Chapter 3
Imaging inclusion complex formation in starch granules using confocal laser scanning microscopy

The tendency of amylose to form inclusion complexes with guest molecules has been an object of wide interest due to its fundamental role in food processing. Here we investigated the interactions between amylose contained in starch granules from different botanical sources and fluorescent lipophilic molecules below gelatinization temperature.

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3.1 Introduction

As already mentioned in Chapter 1.2 starch is the main reserve of carbohydrates for humans[1] and in aggregate the second most prevalent biopolymer present on earth after cellulose.[2] In the last decades starch and its functional properties have been the object of intensive research due to its important role in the food and non-food industries.[3,4]

Native starch, which semi-crystallizes in water-insoluble granules, consists mainly of two polymers of D-glucose, amylopectin and amylose, with varying relative ratios; high amylose starch can present an amylose content up to 85%, while so called waxy starch may reach up to 100% amylopectin content.[5] Depending on the botanical source, amylose and amylopectin are arranged in semi-crystalline granules of specific size, shape and morphology.[6]

It has been demonstrated that the amylose contained in starch in presence of a ligand like iodine or linear alcohols tends to assume a compact helical conformation that can complex with the ligand,[7,8] giving rise to the so-called “V-amylose”. V-amylose is structured as a left-handed single helix with a hydrophobic cavity that is able to host the apolar aliphatic chain of a ligand, while the polar head tends to stay outside the amylose coil.[9,10] The driving forces of this complex formation have been identified as determined by weak intramolecular bonds (van der Waals forces and hydrogen bonds), which take place between the turns of the amylose helix[11,12] and intermolecular forces to stabilize the complex between the amylose chain and the ligand. However, many factors regarding the inclusion complex formations are still under debate. In particular, many studies have investigated the hydrophobic effect, which seems to be the leading force between the guest and the host.[9]

Amylose inclusion complex formation is important for food processing as it can lead to reduced stickiness, improved freeze–thaw stability and an antistaling effect in bread and biscuits due to reduced crystallization (retrogradation) of the amylopectin fraction in starch. Several studies have focused on the guest molecules in the complex formation with starch amylose, such as iodine in solution,[13,14] potassium hydroxide,[15] and dimethyl sulfoxide (DMSO).[10] and have shown that the diameter of the amylose helix is strongly correlated to the ligand participating in the complex.

Among guest molecules, lipids such as fatty acids and phospholipids have been investigated due to their influence on the gelatinization of the granular starch after the complexation with amylose, which is naturally present in the starch granules structure.[16] Although some levels of lipids are present in native starch, they are often added as emulsifiers in order to tailor some properties and to improve the
quality of the starch-related food. Among others, the amylose-lipid inclusion complex has been demonstrated to have an influence on the staling. Recently, the inclusion complex formation between amylose in native wheat starch and lysophosphatidylcholine (LPC) was reported, underlying the effects of such inclusion on the structural properties and the susceptibility to amylase of starch.

A fundamental aspect of the inclusion complex formation is the length of both the amylose and the lipid chains. Long amylose chains can form inclusions with more ligands and the length of the aliphatic chains of the lipids can vary. Recently, a study of the complex formation between amylose brushes on a gold surface and fatty acids showed that octanoic (C8) acid can form inclusion complex more efficiently than myristic acid (C14).

Optical Microscopy is one of the most exploited techniques for the visualization of the starch granules. As already mentioned in the previous Chapter, Confocal Laser Scanning Microscopy (CLSM) is an especially powerful tool for its ability to obtain both 2-dimensional (2D) and 3-dimensional (3D) images of the granules by optically sectioning them and revealing their inner structure. Moreover, fluorescence CLSM can allow for the study of specific interactions when the chromophore used as marker has chemical or biological functionalities. The interaction between different amphiphilic dyes and waxy and regular wheat starch has previously been investigated using CLSM showing that the penetration of amphiphilic dyes is more effective through the waxy starches than through regular ones.

In this chapter we investigated the inclusion complex formation between amylose in starch granules from different botanical sources and lipophilic molecules labeled with fluorescein. These molecules are composed of an aliphatic chain and a polar head, consisting of a carboxylic group and the fluorescein dye. Using Confocal Laser Scanning Microscopy, the fluorescent labels allowed us to study with high spatial resolution the localization on the starch granules of the labeled aliphatic chains. In this way we unraveled the features of different starch granules and established that at low lipid-dye concentration even below the gelatinization point complexation of the lipid with the amylose located at the periphery of the granules is occurring. Control experiments performed with the waxy granules and with fluorescein confirm this finding.
3.2 Materials and Methods

Maize starch, waxy maize starch, potato starch, lugol solution for microscopy, fluorescein (C₂₀H₁₄O₅) with a molecular weight (Mₘ) of 332.31 g/mol and fluorescein octadecyl ester [chromoionophore XI] (C₃₈H₄₈O₅) with a Mₘ of 584.78 g/mol were purchased from Sigma-Aldrich (St. Louis, USA). Waxy potato starch (Eliane 100) was supplied by AVEBE FOOD (Veendam, The Netherlands). 5-Hexadecanoylaminofluorescein (C₃₆H₄₃N₂O₆) with a Mₘ of 585.74 g/mol was purchased from Life Technologies (Waltham, USA). Dimethylformamide (DMF) extra pure was purchased from Acros-Organics (Hampton, USA).

Starch suspensions were prepared at a concentration of 2% in distilled water with 0.02% sodium azide. The starch granules were stained by adding 50 or 100 µl lugol reagent to 1 ml of the starch suspension.

Starch suspensions for CLSM were prepared at a concentration of 2% in distilled water with 0.02% sodium azide as a preservative. 1 mL of the prepared suspensions was stained by 20 µl of one of the molecular probes, as the fluorescence dye by rotating overnight at room temperature in the dark. Dimethylformamide (DMF) was employed as stock solvent for the dye. Consequently, to correct for the difference in molar mass of the molecular probes, a 0.02% dye solution was prepared based on chromoionophore XI or 5-Hexadecanoylaminofluorescein and a 0.0114% dye solution based on fluorescein (respectively 0.02% and 0.0114% dye based on starch). For CLSM imaging 40 µl of the stained samples were transferred to an object glass.
3.3 Results and discussion

In order to obtain insight into the inclusion complex formation between amylose chains in starch granules and lipids, we performed Confocal Laser Scanning Microscopy (CLSM) measurements on samples from different botanical sources: potato, wheat and maize.

Potato starch granules suspended in water solution were exposed at room temperature to two lipophilic fluorescent molecules, 5-hexadecanoylaminofluorescein (lipid-dye 1) and fluorescein octadecyl ester (lipid-dye 2). The lipid-dye 1 and 2 are characterized by a polar group consisting of a fluorescein molecule bound to an aliphatic chain of 15 and 18 carbons, respectively (see Scheme 3.1). By detecting the photoluminescence emission from the dye, the inclusion of the aliphatic chain into the amylose helix could be monitored with high spatial resolution.

![Scheme 3.1: Chemical structures of lipid-dye 1 and lipid-dye 2.](image)

Figure 3.1 shows the normalized photoluminescence spectra of the two lipid-dye molecules in DMF solution.

The lipid-dye 1 emission presents a main peak at 530 nm; the lipid-dye 2 emission is broader and more structured with a main peak at 560 nm and a shoulder at 530 nm.
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**Figure 3.1:** Photoluminescence spectra of 5-hexanoylaminofluorescein (lipid-dye 1) and fluorescein octadecyl ester (lipid-dye 2) in DMF solution. Excitation wavelength 380 nm.

**Figure 3.2** shows CLSM micrographs of potato starch granules in a water-based suspension after addition of 0.02% lipid-dye 1 (a) and 0.02% lipid-dye 2 (b). The spatial resolution of the images was estimated as <300 nm.

In both cases, the images show a bright rim area around the granules, revealing the tendency of lipid-dye molecules to link to the outer part of the granules, rich in amylose chains.

It is important to emphasize that the formation of the bright rim occurs at room temperature, well below the gelatinization temperature of starch, suggesting the high efficiency of this process at low concentration of the lipid-dye used for the complexation (see above). The core of the granules remains dark, inferring that the lipid-dye molecules do not interact with the inner structures of the granules due to the protective coating generated by the lipid-dye molecules already linked to the outer part of the starch granule, or that penetration is hindered by their bulky size and presence of the polar head. From this evidence, we concluded that in the case of un-gelatinized potato starch granules lipid-dye molecules at low concentration selectively complex with the outer amylose chains of the granules, as previously reported by our group as well as others. [8,19–21,28]
Figure 3.2: CLSM images (field of view: 140 µm x 140 µm) of potato starch granules in water suspension after addition of 5-hexadecanoylaminofluorescein (a) and fluorescein octadecyl ester (b). The suspensions were rotated overnight at room temperature in a dark environment. Micrographs were recorded exciting the sample at 488 nm.

In the case of the sample prepared with the lipid-dye 1 (Figure 3.2a) the micrograph shows that the granule’s contours appear bright and sharp. However, when the lipid-dye 2 molecules (Figure 3.2b) were added to the starch suspension the rims appear less defined, revealing also the presence of aggregates in the peripheral zones of the granules. In the second case the background also presents a diffuse emission, suggesting that not all the lipid-dye molecules were involved in the complex formation with the starch granules. Such divergent behaviour of the two molecules can be explained by a better solubility in the water-based suspension of 5-hexanodecanoylaminofluorescein molecules due to the shorter aliphatic apolar chain with respect to fluorescein octadecyl ester. A further possible explanation is the faster complex formation with the lipid-dye 1 due to the relative lengths of the host and lipid-dye chain (15 C).[^23]

Consequently, we focused our attention on the study of the complex formation mechanism between different types of starch granules and the lipid-dye 1.

In order to establish which portion of the lipid-dye molecules is involved in the interaction with the starch granules, we performed a series of experiments and control experiments with the lipid-dye and the fluorescein dye.

Figure 3.3 shows CLSM images of potato (a, b), maize (c, d) and wheat (e, f) starch granules after addition of 5-hexanodecanoylaminofluorescein (a, c, e) and
pure fluorescein dye molecules (b, d, f). These experiments were carried out using the same relative ratios of starch granules and dye molecules for all the samples.

Despite the different features of granules belonging to different botanical sources, Figures 3.3a, 3.3c, 3.3e confirm the ability of peripheral amylose chains to form complexes with the lipid-dye 1 molecules at room temperature. All samples presented a strong emission contrast between the contour and the core of the granules, implying that in all three cases the same process was occurring.

Potato starch granules (Figure 3.3a) are characterized by an oval shape and have diameters ranging between 10 and 100 µm and a smooth surface. In the micrograph, together with the granules complexed only on their surface, a damaged granule can be seen in which the lipid-dye molecules have access to the inner part—spotlighting its internal structure—with alternating rings of amylose and amylopectin. These appear to be of different brightness, probably because of the varying interaction of the lipid-dye with the two macromolecules.

The maize starch granules (Fig. 3.3c) present a typical truncated shape with small channels (~ 1 µm) in which the lipid-dye can be absorbed (the channels are indicated in Fig. 3.3c by an arrow). Wheat starch granules are oval (Figure 3.3e), resembling the potato starch granules; however, Evers revealed in 1971 the disk or lenticular shape of wheat starch granules by means of Scanning Electron Microscopy (SEM).[^29] In this case most of the granules also show the complexation at the peripheral region of the granules, in addition to several damaged granules that show complexation with the lipid-dye inside.

As control experiment we used fluorescein dye instead of the lipid-dye. For all three botanical species (Fig. 3.3b, d and f) we revealed a very different behaviour with respect to that shown by the samples treated with the lipid-dye.

In the case of potato starch (Fig. 3.3b) the granules appeared as dark domains in a bright background, suggesting that the dye molecules remained in the solution without interacting with the granules. This observation is a further confirmation of the inclusion complex formation between lipid-dye 1 and amylose chains, indicating that the discriminant factor is the aliphatic chain present in 5-hexanodecanoylamino fluorescein. Fluorescein molecules are polar and therefore not driven towards the inside of the amylose chains, which is hydrophobic.

In the case of maize (Fig. 3.3d) and wheat (Fig. 3.3f) starch granules the interaction with the fluorescein dye was also dissimilar to the interaction with lipid-dye. In these two types of starch granules, fluorescein molecules appeared to be absorbed by the inner part of the granules, highlighting their structures. This is due to the fact that maize and wheat starch granules are characterized by a different porosity than potato starch, which allows the fluorescein molecules to penetrate inside these granules.[^30] Another interpretation involves the amount of lipids
naturally present in potato, maize and wheat starch. The common cereal starches (maize, wheat, rice) present a higher percentage of lipids (0.6 – 1.0%) compared with potato (0.05%).\cite{31,32} Fluorescein can be attracted and absorbed by the fatty acids due to its lipophilic nature; this can justify the presence of fluorescein inside cereal starch granules.

To obtain a further proof that the amylase in the starch granules is driving the lipid adsorption process, we proceeded in the preparation of identical samples with waxy potato starch. Waxy potatoes are characterized by starch granules that contain only traces of amylase (<1%) and > 99% of amylopectin.\cite{33}
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Figure 3.3: CLSM measurements of potato (a, b), corn (c, d) and wheat (e, f) starch granules after addition of 5-hexadecanoylaminofluorescein molecules (a, c, e) and fluorescein molecules (b, d, f). The suspensions were rotated overnight at room temperature in a dark environment. CLSM micrographs recorded exciting the sample at 488 nm.
Figure 3.4 presents CLSM micrographs of waxy potato starch granules after addition of the lipid-dye 1 (Fig. 3.4a) and the fluorescein dye (Fig. 3.4b). In the sample prepared with lipid-dye 1 molecules, the granules present a bright rim, which is not as defined or as sharp as in the regular inclusion complexed starch (Fig. 3.3a). The rim around the starch granules in this case appears broader (up to 5µm) and fuzzier, with the small granules almost completely stained. This finding can be explained by the lack of amylose in the periphery of the granules in contrast to the regular grains. It was observed that the lipid-dye molecules could penetrate through the granules due to interactions with the amylopectin chains. Experiments carried out on the same sample showed that the rim tends to grow with time, indicating that adsorption and diffusion of the lipid-dye molecules are the most probable phenomenon. Washings of the samples with DMF showed a full removal of the lipid-dye from the waxy starch, while the inclusion of the lipid-dye with regular granules was not affected by the washing.\textsuperscript{34}

The control experiment conducted with the fluorescein molecules (Fig. 3.4b) shows a diffused luminescence, indicating that in the case of the waxy potato starch the fluorescein molecules also does not penetrate into the starch granules. Comparison of the results displayed in Figure 3.3b and Figure 3.4b clearly shows that the lack of the aliphatic chain in the ligand produces the same result in both the regular and waxy starch granules, suggesting that the porosity of the two species are similar.

Figure 3.4: CLSM micrographs of waxy potato starch granules after addition of 5-hexanodecanoylaminofluorescein molecules (a), and fluorescein molecules (b). The suspensions were rotated overnight at room temperature in a dark environment. CLSM micrographs were recorded exciting the sample at 488 nm.
The inclusion complex formation with amylose, indicated by the appearance of a bright and sharp rim around the starch granule, occurs only when in the experiment both the amylose polymers and the aliphatic chain of the lipid-dye are involved.

The ability of amylopectin to form complexes when the chains are long enough has been widely debated.\textsuperscript{[35]} In order to investigate this process we performed CLSM and BFM measurements on waxy maize starch granules. Since waxy maize granules lack amylose, positive staining therein can be attributed to interaction with long chain amylopectin branches. In addition, the high porosity of maize granules enables the ligand to reach the hilum, where the amylopectin chains are known to be longer\textsuperscript{[36,37]} and therefore more suitable for complex formation.

Figure 3.5a presents a CLSM image of waxy maize starch granules treated as described previously with the lipid-dye 1 molecules. The granules show a sharp and bright spot corresponding to the hilum, with a diffuse luminescence all over the granule; this proves the interactions of the lipid dye with the long chain amylopectin branches localized in the hilum. Figure 3.5b shows a BFM image of waxy maize starch granules after addition of iodine molecules.\textsuperscript{[38]} The center of the granules also presents a bright and sharp spot. These findings demonstrate that the long amylopectin chains present in the center of the granules can preferentially generate complex formation with ligands. As a further control experiment, we treated the waxy maize granules with fluorescein (Figure 3.5c), which showed in this case a dark hilum. Therefore the micrographs shown in Figure 3.5 are a strong indication that the lipid chains can selectively interact with longer amylopectin chains.

\textbf{Figure 3.5}: CLSM micrographs of waxy corn starch granules after addition of 5-hexadecanoylamino fluorescein molecules (a) and fluorescein molecules (c), and bright-field micrographs of waxy corn starch granules after addition of iodine molecules (b).
3.4 Conclusions

We investigated the inclusion complex formation between amylose and fluorescent lipophilic molecules in regular and waxy starch granules from different botanical sources. Performing Confocal Laser Scanning Microscopy below gelatinization temperature we evidenced a specific interaction of amylose molecules and the aliphatic chain of the ligand. By studying waxy and not-waxy starch granules, we revealed that the amylose at the periphery of the starch granules interact specifically with aliphatic chains forming inclusion complexes. This phenomenon is occurring efficiently for temperature below the gelatinization point for low lipid concentration. In addition, we confirmed that long amylopectin chains are also able to form complexes with aliphatic ligands.
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