{max} for

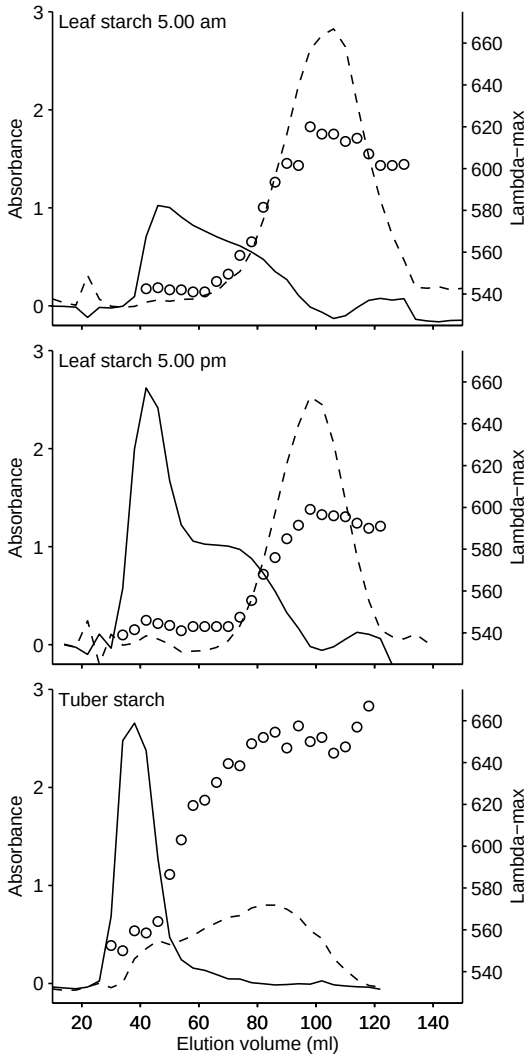

Fig. 17. Resolved chromatograms of amylopectin (—) and amylose (---) from potato leaf, collected in July and tuber starches on Sepharose CL-2B. The λ_{max} of the fractions is shown with open circles.

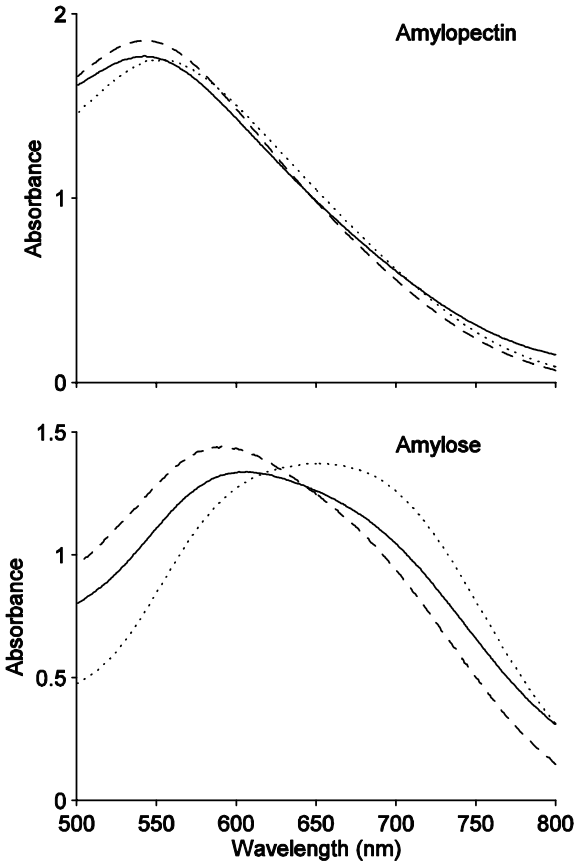

Fig. 18. Spectra of amylopectin and amylose from potato leaf in July and tuber starches stained with iodine. Leaf starch 5.00 a.m. (−), leaf starch 5.00 p.m. (---), tuber starch (···).

amylopectin of *Arabidopsis* leaf starch is 550 nm (Zeeman *et al.*, 2002) and for pea leaves is between 550 and 558 nm (Tomlison, Lloyd & Smith, 1997). The λ_{max} value for amylose of *Arabidopsis* leaf starch is substantially lower than that reported for amylose from other species (585 nm). This information suggests that either amylose from *Arabidopsis* leaves is more branched than that from other species, or that the analysed amylose fraction is contaminated with branched glucans (Zeeman *et al*., 2002). In this section, starches extracted from potato leaves of July were characterised by iodine-staining and compared with potato tuber starch (Paper IV).

Potato leaf starch was extracted with DMSO according to the procedure described in Paper III (Fig. 10) but on a larger scale. Ultrafiltration was introduced to that procedure to reduce the volume of DMSO before starch precipitation (Paper IV).

The DMSO-extracted starches were fractionated on Sepharose CL-2B and analysed by iodine-staining. The results showed that the amylose from both leaf

starches had a larger elution volume compared to the volume for the potato tuber starch sample, suggesting a lower molecular weight (Fig. 17). Resolved chromatograms of amylopectin and amylose were computed using the spectra extracted from their identified pure regions. It is noteworthy that all spectra in the overlapping regions could be accounted for by this method, indicating that the chain length distribution of both amylopectin and amylose was constant over their molecular size ranges. Consequently, no third compound (intermediate material) was needed to explain the spectra of the mixtures.

The tuber starch amylose showed a broader molecular size range than the leaf starch amylose, where the high molecular weight fraction was absent. The amylopectin, on the other hand, had broader size ranges in the leaf starches, but the fraction increasing in proportion during synthesis over the day had a size range more similar to that of tuber starch amylopectin.

Iodine spectra of eluted fractions from leaf and tuber starch samples were chosen from the regions where pure amylose and amylopectin were detected (Fig. 17). Leaf and tuber starches had amylopectins with similar iodine spectra, indicating similar average chain length (Fig. 18). The amylose spectra differed, however, showing that the amylose fraction from the afternoon sample had a higher proportion of short chains than that from the morning sample. The amylose fraction from tuber starch appeared to have a smaller proportion of short chains compared to the leaf starches.

On the presence of starch-bound phosphate in potato leaf starch (Paper V)

According to Bieleski (1968), over 70% of the total phosphorus (P) in plant leaves is in the form of inorganic P, with the remainder being in the form of ribonucleic acids, phospholipids and acid-soluble phosphate esters (Ravindran, Ravindran & Sivalogan, 1994). Small amounts of starch-bound phosphate (glucose-6 phosphate) have previously been detected in potato leaf starch (Blennow *et al*., 2000b). However a more complete characterisation of the starch-bound phosphates in potato leaves is needed.

^{31}P-NMR analysis of the extracted starch according to the procedure described in Paper IV revealed several phosphorus signals (results not shown). Signals corresponding to starch-bound phosphate could not be identified because of overlap with signals of other phosphorus-containing compounds. The material was therefore further purified by ultrafiltration in two steps (Fig. 19). In the first step low molecular weight compounds, which may contain phosphorus, were removed. The retained starch was thereafter degraded with α-amylase and filtered again. High molecular weight non-starch polymers which may also contain phosphorus were retained on the filter, whereas the degraded starch was obtained in the filtrate.

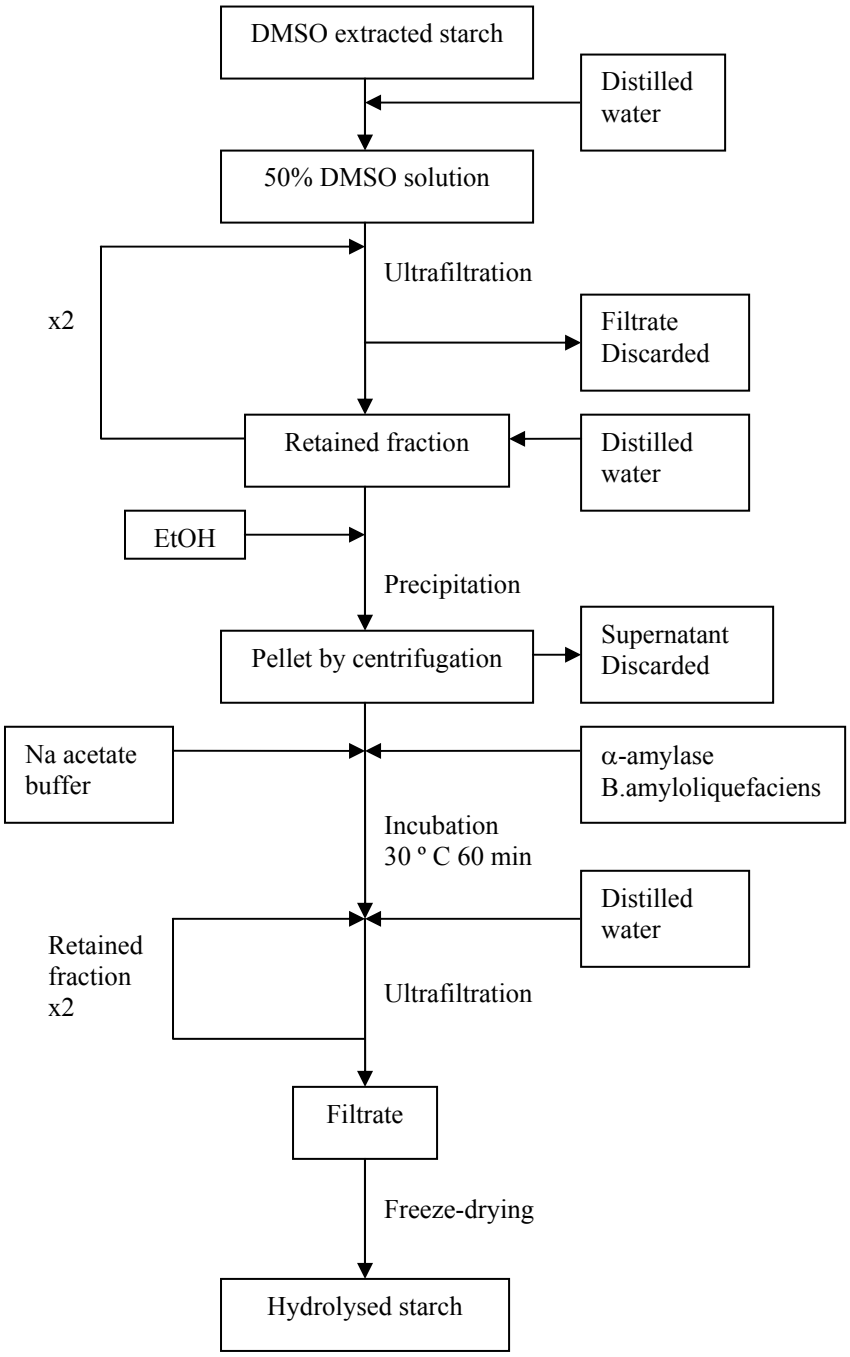

Fig. 19. Flow chart of starch extracted with DMSO and purified with ultrafiltration in two steps in combination with α-amylase hydrolysis.

Purified leaf starch contained no signals corresponding to starch-bound phosphate in ^{31}P-NMR (Fig. 20). One broad signal at a chemical shift of -3.0 ppm was observed. The signal could be attributed to a lecithin carrying phosphodiester linkages (Kasemsuwan & Jane, 1995). Potato tuber starch was degraded and filtered as was done with leaf starch and showed three signals at chemical shifts of 2.21, 1.98 and 1.76 ppm in ^{31}P-NMR. Both up-field signals (1.76 and 1.98 ppm) corresponded to the C_6 positions, whereas the signal at 2.21 ppm was assigned to C_3-bound phosphate as described previously by Frigård (2002). The fact that starch-bound phosphate could be clearly identified in the tuber starch while undetectable in the leaf starch, even though both extracts prepared for NMR-analysis had approximately the same starch concentration, indicated that phosphate monoesters are absent, or present at very low levels, in the potato leaf starch.

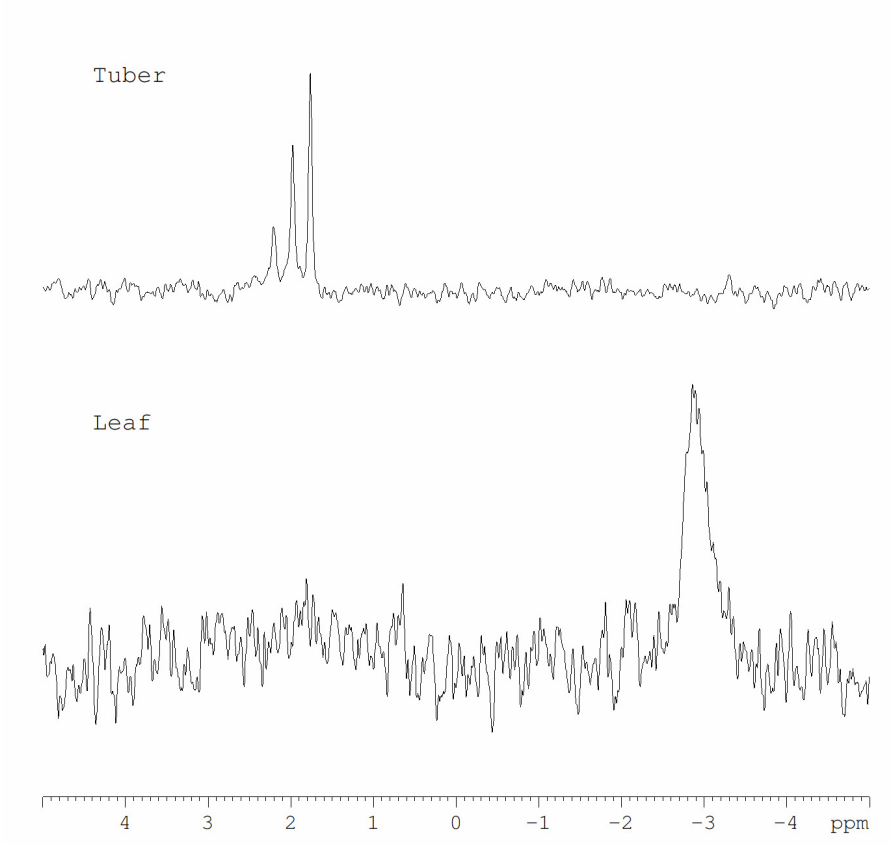

Fig. 20. 400-MHz ^{31}P-NMR spectra of partly purified starch fragments from potato tuber amylopectin starch and from potato leaf starch, both recorded at 30 ºC, pD 8, in D_2O.

Summary of findings

1. a) Starches from *A. xanthorriza*, *C. edulis* and *O. tuberosa* exhibited a B-type X-ray crystallinity pattern, which is typical for almost all root and tuber starches.
   b) Starch granules from *C. edulis* and *O. tuberosa* were oval-shaped with sizes varying between 35-101 µm and 20-55 µm, respectively. *A. xanthorriza* starch displayed an irregular shape with size variation between 7 and 23 µm.
   c) Amylose content for *C. edulis*, *O. tuberosa* and *A. xanthorriza* was 24%, 18% and 4%, respectively. Starch from *A. xanthorriza* with natural low amylose content could be an interesting alternative to other high-amylopectin starches.
   d) Starches from *A. xanthorriza* and *O. tuberosa* had similar weight average chain length but different β-amylolysis values, suggesting a similar degree of branching but a different pattern of branching.
   e) The enthalpy of the transition of the starch-SDS complex was positively correlated to the amylose content, being highest for *C. edulis* (2.7 J g^{-1}), followed by *O. tuberosa* (2.0 J g^{-1}) and *A. xanthorriza* (0.2 J g^{-1}).

2. a) The starch particle volume fraction had a higher effect on the elastic behaviour (G′) of the three starch gels than on the viscous behaviour (G″), which is correlated to gel formation of the amylose fraction.
   b) *A. xanthorriza* starch gels with a low amylose content exhibited only small changes in G′, compared with *C. edulis* and *O. tuberosa* starch gels during three days of storage under refrigeration (+ 4 ºC) and freezing conditions (–20 ºC) and may be used when retrogradation needs to be inhibited.
   c) Higher changes in G′ for the three starches were obtained during repeated freezing-thawing cycles compared to refrigeration conditions. The freezing-thawing transformed the gel into a more elastic one.
   d) The high increase in G′ for *C. edulis* and *O. tuberosa* showed a formation of a stronger gel structure at both refrigeration and freezing conditions.
   e) A decrease in pH from 6.5 to 4.0 resulted in a reduction in G′ for all three starch gels.

3. Extraction of leaf starch with DMSO showed average yields above 80%, which is much higher than for isolation of granules.

4. a) The starch content in potato leaves collected at different times on a day and night in July varied between 1.8% and 12.9%, with a minimum around 5:00 a.m. and a maximum at 2:00-5:00 p.m. A significantly lower starch content (0.6-2%) was found in the leaves collected in August. The morning samples collected in August also had the lowest content of starch and the afternoon/evening samples the highest content. The starch granules had either an oval or a round shape and none bigger than 5 µm was found.
   b) Debranched DMSO-extracted starches analysed by GPC on Sepharose CL-6B showed the presence of a small peak of amylose. A material with intermediate chain length was present in a higher relative amount in the

morning than in the afternoon in a sample from August. Debranched amylopectin of low molecular size had a higher relative amount in the afternoon than in the morning in a sample from July. A peak corresponding oligomers with less than 7 glucose units was observed in all four samples. This peak is a true part of the leaf amylopectin structure but is not present in potato tuber starch.

c) HPAEC analyses of debranched potato leaf starch showed the presence of short unit chains (DP 5), which are not present in potato tuber starch. The afternoon samples showed a similar chain length distribution to that reported for potato tuber starch, where the populations around DP 19 and DP>35 became significant during the day, whereas the chain length distribution between DP 5 and 15 remained basically unchanged. The results indicate that the leaf starch contains a primer of short unit chains (DP 5 to 15) as well as longer unit chains, which are produced during the day and used during the night.

d) Results from iodine-staining indicate that amylose from the afternoon sample had a higher proportion of short chains and a smaller proportion of long chains than the morning leaf starch sample. Amylopectin from both leaf starches and the tuber starch seemed to have similar average chain length. Both leaf and tuber starch seemed to be composed only of amylose and amylopectin, with constant chain length distribution over their molecular weight ranges. The results indicate that the material between the pure amylopectin and amylose is a mixture of the two and not an intermediate material.

5. a) DMSO-extracted starch was further purified by ultrafiltration in two steps. In the first step low molecular weight compounds were removed. The retained starch was thereafter degraded with α-amylase and filtered again. High molecular weight polymers were retained on the filter and the degraded starch obtained in the filtrate.

   b) Degraded leaf starch was analysed with ^{31}P-NMR and showed no signals corresponding to starch-bound phosphate, indicating that phosphate monoesters are absent, or present at very low levels, in the potato leaf starch.

References

Åman, P. & Westerlund, E. 1996. Cell wall polysaccharides: Structural, chemical and analytical aspects. In *Carbohydrates in Food*, edited by Eliasson, A.-C., Marcel Dekker Inc., New York, pp 191-226.

Andersson, L., Rydberg, U., Larsson, H., Andersson R. & Åman P. 2002. Preparation and characterisation of linear dextrins and their use as substrates in *in vitro* studies of starch branching enzyme. *Carbohydrate Polymers 47*, 53-58.

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

Båga, M., Nair, R., Repellin, A., Scoles, G. & Chibbar, R. 2000. Isolation of a cDNA encoding a granule bond 152kD starch branching enzyme in wheat. *Plant Physiology 124*, 253-264.

Ball, S., van de Wal, M. & Visser, R. 1998. Progress in understanding the biosynthesis of amylose. *Trends in Plant Science 3*, 462-465.

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

Banks, W., Greenwood, C. & Muir, D. 1974. Studies on Starches of High-amylose Content. Part 17. A Review of Current Concepts. *Starch 26*, 289-328.

Banks, W. & Greenwood, C. 1975. *Starch and its Components*, Edinburgh University Press. Edinburgh.

Beck, E. & Ziegler, P. 1989. Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology 40*, 95-117

Bertoft, E., Qin, Z. & Manelius, R. 1993. Studies on the Structure of Pea Starches. Part 4: Intermediate Material of Wrinkled Pea Starch. *Starch 45*, 420-425.

Bertoft, E. 2004. On the nature of categories of chains in amylopectin and their connection to the super helix model. *Carbohydrate Polymers 57*, 211-224.

Bieleski, R. 1968. Levels of phosphate esters in spirodella. *Plant Physiology 43*, 1297-1308.

Biliaderis, C. 1983. Differential Scanning Calorimetry in food research: a review. *Food Chemistry 10*, 239-265.

Biliaderis, C., Page, C., Maurice, T. & Juliano, B. 1986. Thermal characterisation of rice starches: a polymeric approach to phase transitions of granular starch. *Journal Agricultural and Food Chemistry 34*, 6-14.

Biliaderis, C., Page, C. & Maurice, T. 1986. On the melting transitions of starch/monoglyceride systems. *Food Chemistry 22*, 279-295.

Biliaderis, C. 1991. The structure and interactions of starch with food constituents. *Canadian Journal of Physiology and Pharmacology 69*, 60-78.

Blauth, S., Yao, Y., Klucinec, J., Shannon, J., Thompson, D. & Guiltinan, M. 2001. Identification of Mutator insertional mutants of starch branching enzyme 2a in corn. *Plant Physiology 125*, 1396-1405

Blennow, A., Bay-Smidt, A., Olsen, C. & Möller, B. 1998. Analysis of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography. *Journal of Chromatography A 829*, 385-391.

Blennow, A., Bay-Smidt, A., Olsen, C. & Møller, B. 2000a. The distribution of covalently bound phosphate in the starch granule in relation to starch crystallinity. *International Journal of Biological Macromolecules 27*, 211-218

Blennow, A., Engelsen, S., Munck, L. & Møller, B. 2000b. Starch molecular structure and phosphorylation investigated by a combined chromatographic and chemometric approach. *Carbohydrate Polymers 41*, 163-174.

Blennow, A., Engelsen, S., Nielsen, T., Baunsgaard, L. & Mikkelsen, R. 2002. Starch phosphorylation: a new front line in starch research. *Trends in Plant Science 7*, 445-450.

Borovsky, D. Smith, E. & Whelan, W. 1976. On the mechanism of amylose branching by potato Q-enzyme. *European Journal of Biochemistry 62*, 307-312.

Buleón, A., Colonna, P., Planchot, V. & Ball, S. 1998. Starch granules: structure and biosynthesis. *International Journal of Biological Macromolecules 23*, 85-112.
Cairns, A., Begley, P. & Sims, I. 2002. The structure of starch from seeds and leaves of the fructan-accumulating ryegrass, *Lolium temulentum L. Journal of Plant Physiology 159*, 221-230.
Caspar, T., Lin, T., Kakefuda, G., Benbow, L., Preiss, J. & Somerville, C. 1991. Mutants of Arabidopsis with altered regulation of starch degradation. *Plant Physiology 95*, 1181-1188.
Chang, C. 1979. Starch and its component ratio in developing cotton leaves. *Plant Physiology 63*, 973-977.
Colonna, P., Leloup, V. & Buleón, A. 1992. Limiting factors of starch hydrolysis. *European Journal of Clinical Nutrition 46*, S17-S32.
CONDESAN-CIP. 1997. Pocket Guide to Nine Exotic Andean Roots and Tubers. CONDESAN-CIP.
Cortella, A. & Pochettino, M. 1995. Comparative morphology of starch of three Andean tubers. *Starch 47*, 455-461.
Davis, P., Supatcharee, N., Khandelwai, R. & Chibbar, R. 2003. Synthesis of Novel Starches in Planta: Opportunities and Challenges. *Starch 55*, 107-120.
Doehlert, D. & Knutson, C. 1991. Two classes of starch debranching enzymes from developing maize kernels. *Journal of Plant Physiology 138*, 566-572.
Donald, A., Kato, L., Perry, P. & Waigh, T. 2001. Scattering studies of the internal structure of starch granules. *Starch 53*, 504-512.
Donovan, J. 1979. Phase transitions of the starch-water system. *Biopolymers 18*, 263-275.
Duwening, E., Steup, M., Willmitzer, L. & Kossman, J. 1997. Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum L.*) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. *Plant Journal 12*, 323-333.
Edwards, A., Marshall, J., Denyer, K., Sidebottom, C., Visser, R., Martin, C. & Smith, A. 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.
Eliasson, A.-C. & Krog, N. 1985. Physical properties of amylose-monoglyceride complexes. *Journal of Cereal Science 3*, 239-248.
Eliasson, A.-C. 1986. On the effects of surface active agents on the gelatinisation of starch-a calorimetric investigation. *Carbohydrate. Polymers 6*, 463-476
Eliasson, A.-C. & Gudmundsson, M. 1996. Physicochemical and functional aspects. In *Carbohydrates in Food*, edited by Eliasson, A.-C., Marcel Dekker Inc., New York, pp. 431-503.
Ellis, R., Cochrane, M., Dale, M., Duffus, C., Lynn, A., Morrison, I., Prentice, R., Swantson, S. & Tiller, A. 1998. Starch production and industrial use. *Journal of the Science of Food and Agriculture 77*, 289-311.
Ferrero, C., Martino, M. & Zaritzky, N. 1993. Stability of frozen starch pastes: effect of freezing, storage and xanthan gum addition. *Journal Food Process Preservation 17*, 191-211.
Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A.-C. & Åman, P. 1998. The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. *Carbohydrate Polymers 35*, 119-134
Frigård, T. 2002. *Amylopectin Starch. Structure, Modification and Properties*. Swedish University of Agricultural Sciences. PhD thesis. ISSN 1401-6249
Gallant, D., Bouchet, B. & Baldwin, P. 1997. Microscopy of starch: evidence of a new level of granule organisation. *Carbohydrate Polymers 32*, 177-191.
Gidley, J. & Bulpin, V. 1987. Crystallisation of malto-oligosaccharides as models of the crystalline forms of starch. *Carbohydrate Polymers 161*, 291–300.
Grange, R., Hammond, B. & Andrews, J. 1989. Characteristics of starch granules isolated from mature pepper leaves grown under different irradiances and day lengths. *Journal of Experimental Botany 40*, 1045-1052.
Guan, H. & Preiss, J. 1993. Differentiation of the properties of the branching isoenzymes from maize (*Zea mays*). *Plant Physiology 102*, 1269-1273.

Gudmundsson, M. & Eliasson, A.-C. 1990. Retrogradation of amylopectin and the effects of amylose and added surfactants/emulsifiers. *Carbohydrate Polymers 13*, 295.
Hermann, H., Uptmoor, R., Freire, I. & Montalvo, J. 1996. *Crop Growth and Starch Productivity of Edible Canna*. Program 6, Postharvest, Management, Marketing. Program Report 95-96, International Potato Centre (CIP), Lima, Perú.
Hermann, M. 1997. Andean roots and tubers: Ahipa, arracacha, maca, yacon. In *Promoting the Conservation and Use of Underutilized and Neglected Crops*. Hermann, M. & Heller, J. eds. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute (IPGRI).
Hizukuri, S., Tabata & Nikuni, Z. 1970. Studies on starch phosphate. *Starch 22*, 338-343
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. & Maehara, Y. 1990. Fine structure of wheat amylopectin: the model of the A to B chain binding. *Carbohydrate Polymers 206*, 145-159.
Hizukuri, S. 1993. Towards an understanding of the fine structure of starch molecules. *Denpun Kagaku 40*, 133-147.
Hizukuri, S. 1996. Starch: Analytical aspects. In *Carbohydrates in Food*, edited by Eliasson, A.-C., Marcel Dekker Inc., New York, pp. 347-429.
Hoover, R. 2001. Composition, molecular structure, and physicochemical properties of tuber and root starches: a review. *Carbohydrate Polymers 45*, 253-267.
Hovenkamp-Hermelink, J., De Vries, J., Adamse, P., Jacobsen, E., Witholt, B. & Feenstra, 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.
Huang, J. & White, P. 1993. Waxy Corn Starch: Monoglyceride Interaction in a Model System. *Cereal Chemistry 70*, 42-47.
Huber, S., Rogers, H. & Mowry, F. 1984. Effects of water stress on phothosynthesis and carbon partitioning in soybean plants grown in the field at different CO2 levels. *Plant Physiology 76*, 244-249.
Hurtado, J., Ortiz, R., Rodríguez, G. & Dufour, D. 1997. Procesamiento de la arracacha (*Arracacha xanthorriza*): Estudio de la factibilidad técnica y económica para la producción de almidón y harina y sus propiedades fisicoquímicas. Seminario Técnico sobre Raíces y Tubérculos autóctonos. Ibagué, Colombia, 27-29 de mayo 1997.
Hussain, H., Mant, A., Seale, R., Zeeman, S., Hinchliffe, E., Edwards, A., Hylton, C., Bornemann, S., Smith, A., Martin, C. & Bustos, R. 2003. Three Isoforms of Isoamylase Contribute Different Catalytic properties for the Debranching of Potato Glucans. *The Plant Cell 15*, 133-149.
Imberty, A., Chanzy, H., Pérez, S., Buléon, A. & Tran V. 1988. The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology 201*, 365–378
Jane, J. & Shen, J. 1993. Internal structure of the potato starch granule revealed by chemical gelatinisation. *Carbohydrate Research 247*, 279-290
Jane, J., Kasemsuwan, T. & Chen, J. 1996. Phosphorus in rice and other starches, *Cereal Foods World 41*, 827-832.
Jenkins, P., Cameron, R. & Donald, A. 1993. A Universal Feature in the Structure of Starch Granules from Different Botanical Sources. *Starch 45*, 417-420.
Jenkins, P. & Donald, A. 1995. The influence of amylose on starch granule structure. *International Journal of Biological Macromolecules 17*, 315-321
Kalichevsky, M., Orford, P. & Ring, S. 1990. The Retrogradation and Gelation of Amylopectins from Various Botanical Sources. *Carbohydrate Research 198*, 49-55.
Kalichevsky, M., Jaroszkiewicz, E., Ablett, S., Blanshard, J. & Lillford, P. 1992. The Glass Transition of Amylopectin Measured by DSC. DMTA and NMR. *Carbohydrate Polymers 18*, 77-88.
Kasemsuwan, T. & Jane, J. 1994. Location of amylose in normal starch granules. II Locations of phosphodiester cross-linking revealed by phosphorus-31 NMR. *Cereal Chemistry 7*, 282-287.

Kasemsuwan, T. & Jane, J. 1995. Location of amylose in normal starch granules. II. Locations of phosphodiester cross-linking revealed by phosphorus ^{31}NMR. *Cereal Chemistry 72*, 169-176.
Klucinec, J., & Thompson, D. 1998. Fractionation of High-Amylose Maize Starches by Differential Alcohol Precipitation and Chromatography of the Fractions. *Cereal Chemistry 75*, 887-896.
Koch, K., Andersson, R. & Åman, P. 1998. Quantitative analysis of amylopectin unit chains by means of high-performance anion-exchange chromatography with pulsed amperometric detection. *Journal of Chromatography A 800*, 199-206.
Kok-Jacon, G., Qin, J., Vincken, J. & Visser, R. 2003. Towards a more versatile α-glucan biosynthesis in plants. *Journal of Plant Physiology 160*, 765-777.
Kossman, J. & Lloyd, J. 2000. Understanding and Influencing Starch Biochemistry. *Critical Reviews in Plant Sciences 19*, 171-226.
Krog, N. & Nybo-Jensen, B. 1970. Interaction of monoglycerides in different physical states with amylose and their anti-firming effects in bread. *Journal of Food Technology 5*, 77.
Kugimiya M. & Donovan J. 1981. Calorimetric Determination of the Amylose Content of Starches Based on Formation and Melting of the Amylose-Lysolecithin Complex. *Journal of Food Science 46*, 765-770, 777.
Kuipers, A., Jacobsen, E. & Visser, R. 1994. Formation and deposition of amylose in the potato tuber are affected by the reduction of granule-bound starch synthase gene expression. *Plant Cell 6*, 43-52.
Kulp, K. & Ponte, J. 1981. Staling of white pan bread: Fundamental Causes. *CRC Critical Reviews in Food Science and Nutrition 15*, 1-49.
Lim, S., Kasemsuwan, T. & Jane, J. 1994. Characterisation of phosphorus in starch by P nuclear magnetic resonance spectroscopy. *Cereal Chemistry 71*, 488-493
Lim, S. & Seib, P. 1993. Location of Phosphate Esters in a Wheat Starch Phosphate by ^{31}P-Nuclear Magnetic Resonance Spectroscopy. *Cereal Chemistry 70*, 145-152.
Longton, J. & LeGrys, G. 1981. Differential scanning calorimetry studies on the crystallisation of aging wheat starch gels. *Starch 33*, 410-414
Lorbert, R., Ritte, G., Willmitzer, L. & Kossmann, J. 1998. Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Natural Biotechnology 16*, 473-477
Manners, D. & Sturgeon, R. 1982. Reserve Carbohydrates of Algae, Fungi and Lichens. In *Plant Carbohydrates I. Intracellular carbohydrates*, edited by Loewus, F. & Tanner, W. Springer-Verlag Berlin Heidelberg, New York, pp 472-507.
Manners, D. 1989. Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers 11*, 87-112.
Matheson, N. 1996. The chemical structure of amylose and amylopectin fractions of starch from tobacco leaves during development and diurnally-nocturnally. *Carbohydrate Research 282*, 247-262.
McCready, R. & Hassid, W. 1943. The Separation and Quantitative Estimation of Amylose and Amylopectin in Potato Starch. *Journal of the American Chemical Society 65*, 1154-1157.
McGrane, S., Cornell, H. & Rix, C. 1998. A Simple and Rapid Colorimetric Method for the Determination of Amylose in Starch Products. *Starch 50*, 158-163.
Miles, M., Morris, V., Orford, P. & Ring, S. 1985. The roles of amylose and amylopectin in the gelation and retrogradation of starch. *Carbohydrate Research 135*, 271-278.
Morrison, W. 1981. Starch Lipids: A Reappraisal. *Starch 33*, 408-410.
Morrison, W. & 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. 1988. Lipids in Cereal Starches: A Review. *Journal Cereal Science 8*, 1-15.
Morrison, W. & Karkalas, J. 1990. Starch. In *Methods in Plant Biochemistry*, vol 2, edited by Dey, P. Academic Press Ltd., London, UK, pp 323-352
Morrison, W. 1995. Starch lipids and how they relate to starch granule structure and functionality. *Cereal Foods World 40*, 437-446.

Mouille, G., Maddelein, M., Libessart, N., Tagal, P., Decq, A., Deirue B. & Ball S. 1996. Pre-amylopectin processing. A mandatory step for starch biosynthesis in plants. *Plant Cell 8*, 1353-1366.

Muhrbeck, P. & Tellier, C. 1991. Determination of the Phosphorylation of Starch from Native Potato Varieties by ^{31}P-NMR. *Starch 43*, 25-27.

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

National Research Council. 1989. *Lost Crops of the Incas: Little-known Plants of the Andes with Promise for Worldwide Cultivation*. National Academy Press, Washington, D.C. pp. 27, 47, 83.

Nelson, O. & Rines, H. 1962. The enzymatic deficiency in the waxy mutant of maize. *Biochemical and Biophysical Research Communications 9*, 297-300.

Nielsen, T., Wischmann, B., Enevoldsen, K. & Møller, B. 1994. Starch phosphorylation in potato tubers proceeds concurrently with de novo biosynthesis of starch. *Plant Physiology 105*, 111–117.

Oostergetel, G. & Bruggen, E. 1993. The crystalline domains in potato starch granules are arranged in a helical fashion, *Carbohydrate Polymers 57*, 211-224

Orford, P., Ring, S., Carroll, V., Miles, M. & Morris, Y. 1987. The effect of concentration and botanical source on the gelation and retrogradation of starch. *Journal Science of Food and Agriculture 39*, 169-177.

Peng, M., Gao, M., Båga, M., Hucl, P. & Chibbar, R. 2000. Starch branching enzymes preferentially associated with A-type starch granules in wheat endosperm. *Plant Physiology 124*, 265-272.

Pereira, A. 1995. O valor nutritivo da mandioquinha-salsa. V Encontro Nacional Sobre Mandioquinha-Salsa. Palestras e Trabalhos Técnicos, Fornazier, M., & Carmo, A. eds. Venda Nova do Imigrante-Espírito Santo, Sociedade de Olericultura do Brasil.

Pérez, E., Breene, W., & Bahnassey, Y. 1998. Gelatinisation profiles of Peruvian carrot, cocoyam and potato starches as measured with the Brabender visco-amylograph, rapid visco-analyzer, and differential scanning calorimeter. *Starch 50*, 14-16.

Preiss, J. & Levi, C. 1980. Starch biosynthesis and degradation. In *The Biochemistry of Plants, Carbohydrates Structure And Function*, edited by Preiss J. Academic Press, San Diego, vol 3, pp 371-423.

Radwan, M. & Stocking, C. 1957. The isolation and characterization of sunflower leaf starch. *American Journal of Botany 44*, 682-686.

Ravindran, V., Ravindran, G. & Sivalogan, S. 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. *Food Chemistry 50*, 133-136.

Riaz, M. 1999. Processing biodegradable packaging material from starches using extrusion technology. *Cereal Foods World 44*, 705-709.

Ridout, M., Gunning, A., Parker, M., Wilson, R. & Morris, V. 2002. Using AFM to image the internal structure of starch granules. *Carbohydrate Polymers 50*, 123-132.

Ring, S. 1985. Some Studies on Starch Gelation. *Starch 37*, 80-83.

Ring, S., Colonna, P., I'Anson, K., Kalichevsky, M., Miles, M., Morris, V. & Orford, P. 1987. The gelation and crystallisation of amylopectin. *Carbohydrate Research 162*, 277-293.

Ritte, G., Eckermann, N., Haebel, S., Lorberth, R., Steup, M. 2000. Compartmentation of the Starch-Related R1 Protein in Higher Plants. *Starch 52*, 179-185.

Rutschman, M. & Solms, J. 1990. Formation of inclusion complexes of starch with different organic compounds. II. Study of ligand binding in binary model systems with decanal, 1-naphtol, monostearate and monopalmitate. *Lebensmittel-Wissenschaft und Technologie 23*, 70-79.

Rydberg U., Andersson L., Andersson R., Åman P. & Larsson, H. 2001. Comparison of starch branching enzyme I and II from potato. *European Journal of Biochemistry 268*, 6140-6145.

Schwall, G., Safford, R., Wescott, R., Jeffcoat, R., Tayal, A., Shi, Y.-C., Gidley, M. & Jobling, S. 2000. Production of very-high-amylose potato starch by inhibition of SBE A and B. *Nature Biotechnology 18*, 551-554.

Shi, Y. & Seib, P. 1992. The structure of four waxy starches related to gelatinization and retrogradation. *Carbohydrate Research 227*, 131-145.
Shi, Y., Seib, P. & Bernardin, J. 1994. Effects of temperature during grain-filling on starches from six wheat cultivars. *Cereal Chemistry 71*, 369-383
Shogren, R., Fanta, G. & Doane, W. 1993. Development of Starch-Based Plastics – a Re-examination of Selected Polymer Systems in Historical Perspective. *Starch 45*, 276-280.
Silverio, J., Svensson, E., Eliasson, A.-C. & Olofsson, G. 1996. Isothermal calorimetric studies on starch retrogradation. *Journal of Thermal Analysis 47*, 1179-1200.
Slattery, C., Kavakli, H. & Okita, T. 2000. Engineering starch for increased quantity and quality. *Trends in Plant Science 5*, 291-298.
Smith, A. & Denyer, K. 1992. Transley review No. 39: Starch synthesis in developing pea embryos. *New Physiology 122*, 21-33.
Smith, A. 1999. Making starch. *Current Opinion in Plant Biology 2*, 223-229.
Smith, A. 2001. The biosynthesis of starch granules. *Biomacromolecules 2*, 335-341.
Smith, A., Zeeman, S., Thorneycroft, D. & Smith, S. 2003. Starch mobilization in leaves. *Journal of Experimental Botany 54*, 577-583.
Snyder, E. 1984. Industrial microscopy of starches. In *Starch - Chemistry and Technology*, eds. Whistler, R., BeMiller, J. & Paschall, E. Academic Press, London, pp 662-663.
Steeneken, P. 1989. Rheological properties of aqueous suspensions of swollen starch granules. *Carbohydrate Polymers 11*, 23-47
Steup, M. 1988. Starch degradation. In *Biochemistry of Plants*, edited by Preiss, J., vol 14, Academic Press, New York, pp 255-296.
Steventon, A., Parkinson, C., Fryer, P. & Bottomley. 1990. The Rheology of Yogurth. In *Rheology of Food, Pharmaceutical And Biological Materials With General Rheology*, edited by Carter, R. Elsevier Applied Science, London and New York, pp 196-210.
Svegmark, K. 1992. Potato *Starch Dispersions. Structure and Rheology*. PhD Thesis. Chalmers Biblioteks Reproservice. Göteborg.
Swinkels, J. 1985. Composition and properties of commercial and native starches. *Starch 37*, 1-5
Takaha, T., Yanase, M., Okada, S. & Smith, S. 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.
Takeda, Y. & Hizukuri, S. 1981. Re-examination of the action of sweet potato beta-amylase on phosphorylated (1-4)-α-D-glucan. *Carbohydrate Research 89*, 174-178.
Takeda, Y. & Hizukuri, S. 1982. Location of phosphate groups in potato amylopectin. *Carbohydrate Research 102*, 321-327.
Tester, R. 1997. Starch: the polysaccharide fractions. In *Starch Structure and Functionality*, eds. Frazier, J., Richmond, P. & Donald, A. The Royal Society of Chemistry, Bookcraft, Cambridge, UK, pp 163-171.
Thitipraphunkul, K., Uttapap, D., Piyachomkwan, K. & Takeda, Y. 2003. A comparative study of edible canna (*Canna edulis*) starch from different cultivars. Part I. Chemical composition and physicochemical properties. *Carbohydrate Polymers 53*, 317-324.
Thompson, D. 2000. On the non-random nature of amylopectin branching. *Carbohydrate Polymers 43*, 223-239.
Tomlison, K., Lloyd, J. & Smith, A. 1997. Importance of isoforms of starch branching enzyme in determining the structure of starch in pea leaves. *The Plant Journal 11*, 31-43
Tomlison, K., Craig, J. & Smith, A. 1998. Major differences in isoform composition of starch synthase between leaves and embryos of pea (*Pisum sativum L.*). *Planta 204*, 86-92.
Torneport, L., Salomonsson, B. & Theander, O. 1990. Chemical characterization of bromine oxidized potato starch. *Starch 42*, 413-417.
Trethewey, R. & Smith, A. 2000. Starch metabolism in leaves. In *Advances in Photosynthesis. Photosynthesis: Physiology and Metabolism*, eds. Leegood, R., Sharkey, T. & von Caemmerer, S., vol 9, Kluwer Academic Publishers, Dordrecht, pp 205-231.
Upmeyer, J. & Koller, H. 1973. Diurnal trends in net photosynthesis rate and carbohydrate levels of soybean leaves. *Plant Physiology 116*, 341-381.

Villwock, V., Eliasson, A.-C., Silverio, J. & BeMiller, J. 1999. Starch-Lipid Interactions in Common, Waxy, ae du, and ae su2 Maize starches examined by Differential Scanning Calorimetry. *Cereal Chemistry 76*, 292-298.

Visser, R., Suurs, L., Bruinenberg, P., Bleeker, I. & Jacobsen, E. 1997. Comparison Between Amylose-free and Amylose Containing Potato Starches. *Starch 49*, 438-443.

Waigh, T., Donald, A., Heidelbach, F., Riekel, C. & Gidley, M. 1999. Analysis of the Native Structure of Starch Granules with Small Angle X-Ray Microfocus Scattering. *Biopolymers 49*, 91-105.

Waigh, T., Kato, K., Donald, A., Gidley, M., Clarke, C. & Riekel, C. 2000. Side-Chain Liquid-Crystalline Model for Starch. *Starch 52*, 450-460.

Ward, K., Hoseney, R. & Seib, P. 1994. Retrogradation of amylopectin from maize and wheat starches. *Cereal Chemistry 71*, 150-155.

Watanabe, Y., Nakamura, Y. & Ishii, R. 1997. Relationship between Starch Accumulation and Activities of the Related Enzymes in the leaf Sheath as a Temporary Sink Organ in Rice (*Oryza sativa*). *Australian Journal of Plant Physiology 24*, 563-569.

Wattebled, F., Ral, J.-P., Dauvillée, D., Myers, A., James, M., Schlichting, R., Giersch, C., Ball, S. & Hulst, C. 2003. STA11, a *Chlamydomonas reinhardtii* Locus Required for Normal Starch Granule Biogenesis, Encodes Disproportionating Enzyme. Further Evidence for a Function of α-1,4 Glucanotransferases during Starch Granule Biosynthesis in Green Algae. *Plant Physiology 132*, 137-145.

White, P. Abbas, I. & Johnson, L. 1989. Freeze-thaw stability and refrigerated-storage retrogradation of starches. *Starch 41*, 176-180

Wong, R. & Lelievre, J. 1982. Effects of storage on dynamic rheological properties of wheat starch pastes. *Starch 34*, 231-233

Yoo, S.-H., Jane, J.-L. 2002. Molecular weight and gyration radii of amylopectin determined by high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors. *Carbohydrate Polymers 49*, 307-314

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

Zeeman, S., Northrop, F., Smith, A. & Rees, T. 1998b. A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch-hydrolysing enzyme. *The Plant Journal 15*, 357-365.

Zeeman, S., Tiessen, A., Pilling, E., Kato, L., Donald, A. & Smith, A. 2002. Starch synthesis in Arabidopsis. Granule Synthesis, Composition and Structure. *Plant Physiology 129*, 516-529.

Zhu, Z., Hylton, C. Rossner, U. & Smith A. 1998. Characterisation of starch-debranching enzymes in pea embryos. *Plant Physiology 118*, 581-590.

Zobel, H., French, A. & Hinkle, M. 1967. X-ray diffraction of oriented amylose fibres. II. Structure of V amyloses. *Biopolymers 5*, 837-845.

Zobel, H. 1988. Starch crystalline transformations and their industrial importance. *Starch 40*, 1-7.

Acknowledgements

I want to thank Per Åman, my main supervisor, for giving me the opportunity to continue my education and make my view of the world of research much wider. Moreover, besides my work, the personal experiences of these four years have enriched my life and those memories will stay in my mind and my heart for a long time.

Roger, my other supervisor. You always had time when I came with questions. You were always in a nice mood.

My colleagues at the department for the everyday life during working hours.

PhD students Lena A., Lena S., Viktoria, Karin, Lars (Stora pojke), Anders, Eva G., Galia, Sabine, Anke, Madelene, Ali, Jan, Maria Å., Anna W., Anakalo, Elin, Linnea, Susanne, Maria L., Maria S., Jan, Tuomo, Maria S.

Lotta for helping me with my Swedish, especially for making an effort and not speaking skånska.

Einar, such a nice and warm person.

Eva Eriksson, thanks for your sincerity and your friendly smile.

Rolf Andersson from the Department of Chemistry for your kindness during my work with the NMR.

To my roommates: Alastair, Lena R. and Christina. Lena, thanks for your friendship, the smiles, conversations, remember to keep in touch. Christina (lorita), a genuine smile, I like you very much my friend.

Tebe, Shahid, Nina, Bee, Tuk, for the nice times when we met in the corridor.

Every person needs something to get balance in life. One of the things that complemented my life during these four years was the time I worked on radio. Thanks to my colleagues from the Latin-American radio: Cheo, for giving me the opportunity to work there, Cheli, Alvaro, Martín, Ramiro, Alex, Francisco. To the people who helped me make the radio programme, coming even when the weather was cold, snowy or rainy. Thanks Celia, Luis, Manu, Åsa, Rebecka, Mariana, Natasha, Sofia, Cecilia.

I was lucky to find persons who care about me. Kristine, Patricia T., Annu, Catalina, Ingrid. Thanks for being there, always ready to listen to me, ready to help me with your words. I think I was too egoist, thinking just about my problems and not yours. You are in a special place of my heart.

Thanks to my Swedish family: Ingrid, Gunnar, Barbro, Kerstin, Anders, Gustav and Melker. I felt at HOME when I stayed with you. Ingrid, your friendship, your big heart, your sincerity; I do not want to lose them! Let's be in touch always.

Some of my friends in Uppsala: Natasha, Carina, Octavio, Kristin, Adrian, Luca, Steffan, Marco, Susanna. Your friendship means very much to me.

Maja.

Ece and Jenny. Thanks for making a place in your home whenever I needed it.

Joachim and Anna Karin. I had a lot of fun with you. Thanks for the great times we had in Lund, Karlskoga, Uppsala. I especially remember the time we were driving around in Karlskoga with your brother trying to see an elk.

Mi prima Patricia. Que hubiese hecho sin ti, sin tu ayuda, sin tus palabras. Gracias por compartir mi felicidad y mi tristeza.

My sister Priscila, the sweet company during all the time I have been abroad. You are the one who kept my feet on the ground, my little sister; I love you and miss you so much. Cristian, my little brother and my friend. I miss you a lot. I miss the times we shared in Uppsala during the spring of 2003. Hubiese querido compartir mas tiempo con los dos, estar allí cuando me necesitaban. Pero sepan que tienen un hermano, aunque lejos, los ama y quiere su felicidad. Estoy siempre aquí para los dos.

Mamá y papá, se que debe ser duro ver a un hijo partir, pero quiero que sepan que los tengo en mi corazón. Cualquiera sea el lugar donde este, siempre seguiremos siendo una familia. Gracias por enseñarme a ser quien soy. Los amo con todo mi corazón.