SUMMARY

Starch comprises two carbohydrate polymers: amylose and amylopectin. Amylose consists of a largely linear chain of α-1,4-linked glucose units, whereas amylopectin is highly branched. Amylopectin is built of short linear chains which are interconnected by α-1,6-bonds, forming branch points. The branch points are clustered along the molecule. Since the branch points are concentrated in one half of the cluster, the other half comprises the linear parts of the chains. The molecules are radially oriented in the granule with their growing ends pointing to the surface. Linear chains of adjacent amylopectin molecules are crystallized into a superhelical structure.

Starch, as found in potato tubers and cereals, is synthesized in amyloplasts, an organelle bounded by two membranes. Inside the amyloplasts the starch granules are formed, surrounded by a thin layer of stroma.

The final steps in the biosynthesis of starch are catalyzed by ADPglucose pyrophosphorylase (AGPase), starch synthase and branching enzyme. AGPase catalyzes the formation of ADPglucose. Starch synthase elongates linear chains with the use of glucose residues from ADPglucose. Two forms of starch synthase are distinguished in higher plants: soluble starch synthase (SSS) and granule bound starch synthase (GBSS). Although in essence both enzymes catalyze the same reaction, they play distinct roles in starch synthesis. Most plants contain two soluble starch synthases (SSS-I and SSS-II) and one GBSS. Finally, branching enzyme catalyzes the formation of branch points by breaking one of the α-1,4-bonds in a chain and subsequently linking the severed fragment to a similar chain via an α–1,6-bond.

The formation of amylose is dependent on the presence of GBSS only. Starch mutants lacking GBSS do not form amylose, although the formation of amylopectin is not hampered. Amylopectin is formed by the concerted action of soluble starch synthases and branching enzymes. Amylose and amylopectin are therefore formed via separate routes.

Although amylose is not branched *in vivo*, branching enzyme is capable of branching amylose *in vitro*. This raises the question, therefore, why amylose is not branched *in vivo* as well. A possible explanation would be that the synthesis of amylose is spatially separated from the synthesis of amylopectin.

Evidently, there are still questions concerning the synthesis of starch *in vivo*, which cannot be answered by studying the activity of isolated enzymes *in vitro*. One of the questions we tried to solve is where the starch synthesizing enzymes are active, by determining their subcellular location. First, the localization of AGPase, branching enzyme and GBSS was studied in potato tubers. In addition the localization of AGPase in barley endosperm was studied.

The enzymes were localized with the use of immunoelectron microscopy (IEM). With this technique proteins in tissue sections are labeled with the use of specific antibodies

coupled to colloidal gold as a probe. The resolution of this technique is 25-40 nm, which enables us to determine the localization of proteins inside the amyloplasts. For IEM not only specific antibodies are needed, but also ultrathin tissue sections with preservation of ultrastructure as well as antigenicity. A suitable method for the preparation of starch storage tissue was not yet available and had to be developed. Specific antibodies were kindly provided by others.

In chapter 2 the suitability of different methods for the preparation of ultrathin sections of starch storage tissue is discussed. Ultrathin sections for electron microscopy (~100 nm in thickness) must withstand the electron beam and the vacuum in the microscope. This usually means that the ultrastructure of fresh tissue is stabilized (fixation), dehydrated with an organic solvent (dehydration), and finally embedded in a resin. From the resultant hard block, containing the tissue, ultrathin sections are then cut. For every tissue and concomitant problem under study, a suitable preparation method has to be developed.

Starch granules consist largely of carbohydrates. However, for starch no chemical fixatives are known which are also compatible with IEM. Starch granules swell and shrink, dependent on the amount of water incorporated. During a conventional preparation method, as described above, the granules shrink upon dehydration and become embedded in this state. Upon sectioning, however, the starch granules come into contact with water and swell again. The swollen granules will then fold and cover other parts of the section. The result can be very messy and unsuitable for further research. In developing a preparation method for starch storage tissue we have tried to avoid or minimize shrinking and swelling of the starch granules.

The problems associated with starch are directly related to the size of the starch granules. Mature potato tubers contain starch granules up to 100-200 µm. Using small developing potatoes, containing smaller granules, would therefore be an advantage. In addition, we used potato microtuber. Microtubers are *in vitro* grown tuber of approximately 1 cm in diameter, containing smaller cells and also smaller starch granules (10-20 µm). These microtubers were very suitable for electron microcopy. Small developing tuber, with a fresh weight of approximately 20 g, could also be used. Tissue of large tubers was difficult to embed. Further studies were, therefore, done with small developing tubers and potato microtubers.

For ultrastructural research, fresh tissue was fixed with glutaraldehyde and osmiumtetroxide, dehydrated in a graded series of ethanol and embedded in Epon. The embedded material had to be trimmed down carefully to avoid pulling starch granules from the block. Cutting ultrathin sections of material embedded in Epon was relatively easy. The ultrastructure of the cells was well preserved. Subsequently a suitable embedding method for IEM was developed. The use of freeze-substitution and cryosectioning was compared to more conventional embedding methods. Material was embedded in Lowicryl K4M or LR White.

The first step in freeze-substitution is a physical fixation by means of freezing. The ice in the tissue is then substituted by an organic solvent (dehydration), followed by embedding in the resin of choice. Additional chemical fixation is optional. The advantage of this method is an excellent preservation of ultrastructure. Membranes in freeze-substituted tissue usually appear smooth and turgid. Freezing the samples is, however, a critical step. It is essential that upon freezing the water in the cells is vitrified. Vitrification means that water turns into amorphous ice and that no ice crystals are formed. Freezing is therefore done by ultrarapid freezing at atmospheric pressure or by high-pressure freezing. Recrystallization of amorphous ice during the follow-up procedure must be avoided. For freezing potato tuber tissue we have used high-pressure freezing. With this method, in principle, tissue blocks up to 500 µm in thickness may be vitrified. The frozen tissue was then substituted according to different protocols. Unfortunately the tissue was badly damaged, due to ice crystal formation. Whether crystallization of ice had occurred during freezing or the follow-up procedure, is not known. It is probable, however, that the large watery cells of potato tubers cannot be frozen without freezing-damage.

For cryosectioning, fresh tissue is chemically fixed, infused with sucrose, frozen in liquid nitrogen and cut at -100°C. The sucrose works as a cryoprotectant and facilitates ultrathin sectioning at low temperatures. The frozen sections are subsequently thawed, labeled, and embedded on-grid. This method does not involve dehydration and is therefore, in theory, ideal for starch. It proved to be impossible, however, to cut intact cryosections of potato tuber tissue. The cells, consisting of amyloplasts surrounded by a large vacuole, are probably unsuitable for cryosectioning. Cryosections of isolated starch granules did stay intact and could be used for ultrastructural research.

Tissue embedded at low temperature in Lowicryl K4M resulted in suitable sections. Tissue was fixed in glutaraldehyde, dehydrated in alcohol under progressive lowering of the temperature to -30°C, an embedded in Lowicryl K4M at that temperature. The ultrastructure of the cells was well preserved, although the sections were fragile. The starch granules tended to detach from the surrounding tissue. This problem could be largely overcome by cutting slightly thicker sections, i.e. 150 nm in thickness, and retrieving the sections quickly form the water surface. Tissue embedded in LR White at 4°C was also suitable for ultrathin sectioning. Structural details were, however, less well preserved in LR White than in Lowicryl. LR White, on the other hand, is user friendly, easy to section, and the starch does not detach as easily. For localization of starch granule bound proteins, therefore, LR White may be a better choice.

Chapter 3 describes the localization of branching enzyme and AGPase in potato tubers, embedded in Lowicryl K4M, with antibodies raised against denatured potato branching enzyme (kindly supplied by dr. G. Vos-Scheperkeuter and dr. A.S. Ponstein) and native spinach leaf AGPase (kindly supplied by dr. J. Preiss). Branching enzyme was located in the stroma of the amyloplasts, concentrated at the surface of the growing starch granule.

AGPase, on the other hand, was evenly distributed over the stroma. Branching enzyme was also found in a membrane-bounded inclusion body in the stroma. The specific location of branching enzyme at the granule surface indicates that branching enzyme is active at that surface.

In chapter 4 the localization of GBSS is described. The crude antiserum, raised against denatured GBSS (kindly supplied by dr. R. Visser), reacted strongly with GBSS on a Western blot. The corresponding preimmune serum showed no reaction. On sections of wild-type tubers a strong labeling of the starch was seen and, to a lesser extent, also of the cytoplasm and the phytoferritin in the stroma. Furthermore, a strong labeling of a crystalline structure in the cytoplasm was found. However, the crude antiserum also reacted strongly with the starch granules of amylose-free tubers. The starch of this mutant does not contain GBSS and therefore labeling of amylose-free starch, and possibly also of wild-type starch, could not be specific. To check whether labeling of wild-type starch was specific for GBSS, the crude antiserum was purified. Affinity-purified antibodies, specific for GBSS, did not react with the starch, although labeling in the rest of the cell was similar to that found with crude antiserum. This result suggested that the crude antiserum contained antibodies with affinity for another granule bound protein or the starch matrix. Since the crude antiserum did not react with other proteins on a Western blot, the second possibility was more likely. This hypothesis was then tested by incubating crude antiserum with amylose-free starch. After pelletting the starch granules, the supernatant was used for labeling experiments. The results with starch-purified antiserum were comparable to those found with affinity-purified antibodies. We concluded, therefore, that the crude antiserum contained antibodies with affinity for starch as well as GBSS, leading to a false positive result. The implications of these results for the work of others is discussed. We have not been able to locate GBSS with purified antibodies.

In chapter 5 the localization of AGPase in barley endosperm is described. Recent publications indicate that AGPase in the endosperm of cereals is located in the cytoplasm, although it was assumed that all AGPases are located in plastids. Barley seeds of different developmental stages were embedded in Epon. It proved that barley seeds harvested 15 days after flowering (15 DAF) were good material for electron microscopy. For immuno-localizations 15 DAF barley seeds were embedded in Lowicryl K4M at -30°C with the use of progressive lowering of the temperature. Sections were labeled with antibodies specific for the small subunit of spinach leaf AGPase (kindly supplied by dr. J Preiss and dr. L.A. Kleczkowski). Label was found in the stroma of amyloplasts in the endosperm but also in the cytoplasm of endosperm cells. Since the corresponding preimmune serum was not available, other controls were sought. For comparison, cells in the pericarp of barley seeds, present in the same section as the endosperm cells, were used as controls. In addition, the antibodies were tested on potato tuber cells. Only in cells of the endosperm, label was found in the stroma as well as the cytoplasm. In cells of the pericarp and in potato tuber

cells, label was confined to the plastids. We conclude, therefore, that in the endosperm of barley seeds AGPase is located in the amyloplast stroma as well as in the cytosol. This means that ADPglucose is probably formed in the cytosol and transported into the amyloplasts.

In chapter 6 a preliminary model for the biosynthesis of starch *in vivo* is presented using the results of the localization experiments and our present knowledge of starch ultrastructure and biosynthesis. From the localization of branching enzyme we conclude that the synthesis of amylopectin occurs predominantly at the surface of the growing granule. The soluble starch synthases, providing the substrate for branching enzyme, are probably also active at the granule surface.

Amylopectin has a polymodal chain length distribution, which means that it contains chains with specific lengths. The formation of different chain lengths was always attributed to the specific action of branching enzymes. However, recent publications on starch mutants of the green alga *Chlamydomonas reinhardtii,* indicate that the soluble starch synthases are responsible for a polymodal chain length distribution.

Although we were not able to localize GBSS in the starch granule it is probable that GBSS is more or less evenly distributed throughout the granule. The synthesis of amylose, however, is likely to be confined to the outer layer of the growing granule, since ADP-glucose is formed in the stroma. GBSS may elongate amylopectin molecules inside the granule. The granule bound location of GBSS makes the newly formed amylose inaccessible to branching enzyme.

We propose that the synthesis of amylopectin and the crystallization of the linear chains is one process. The concerted action of the different starch synthesizing enzyme is therefore directly responsible for the formation of starch ultrastructure. Changes in the synthesis of amylopectin influences the structure of starch. When the formation of a proper granule is disturbed, the synthesis of amylose and amylopectin is probably also affected.