Unraveling structure and dynamics by confocal microscopy
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Chapter 4

Localization of amylose-lipophilic molecules inclusion complex formation in starch granules

By combining Confocal Laser Scanning Microscopy and spatial resolved photoluminescence spectroscopy, we are able to discriminate the presence of amylose in the peripheral region of regular and waxy granules from potato and corn starch, associating a clear optical fingerprint of the interaction between starch granules and lipophilic dye molecules. Measurements performed on samples that have been extensively washed provide strong proof of the specific interaction between lipid dye molecules and amylose chains in regular starch.

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4.1 Introduction

As already discussed in Chapter 3, starch constitutes the main source of carbohydrates for humans and many animal species and consequently is one of the main ingredients of foods.\textsuperscript{1-3} We have already described how starches from different botanical sources are found in the form of granular structures, ranging in size between \(\sim 1\) and over \(100\ \mu\text{m}\),\textsuperscript{4} which are characterized by a variable content of amylose and amylopectin. They consist typically of 10-30\% amylose and 90-70\% amylopectin, but high-amylose starches with a content up to 85\% exist, and the so-called waxy starches are constituted of nearly 100\% amylopectin.\textsuperscript{2,5}

Several models have been proposed in order to explain the architecture of these semi-crystalline structures.\textsuperscript{6} A cluster-like arrangement of the amylopectin polymers generates alternating stacks of amorphous and crystalline lamellae. The amorphous regions consist of branching zones of the amylopectin chains, while the crystalline lamellae are constructed by an ordered packing of parallel linear glucan chains.\textsuperscript{7-9} Figure 4.1 shows the schematic diagram of starch granule structure. Amylose chains appear generally interspersed among the amylopectin clusters\textsuperscript{10}, although it has been commonly assumed that amylose resides mainly in the amorphous regions.\textsuperscript{11,12}

![Figure 4.1: Amorphous and semi-crystalline phases in alternating rings in a single granule (a). Scheme of the composition of the semi-crystalline compositions, emphasizing the alternation of crystalline and amorphous lamellae (b), resulting respectively from branching amylopectin and regular linear glucan chains zones (c) [based on ref. 8].](image-url)
Jane and co-workers demonstrated that amylose bundles are more concentrated near the granule surface,\cite{13} which in native starch and according the Lineback’s model\cite{14} (“hairy billiard ball” model) and later implementations (by Stark and Lynn),\cite{15} has been characterized by the terminations of the amylose chains protruding from amylopectin clusters.

The tendency of amylose chains to generate inclusion complexes in presence of an appropriate guest molecule has been object of wide interest,\cite{10,16,17} and already debated in Chapter 3. The amylose helix presents a hydrophobic cavity that shows a high affinity for apolar guest molecules.\cite{18} In the process of inclusion complex formation, amylose coils undergo a conformational change, assuming the structure of a left-handed helix that allows inclusion of guest molecules such as iodine,\cite{19} dimethyl sulfoxide (DMSO)\cite{20} or potassium hydroxide.\cite{21} Among others, inclusion complexes between amylose and lipids such as fatty acids and phospholipids have been widely investigated.\cite{22-24} It has been demonstrated that amylose-lipid inclusion complexes affect the staling,\cite{25} digestibility,\cite{10} and the rheological properties of food.\cite{26} It is therefore important to understand the nature of the process and its dynamics.

The most accepted model describes the aliphatic chain of the fatty acid molecule hosted inside the hydrophobic cavity of the amylose helix,\cite{27} while the polar group, including the carboxylic acid, remains outside the helix due to electrostatic interactions.\cite{27-30} Several studies have been carried out in order to validate the models. Karkalas and co-workers, for example, determined the thermal properties of the inclusion complexes, investigating their dissociation temperature and dissociation enthalpies.\cite{31} Lately, we have shown by Confocal Laser Scanning Microscopy the formation of specific inclusion complexes between lipophilic molecules with regular and waxy starch granules from different botanical sources.\cite{32} Importantly, and in contrast to what is commonly believed, we demonstrated that the inclusion occurs also at temperatures below the gelatinization point for very low lipid concentrations.

Although there is a great interest for the inclusion complex formation, very limited data are available concerning the dynamics of the process. Recently, Cao and co-workers presented a study on the dynamics of complex formation involving well-defined amylose brushes and fatty acids characterized by a different length of the aliphatic chain (C8 and C14), showing that octanoic acid (C8) includes more efficiently than longer fatty acids.\cite{33}

The aim of the work presented in this Chapter is to associate an optical fingerprint of the interaction between starch granules and lipids, thus determining the role of the amylose chains present in the peripheral region of the granules. Combining Confocal Laser Scanning Microscopy (CLSM) with spatial resolved
Unraveling structure and dynamics by confocal microscopy

photoluminescence (PL) spectroscopy, we established that the presence of amylose in the starch granules modifies the PL signature of the lipid-dye, which is used as a nano-probe of its interaction with the surroundings. We demonstrate in this way that in regular starch, as opposed to waxy starch, the hydrophobic head remains outside the helix. Furthermore, measurements performed on samples that have been extensively washed provide a strong proof of the specific interaction between lipid dye molecules and amylose chains in regular starch. These measurements also confirmed the tendency of longer amylopectin chains, located in the hilum of waxy starch granules, to form inclusion complexes with ligands.

4.2 Materials and Methods

Corn starch, waxy corn starch, potato starch, and lugol solution for microscopy were purchased from Sigma-Aldrich. Waxy potato starch (Eliane 100) was supplied by Avebe Food. 5-Hexadecanoylaminofluorescein (C_{36}H_{43}NO_{6}) with a M_w of 585.74 g/mol was purchased from Life Technologies. Dimethylformamide (DMF) extra pure was purchased from Acros-Organics.

Starch suspensions for Confocal Laser Scanning Microscopy (CLSM) were prepared at a concentration of 2% in distilled water with 0.02% sodium azide as a preservative. 1 mL of the prepared suspension was stained with additional 20 µl of a 0.02% solution of the fluorescence dye 5-Hexadecanoylaminofluorescein in DMF, by rotating overnight at room temperature in the dark (stock suspension).

For CLSM measurements 40 µl of the stained samples were transferred to an object glass.

Washed starch suspensions for CLSM were prepared at a concentration of 2% in distilled water with 0.02% sodium azide as a preservative. 10 mL of the prepared suspensions was stained with 200 µl of 0.02% DMF solution of the fluorescence dye 5-Hexadecanoylaminofluorescein, by rotating the preparation for 7 days at room temperature in the dark. The suspensions were centrifuged for 5 min at 2000 rpm in a Heraeus Labofuge 400R with swing out rotor. The supernatants were decanted and an equal amount of distilled water with 0.02% sodium azide was added. 1 ml of the suspension was taken as a reference unwashed sample. For the washing procedure, several other aliquots of 1 ml from the suspension were transferred in separate micro tubes and centrifuged for 5 min at 2000 rpm. The supernatant was decanted and 1 ml of 50%, 80%, 90% or 100% DMF was added, followed by rotating for 1 hour in the dark. The suspensions were centrifuged for 5 min at 2000 rpm and the residues were washed two additional
times under similar conditions. The residues washed with different percentages of DMF were suspended in 1 ml distilled water with 0.02% sodium azide. For CLSM imaging 40 µl of the washed and unwashed samples were transferred to an object glass.

### 4.3 Results and discussions

Confocal Laser Scanning Microscopy (CLSM) combined with spatially resolved photoluminescence (PL) spectroscopy are ideal tools to investigate the nature and the properties of the inclusion complex formation between starch granules from different botanical sources and lipids. The samples were prepared by suspending starch granules in water and exposing them to 5-hexanodecanoylaminofluorescein, a lipophilic fluorescent molecule (lipid-dye) characterized by an aliphatic chain of 15 carbon atoms and a polar head based on a carboxylic acid and a fluorescein molecule. The exposure of the starch granules to the lipid-dye molecules, as well as the CLSM measurements, were performed at room temperature, well below the gelatinization temperature of starch, using lipid-dye concentrations as low as 0.02% respect to starch. The amylose content in starch have been estimated to be roughly 30%.[34,35] Therefore the amount of complex in our case is limited by the lipid-dye concentration we have used. It is therefore evident that techniques such as DSC and x-rays are not sensitive enough to detect such a low percentage of complexes. While photoluminescence based techniques are the only which may allow this level of sensitivity.

**Figure 4.2** shows the CLSM images of starch granules in water-based suspension after addition of 0.02 % (based on starch) lipid-dye molecules at room temperature. The starch granules are from regular potato (Fig. 4.2a), waxy potato (Fig. 4.2b), regular corn (Fig. 4.2c) and waxy corn (Fig. 4.2d). The CLSM images were recorded after rotating the starch-lipid-dye suspensions overnight in a dark environment at room temperature.

The micrographs reveal a bright rim area around the granules from both the regular potato and regular corn starch (Fig.4.2a, 4.2c), which highlights the typical shape and the structure of the starch granules.[36] Potato starch granules appear oval with smooth surfaces with dimensions ranging between 10 and 100 µm. Corn starch granules present a truncated shape with dimensions ranging between 5 and 30 µm.[37]

The sharpness and the thickness of the rim (dimension smaller than 1 µm) indicate the formation of inclusion complexes between the amylose chains present
Unraveling structure and dynamics by confocal microscopy

in the outer region of the granules and the lipid-dye molecules. In agreement with the above mentioned theories, the aliphatic chain of the lipid-dye can be included in the amylose polymer, while the fluorescent polar head remains outside. In both regular corn and potato starches the background remains dark, suggesting that all lipid-dye molecules are involved in the inclusion complex process leaving no molecules in the water-based solution.

![Image](image-url)

**Figure 4.2:** CLSM micrographs of potato (a) and corn (c) regular starch granules; potato (b) and corn (d) waxy starch granules, in suspension after addition of 0.02% lipid-dye molecules to starch ratio. The suspensions were rotated overnight at room temperature in a dark environment.

The micrographs of the waxy potato and waxy corn starches (Fig. 4.2b and 4.2d) show a contour area around the granules not as defined as in the case of the regular starch granules. The rims appear in these cases much broader, with a variable thickness up to 5µm after one day of incubation, which is the same incubation-time of the non-waxy granules in (Fig. 4.2a and 4.2c). The diffuse emission might be considered a sign that the lipid-dye molecules are involved only in weak interactions with the components of the starch granules.

We have shown in Chapter 3 the investigation of the interaction of the lipid-dye molecules (5-hexanodecanoylaminofluorescein) with potato starch granules in their regular and waxy form, demonstrating that the absence of amylose in the waxy starch granules affects the size and shape characteristics of the luminescent
rim, due to the weak interactions of the lipid-dye with the components of the waxy starch. In the present Chapter, our goal is to find a quantitative fingerprint characteristic of the interaction of the lipid-dye with amylose and amylopectin by recording point by point the emission spectra in different samples.

**Figure 4.3** shows the CLSM images with a 50 µm field-of-view of regular potato (Fig. 4.3a) and waxy potato (Fig. 4.3b) starch granules in water-based suspensions after addition of the lipid-dye solution. Figure 4.3c shows the spatially resolved PL spectra recorded in the regions highlighted by white circles in Figure 4.3a and 4.3b and the spectrum of the lipid-dye molecules in DMF/water solution (20 µL 0.02% lipid-dye in DMF solution in 1 mL water-based solution). The regions are specifically selected focusing the laser at these points after image acquisition. The spectrum recorded exciting the regular potato starch sample in the region A (Fig. 4.3a) shows the emission of 5-hexadecanoylaminofluorescein molecules, with the main emission peak at ~530 nm.

![CLSM images](image)

**Figure 4.3**: CLSM images of regular potato (a) and waxy potato (b) starch granules after addition of 5-hexadecanoylaminofluorescein molecules. PL spectra (c) recorded in the regular potato rim area (circle A) and in the waxy potato rim area (circle B). The emission of the reference lipid-dye molecules in solution is also shown. Excitation wavelength 488 nm. The lipid-dye to starch ratio used is 0.02%.
The spectrum associated to the region B in the rim of a waxy potato starch granule (Fig. 4.3b) presents the main photoluminescence peak also at 530 nm, without spectral shift in respect to the spectrum recorded exciting the regular potato starch; in this case, however, the emission appears narrower (the difference in full width at half maximum (FWHM) is ~10 nm between spectra A and B). The emission resulting from the free lipid-dye molecules in DMF/water solution shows the same spectral characteristics of the emission recorded exciting regular starch granules.

**Figure 4.4**: CLSM images of regular corn (a) and waxy corn (b) starch granules in suspension after addition of 5-hexadecanoylaminofluorescein molecules. PL spectra (c) recorded in the regular corn rim area (circle A) and in the waxy corn rim area (circle B). The emission of the reference lipid-dye molecules in DMF/water solution is also shown. Excitation wavelength 488. The lipid-dye to starch ratio used is 0.02%.

In **Figure 4.4** we present the CLSM micrographs with a field-of-view of ~ 30 µm of regular (Fig. 4.4a) and waxy corn (Fig. 4.4b) starch granules, in water-based suspension after exposure to 5-hexadecanoylaminofluorescein molecules in solution. As in the case of all samples reported, exposure to the lipid-dye was
performed at room temperature, well below the gelatinization temperature of starch. Figure 4.4c shows the spatially resolved photoluminescence spectra recorded in the two regions indicated by a white circle in the CLSM micrographs and the spectrum recorded exciting the lipid-dye molecule in DMF/water solution as a reference. In this case we also found that a narrower emission spectrum is associated to the fluorescent rim of the waxy starch granule and a superposition of the spectra coming from regular starch and free lipid-dye molecules.

The consistent finding of different spectra for the same dye in regular and waxy forms of starch suggests that the presence of amylose chains in the peripheral regions of the granules affects the nature of the interaction and consequently the photoluminescence properties between the lipid-dye molecules and the starch.

The difference between the spectra associated to the regular and waxy starch granules can be explained by the different environment experimented with the lipid-dye, depending on the chemical composition of the granule. In the case of the regular potato starch, the aliphatic chains of the lipid is included inside the amylose chains and the polar groups stay outside due the hydrophobic nature of the interaction, while in the case of the waxy form the lipid-dye molecule (both the aliphatic chain and the polar group) enters the inner structure of the granule giving rise to a broader and less defined rim. The embedding of the lipid-dye molecule inside the granule structure generates the suppression of vibrational modes of the molecule: for example, C-H stretch and bendings and C-C and C-H bending, which are reflected into a narrower and less structured emission spectrum compared to the spectrum of the lipid-dye molecule complexed with the regular starch granules.

The emission fingerprint of the free lipid-dye molecules in DMF/water solution coincides with that from the regular potato starch, indicating in both cases that the chromophore is in contact with water. These kinds of chromophores are extremely sensitive to the medium in which they are embedded (dielectric constant); when the polar group of the lipid-dye molecule is in water and free to move, the emission spectra results are broader and more structured than those generated by an embedded lipid-dye molecule, as in the case of waxy starch.
Unraveling structure and dynamics by confocal microscopy

Figure 4.5: CLSM images of regular potato (a), waxy potato (c), regular corn (e) and waxy corn (g) starch granules in suspension 7 days after the addition of 5-hexadecanoylaminofluorescein molecules. The images b, d, f, h refer to the same starch species acquired after the “washing” procedure with 80% DMF. The lipid-dye to starch ratio used is 0.02%.
To confirm that there is an actual difference in strength and specificity between the interactions in the case of regular and waxy starches, we performed a “washing” process on regular and waxy potato and regular and waxy corn starch granules, with different concentrations of DMF in water (50, 80, 90 and 100% v/v). Since the lipid-dye molecules are soluble very little in water but very well soluble in DMF, it may be expected that washing with a high DMF concentration might remove the lipid-dye molecules from the starch granules in the case of non-specific interactions with the starch granules.

When the exposure time of the starch granules with the lipid-dye solution is increased the luminescent rim around the granule becomes broader, especially in the case of the waxy starch granules. We therefore increased the exposure time with the lipid-dye solution from 1 day to 7 days.

**Figure 4.5** a, c, e, g show the CLSM micrographs of regular potato, waxy potato, regular corn and waxy corn starch granules acquired after 7 days of exposure to the lipid-dye solution.

The regular starch granules (Fig. 4.5 a, e) present a bright rim, which is identical in dimension and shape to the rim imaged after one day of exposure to lipid-dye molecules as reported in Figure 4.2a and 4.2c. The longer incubation time clearly increased the absorbance of the lipid-dye molecules into the waxy potato and waxy corn starch granules. Figure 4.5c and 4.5g evidences that the starch granules are completely stained. In comparing these with Figure 4.2b and 4.2d, it is clear that the lipid-dye goes through a time-dependent diffusion process inside the waxy starch granules. We can also observe (Figure 4.5 c and g) that the hilum of the waxy starch granules are brighter compared to the rest of the granule, which is most probably due to the complex formation between the lipid-dye molecules and the longer amylopectin chains located in the inner part of the granules.

**Figure 4.5** b, d, f, and h show the CLSM images of in order, regular potato, waxy potato, regular corn and waxy corn starch granules washed with 80% DMF in water (v/v) solution, after 7 days of exposure to lipid-dye molecules. While the regular starch granules (Fig. 4.5 b, f) do not present substantial difference with respect to the unwashed granules (Fig. 4.5a, e), the micrographs in Figure 4.5d and 4.5e reveal, very interestingly, that the washing process with the DMF-based mixture removes dramatically the lipid-dye molecules from the granules.

This finding confirms that the presence of amylose in the regular starch granules generates specific interactions between the host and the ligand. The nature of such bonds has been extensively debated, the general conclusion being that the hydrophobic effect seems to be the main force responsible for the specific interaction. Conversely, we can infer that in the case of waxy starch the lipid-dye molecules seems not to undergo a specific interaction with amylopectin, and
weaker interactions explain the experiments in Figure 4.5c and 4.5g. However, after washing only in the hilum of the waxy starch granules, some photoluminescence emission from the lipid-dye molecules appears to persist (see Figure 4.5h and Fig. 4.6e). This finding confirms our previous hypothesis of interaction between the lipid-dye molecules and the longer amylopectin chains present in the hilum. However, cannot excluded that a very small amount of amylose chains are still present in the hilum of waxy starches.

Figure 4.6: CLSM images of regular potato (a), waxy potato (d), regular corn (g) and waxy corn (l) starch granules in suspension 7 days after the addition of 5-hexadecanoylaminofluorescein molecules. The images b, e, h, m refer to the same starch species acquired after the “washing” procedure with 50% DMF. The images c, f, i, n refer to the same starch species acquired after the “washing” procedure with 80% DMF.
Washing procedures with 50% DMF were also performed (see Figure 4.6), revealing that the increasing percentage of DMF has a proportionally stronger capacity to remove the lipid-dye molecules from the waxy starch compared to the regular starch granules. Washing with 90% and 100% DMF solutions resulted also in a gradual loss of staining in the case of regular starch granules.

4.4 Conclusions

In the present Chapter we presented our investigation on the role of the amylose chains in the inclusion complex formation between starch granules and lipophilic fluorescent molecules. By performing Confocal Laser Scanning Microscopy we explored the inclusion complex formation involving regular and waxy forms of starch from two different botanical sources. In particular, by correlating Confocal Laser Scanning Microscopy and spatially resolved spectroscopy we were able to discriminate the presence of amylose polymers in starch granules using the photoluminescence spectra of the dye as a nano-probe of the interaction experienced by the lipid-dye molecule with the surroundings, demonstrating that in regular starch the hydrophobic head remains outside of the amylose chain.

By performing a “washing” process, we proofed the specific interaction of the lipid-dye with amylose while the adsorbed lipid-dye in the waxy starches was easily reversed by washing. Only in the hilum do traces of complexation remain with the longer amylopectin chains or with eventual traces of amylose.
Unraveling structure and dynamics by confocal microscopy

References

Chapter 4

Unraveling structure and dynamics by confocal microscopy