Chapter 5

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

This Chapter presents the investigation of the dynamics of inclusion complex formation occurring between lipids and amylose polymers located at the periphery of starch granules. Through real-time recording of CLSM micrographs, a time frame within tens of seconds is estimated for the inclusion complex to occur.

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**5.1 Introduction**

In Chapter 3 and Chapter 4 we discussed the tendency of the amylose chains in starch granules to generate inclusion complexes in presence of a guest molecule.\cite{1-3} This is generally attributed to its hydrophobic cavity that shows a high affinity for apolar ligands.\cite{4} 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 guests.\cite{5-7} Among others, inclusion complexes between amylose and lipids such as fatty acids and phospholipids have been widely investigated.\cite{8-10} It has been demonstrated that amylose-lipid inclusion complexes affect the staling,\cite{11} the digestibility,\cite{1} and the rheological properties of food.\cite{12} It is therefore important to understand the nature of the process and in particular its dynamics.

Several studies have been carried out in order to get insight on different properties of the inclusion complex formation between lipids and amylose, but so far the dynamics of this process has been not investigated.

Recently, Cao and co-workers published a study on the dynamics of complex formation involving 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{13} In this case the amylose chains were “grafted” on a surface, resulting in a molecularly precise definition of the length and density of the amylose molecules, but because of this precision far from the situation occurring in real starch.

In this Chapter real time observations of the inclusion complex formation in starch granules are performed with CLSM microscopy. From the images of the starch granules taken in real time in presence of the solution containing the chromophore functionalized fatty acid we estimate a upper time for complexation of about 10 s. This experiment confirms again the efficiency and specificity of the inclusion process between the amylose in the periphery of the starch granules and lipids.

**5.2 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
Mₙ of 585.74 g/mol was purchased from Life Technologies. Dimethylformamide (DMF) extra pure was purchased from Acros-Orgnics.

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.

For real time observations, 10 µl (potato starches) and 5 µl (corn starches) of the stock suspensions (see above) were added to 200 µl of the starch suspensions without dye-lipid. From the mixed solutions a drop (37 µl) was placed on the microscope object glass and 3 µl of lipid-dye solution (molecular probe 0.02% 5-Hexadecanoylaminofluorescein in DMF) was added during the on-going CLSM measurements.

The real time CLSM measurements were performed acquiring image frames with 1002 ms time intervals and dwell time line of 1.68 µs.

5.3 Results and discussion

In order to investigate the time scale of the inclusion complex formation at room temperature, we performed CLSM measurements and real-time CLSM measurements of the process. The experiment was performed exposing potato and corn starch granules in the regular and waxy form and wheat starch granules to 5-hexadecanoylaminofluorescein, 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. Figure 5.1 shows CLSM micrographs of starch granules in water-based suspension after the exposure to 0.02% lipid-dye solution.
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Figure 5.1: CLSM micrographs of potato starch (a) and corn starch (b) in the regular form, potato starch (d) and corn starch (e) in the waxy form, and (c) regular wheat starch. Chemical structure of 5-hexanodecanoylaminofluorescein (f).

The micrographs present potato starch granules in the regular form (Fig. 5.1a) and in the waxy form (Fig. 5.1d), corn starch granules in the regular form (Fig. 5.1b) and in the waxy form (Fig. 5.1e) and wheat starch granules (Fig. 5.1c). Figure 5.1f presents the chemical structure of 5-hexanodecanoylaminofluorescein molecule.

The micrographs in figure 5.1 are recorded after rotating the starch-lipid-dye mixture overnight at room temperature, well below the gelatinization temperature, in a dark environment. The bright rim (thickness ~ 1 µm) around the granules from the different botanical sources (Fig. 5.1a, 5.1b, 5.1c) makes us infer that the inclusion complex formation between the amylose chains in the peripheral region of the granules and the lipid-dye ligands, has already occurred in the case of the regular form of potato, corn and wheat starch.\textsuperscript{14} The waxy starch granules undergo a different process, due to the absence of amylose chains in the outer region of the granules\textsuperscript{15} resulting in a fuzzier and broader rim (thickness up to 5 µm) around the starch granules. The rim tends to increase in time, resulting in a complete staining of the granules.\textsuperscript{14}

The inclusion complex formation could be observed in real time from the initial stage by adding a small amount of the lipid-dye solution (3 µL, see the
experimental section) directly to the starch suspension already present on the sample stage of the confocal microscope. The effect of the addition of the lipid-dye solution was monitored in real time without stirring or heating. Here is important to notice that the ratio between the concentration of the lipid-dye and the starch used for the dynamics is 4 times higher (0.08%) than what has been used for the previously reported measurements in Figure 4.1 (0.02%).

A small amount (0.05%) of overnight complexed starch granules were added to the starch suspensions as reference starch granules, in order to facilitate the focusing operations in the microscope stage.

In Figure 5.2 we present a sequence of micrographs of the complexation of potato starch granules in the regular and in the waxy form, extracted from CLSM live records.

![Micrographs of Starch Complexation](image)

**Figure 5.2:** Photograms extracted from CLSM videos performed on regular potato starch (a, b and c) and waxy potato starch (d, e and f), (a and d) were performed before the addition of the lipid-dye solution (t = 0), (b and e) are recorded 1 sec after the addition of the lipid-dye solution and (c and f) at 40 sec after the addition of lipid-dye solution. The lipid-dye to starch ratio used for the experiment is 0.08%.

Figure 5.2a and Figure 5.2d present the initial situation at t = 0 sec, when the lipid-dye molecules are added to the water-based suspension of the starch granules of regular potato and waxy potato located on the microscope stage, respectively.
These micrographs (Fig. 5.2a and 5.2d) are characterized by a dark background except for a few reference granules, which were inserted in the sample composed of not-complexed granules with the aim to finding the focal plane. In Figure 5.2d the presence of the starch granules is very faintly visible before addition of the lipid-dye. This phenomenon can be explained by the fact that the waxy starch interaction with the lipid-dye in this case is not specific, and that the bright rim is due to adsorption and diffusion of the lipid-dye molecules. The lipid-dye molecules in this case can easily be released in the solution and be re-adsorbed by other granules in water-based suspension.

Figure 5.2b presents the micrograph recorded 1 sec after exposure of the regular starch granules to the lipid-dye molecules. The background appears luminescent, indicating that the fluorescent lipid-dye molecules are homogeneously distributed in the sample water-based solution. At this time a weak luminescent rim starts appearing, revealing the presence of several starch granules. At 40 sec after the exposure of the granules to the lipid-dye molecules (Fig. 5.2c) the rim around the granules appears brighter and more defined, while the background appears darker, indicating that most of the added lipid-dye molecules are involved in the complex formation with the amylose chains present in the outer region of the starch granules.

Figure 5.3 presents a sequence of photograms extracted from live CLSM recordings on regular and waxy corn starch granules before and during the exposure to the lipid-dye molecule. Also in this case the photograms show different phases of the process initialized by the addition of the lipid-dye to the solution.

Figure 5.3a and Figure 5.3d present the situation at t=0 sec, when the addition of lipid-dye solution to the granules water suspension takes place. Also in this case a few granules already complexed with lipid-dye molecules were added before to
the not-complexed suspension of starch granules in order to facilitate the localization of the unstained starch granules.

![Figure 5.3: Photograms extracted from CLSM recordings performed on regular corn starch (a, b and c) and waxy corn starch (d, e and f), (a and d) before the addition of the lipid-dye solution (t = 0), at 5 sec after the addition of lipid-dye solution (b and e) and at 40 sec after the addition of lipid-dye solution (c and f). The lipid-dye to starch ratio used for the experiment is 0.08%.

At t = 5 sec after the exposure of the not complexed granules to the lipid-dye molecules, a defined and bright rim around a multitude of regular corn starch granules appears (Fig. 5.3b). At 40 sec after the exposure (Fig. 5.3c), a large assembly of fully complexed granules appears. The background is very dark, as are the inner regions of the granules where the lipid-dye is not present. This observation suggests that most of the added lipid-dye molecules are involved in the inclusion complex formation.

As expected, the waxy corn starch shows a totally different behavior. At t = 5 sec after the exposure (Fig. 5.3e), the profile of the granules becomes highlighted by the presence of the lipophilic fluorescent molecules in a rim which is less defined and fuzzier than the one which characterizes the regular starch granules, confirming also in this case that the presence of amylose chains at the peripheral
regions of the granules is a discriminant for the inclusion complex formation. Figure 5.3f shows the waxy corn starch granules 40 sec after the addition of the lipid-dye molecules. In this case the whole starch granules look more or less stained by the lipid-dye, indicating that the lipid-dye molecules are able to penetrate the whole granule and confirming again that the presence of the amylose chains affects the interaction of the starch granules with the lipid-dye molecules, and that it is the necessary element for the inclusion complex formation in the peripheral region of the granules at room temperature. Furthermore, a diffuse luminescence is present in all samples, suggesting that a significant amount of lipid-dye molecules are still not adsorbed within the waxy corn starch granules at this time scale.

The experiments carried out on potato and corn starch demonstrate that at very low lipid-dye concentration inclusion complex formation occurs at room temperature in a quantity detectable only by CLSM within tens of seconds.

As a further confirmation, we performed CLSM measurements also on wheat starch granules in water-based solution. In Figure 5.4 we present a sequence of micrographs recorded on wheat starch granules extracted from CLSM live records.

**Figure 5.4:** Photograms extracted from CLSM recordings performed on wheat starch. At the addition of the lipid-dye solution (t = 0), at 5 sec after the exposure (Fig. 5.4b) the rim around the granules become visible, showing as in the previously reported samples that the inclusion complex formation start occurring. The last presented

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Figure 5.4a presents the wheat granules at t=0 sec, when the lipid-dye molecules are added to the starch suspension. 10 sec after the exposure (Fig. 5.4b) the rim around the granules become visible, showing as in the previously reported samples that the inclusion complex formation start occurring. The last presented...
photogram (Fig. 5.4c), taken 40 sec after the addition of lipid-dye molecules, shows that a large amount of granules are complexed with the lipid-dye molecules. The experiment carried out on wheat starch confirm that the inclusion complex formation takes place in tens of seconds, driven by pure intermolecular type of bonds (van der Waals forces and hydrogen bonds) between the amylose helix and the ligand \textsuperscript{16,17} which allow to stabilize the complex, since the addition of lipid-dye molecules occurred without any stirring or any kind of extra support for the process as enhanced temperature.

### 5.4 Conclusions

In this Chapter we presented an investigation of the dynamics of the inclusion complex formation occurring between amylose polymers in the peripheral region of starch granules and lipophilic molecules marked with a chromophore. By performing real time CLSM measurements, below the gelatinization temperature of starch, we were able to estimate within tens of seconds the time necessary for the inclusion complex formation to occur. This is a further confirmation of the power of fluorescence CLSM, being not only as tool to analyse structural properties but also to study dynamical process, especially when using fluorescence as observable, which allows reaching very high detection sensitivity.
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References