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On the effect of surface active agents and their structure on the temperature-induced changes of normal and waxy wheat starch in aqueous suspension. Part II: A confocal laser scanning microscopy study

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Abstract

The location and penetration patterns of two fluorescently labelled, surface active molecules into normal and waxy wheat starch granules prior, during and after the temperature-induced gelatinization were studied by means of confocal laser scanning microscopy (CLSM). Amphiphilic dyes were found to have a tendency to penetrate wheat starch granules in aqueous suspension. The penetration patterns were however found to be dependent on the contact time, type of starch and the chain length (C_{12} vs. C_{16}) of the amphiphilic dye. The penetration of amphiphilic dyes through the starch granule matrix proved to be less restricted in waxy than in normal wheat starch. For a given type of starch, the penetration of the longer chain dye was more constrained than that of the shorter chain one. The extent to which the dye diffuses into the granule matrix as it gelatinizes is also affected by the chain length of the dye, diffusion of the shorter chain dye occurring more profusely and at lower temperatures than for the longer chain one. These differences are suggested to be related to the dissociation temperature of the AM-amphiphilic dye complexes.
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1. Introduction

Amphiphilic, surface active molecules such as surfactants and emulsifiers are well-known for their ability to affect different aspects of the starch gelatinization process and to ultimately alter the properties of starch-based products. In addition to the numerous studies that have aimed at the characterization of their effects on the thermal and rheological properties of starch suspensions, the effect of surfactants on the morphological changes that starch granules undergo during gelatinization has also been extensively characterized. In wheat starch, the typical morphological changes occurring when granules are heated have been reported to occur at lower temperatures in the presence of some surfactants [sodium dodecyl sulphate (SDS)] (Eliasson, 1985; Svensson, Autio, & Eliasson, 1998) and at higher temperatures in the presence of others [glycerol monostearate and sodium stearoyl lactylate] (Eliasson, 1985). Light microscopy observations of iodine-stained wheat starch suspensions at different stages of starch gelatinization have revealed, that the presence of a mixture of polyglycerol ester and monoglycerides preserves the granule structure up to much higher temperatures than in the control sample, restricting granule swelling and inhibiting the leaching of amylose (AM) (Richardson, Langton, Bark, & Hermansson, 2003).

Much of the overall effect of surfactants on the gelatinization of starch has been explained in terms of the formation of helical inclusion complexes with the starch polysaccharides, specially amylose (AM). Yet, for many

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years it was not possible to directly address the questions on where such complexation might take place and which regions of the starch granules were accessible to the amphiphilic molecules.

The development of novel microscopy techniques, in particular confocal scanning laser microscopy (CLSM), has enabled the study of the morphology and internal structure of starch granules without the use of complicated or invasive sample preparation methods, thus opening the door to countless new possibilities. Over the past ten years and in combination with the use of fluorescence probes, CLSM has been extensively used in the study of many structure and granule-composition-related issues. Considerable progress has been made in topics concerning the identification of pores and channels in some cereal and tuber starches (Fannon, Gray, Gunawan, Huber, & BeMiller, 2003, 2004; Huber & BeMiller, 1999), the location of granule-associated proteins both in gelatinized (Han & Hamaker, 2002a) and ungelatinized starch granules (Han & Hamaker, 2002b) and the identification of accessible regions in starches under different swelling conditions (Gray & BeMiller, 2001), just to mention a few.

The present study is the second of a series of two papers which seek to gain insight into the effect of the structure (in particular, the length of the *n*-alkyl chain) of surface active agents on the temperature-induced changes of wheat starch granules in excess of water. By means of CLSM and the use of fluorescently labelled surface active molecules, this investigation aims at determining the location and penetration patterns of amphiphilic molecules into normal and waxy wheat starch granules prior, after and even during the temperature-induced gelatinization. In combination with what is already known regarding the molecular events involved, this information may contribute to a better understanding of the processes in which starch and amphiphilic molecules interact.

In order to make the results from this study relevant to actual practical situations, the experimental conditions have been set up to be as similar as possible to the ones encountered when interactions between starch and amphiphilic molecules are studied. Thus, the penetration patterns of amphiphilic molecules into starch granules are studied in granules suspended in excess water, without any further preparation, dye removal or filtration steps. Furthermore, *in situ* gelatinization studies in the presence of the amphiphilic dyes are carried out after short contact times, increasing the temperature at a rate of 1.5 °C/min (a heating rate commonly used in starch pasting studies).

Short and long (12 and 16 carbon atoms, respectively) *n*-alkyl chain amphiphilic, fluorescein-based dyes have been chosen as surfactant models. In combination with fluorescent recovery after photo bleaching (FRAP) techniques, these type of dyes have proved very useful in the characterization of the structure and diffusion kinetics of macromolecular assemblies of amphiphilic molecules (Chatenay, Urbach, Messager, & Langevin, 1987; Maldonado, Urbach, & Langevin, 1997; Podhajecka, Stepanek, Prochazka, & Brown, 2001). Though different with respect to common surfactants or emulsifiers in terms of their hydrophilic–lipophilic balance, the structural differences of these two dyes with respect to each other could reveal the existence of possible differences in granule accessibility relevant to real short- and long-chain emulsifiers as well as other potential hydrophobic guest molecules of interest.

2. Experimental section

2.1. Materials

2.1.1. Starches and starch polysaccharides

Two wheat starches (normal and waxy) were used in this study. Both samples were prime starches prepared from flour. The normal wheat flour used was a commercial flour milled from the Swedish winter wheat cultivar Gnejs provided by Nord Mills (Sweden). The waxy wheat flour was kindly provided by Dr. Craig F. Morris (USDA/ARS Western Wheat Quality Laboratory, Pullman, WA, USA). This flour was milled (Buhler MLU-202) from a spring habit, hexaploid waxy wheat, produced from a cross of 'Bai Huo' and 'Kanto 107' grown in California in 1997.

Amylose, type III from potato, was purchased from Sigma Chemicals (Schnelldorf, Germany) and used as received.

2.1.2. Amphiphilic dyes

Hexadecanoyl and dodecanoyl conjugates of 5-aminofluorescein were purchased from Molecular Probes-Invitrogen (Eugene, OR, US). The structure and molecular weights of the dyes as well as the abbreviations used to refer to these throughout this study are summarized in Table 1.

2.2. Methods

2.2.1. Starch preparation

Prime starch was extracted from flour according to a gluten washing procedure based on the method of Wolf (Wolf, 1964) as described elsewhere (Mira, Persson, & Villwock, 2007).

2.2.2. Starch analysis

2.2.2.1. Moisture content. Starch moisture content was taken as weight loss after heating at 120 °C for 2 h and was found to be 11.0% and 11.6% for normal and waxy wheat starch, respectively.

2.2.2.2. Structural characterization – scanning electron microscopy (SEM). Initial characterization of the three-dimensional structure of the normal and waxy wheat starch samples was performed by means of a scanning electron microscope (SEM) (XL30 ESEM TMP, FEI/Philips, The Netherlands). Starch granules were mounted onto double-sided carbon tape on aluminum stubs and coated with gold in a sputter coater (SCD 050, Balzers). Micrographs were then obtained in the SEM under low vacuum mode at 20 kV.

2.2.2.3. Particle size distribution. The particle size distribution of the two wheat starches used in this study was determined by means of a Malvern mastersizer light scattering instrument, model 2000 (Malvern instruments Inc., UK) as described elsewhere (AACC, 1995; Raeker, Gaines, Finney, & Donelson, 1998). Both starch samples exhibited the typical bimodal size distribution of wheat starches. The normal wheat starch was found to have two main populations of granules: one with a peak value in the range 2.4–3.2 μm and the other one with a peak value in the range 17.4–23.6 μm, which represented the 0.705 and 13.9 vol% of the sample, respectively. The waxy wheat starch exhibited a very similar particle size distribution, with one of the main population of granules with a peak value in the range 2.5–3.4 μm (1.1 vol%) and a the other one with a peak value in the range 18.3–24.2 μm (10.69 vol%).

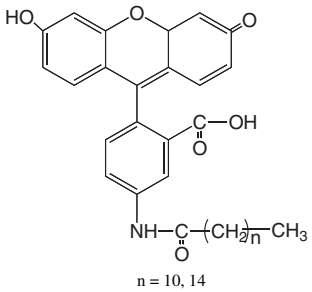
2.2.3. Amphiphilic dyes

2.2.3.1. Complex formation ability with amylose (AM). The ability of the two amphiphilic dyes to form complexes with amylose (AM) was assessed by means of differential scanning calorimetry (DSC). The thermal transitions associated with the dissociation of AM-amphiphilic dye complexes were examined with a calorimeter of the heat flux type (DSC 821^e, Mettler-Toledo, Switzerland). The measurements were done according to a procedure based on the method used by (Tufvesson, Wahlgren, & Eliasson, 2003b) to investigate the formation of complexes between AM and fatty acid salts. The dyes, the AM and a 0.1 M NaOH solution were thus mixed in a 0.2:1:3 weight ratio in 40 μL-aluminium pans (Mettler-Toledo, Switzerland). As in the study by Tufvesson et al. (2003b), the aqueous alkaline solution was used in order to convert the dyes into their anionic salts. Before sealing the pans, the samples were carefully mixed with the aid of a pin to guarantee an even water distribution. The samples were then heated from 20 °C to 125 °C at a rate of 10 °C/min using an empty aluminium pan as a reference sample. The DSC traces of the amphphilic dyes in the absence of AM (using a dye–alkaline solution weight ratio of 0.2:3) were also recorded under the same experimental conditions.

The temperatures [onset (T_O) and peak (T_{PEAK})] and enthalpies (ΔH) of the endothermic transitions observed in the DSC traces were determined by means of the instrument analysis software. The onset temperature is defined as the point at which a straight line drawn up the leading edge of the endotherm intersects the baseline while the peak temperature corresponds to the point of maximum endothermic heat flow relative to the baseline. Enthalpy values are calculated by estimation of the area under the endotherm with a fitted straight baseline. These values are reported on an AM (as is) basis. All tests were conducted in triplicate. Analysis of variance (ANOVA) with significance defined at $p < 0.05$ was performed on the calorimetry data. Significant differences among mean values were determined by Tukey's test using a family-wise error level of 0.05.

2.2.3.2. Dye solutions for CLSM. In their neutral form, the two amphiphilic dyes are essentially insoluble in water. Given the nature of the study to be undertaken, the use of alkaline conditions or solvents other than water to solubilize the dyes was not a viable option, as they would affect the course of the starch gelatinization. The aqueous solubility of both dyes was thus improved by converting the neutral fluorescein group into its monoanionic form, which results from the deprotonation of the carboxylic acid group (see Table 1). This was done by reacting the dyes (0.2 mM) with 1 equivalent of sodium hydroxide (NaOH) at room temperature. After 24 h, a fraction of the dyes still remained insoluble and the pH of the solution high (ca. 8), meaning that the reaction had not proceeded to completion. Prolonging the reaction time and increasing the temperature (up to 60 °C) did not have any effect. This was taken as an indication that the solubility limit of the monoanion was lower than 0.2 mM. The insoluble dye was then recovered by filtration, dried and weighed. The difference between the originally added and the recovered dye gave an indication of the amount of dye that had been converted to its monoanion and about its solubility limit in solution. The solubility limits of the monoanionic forms of the dyes in water at room temperature were found to be ~0.05 and 0.02 mM for

Table 1
Amphiphilic dyes used

Dye	Structure	Abbreviation[a]	M_W	
			C_{12}Fluor	C_{16}Fluor
n-Alkanoyl 5-amino-fluorescein		C_{n+2}Fluor	529.6	585.7

n = 10, 14

[a] *n*, number of CH_2 groups in the alkyl chain of the dye (see the schematic of the dye molecular structure in the table).

C_{12}Fluor and C_{16}Fluor, respectively. A dye concentration in solution of 0.02 mM was thus chosen and used for all experiments. Dye solutions to be used for CLSM experiments were prepared by reacting the dyes (0.02 mM) with 1 equivalent of NaOH. After being kept under stirring for 24 h at room temperature essentially all the dye had dissolved and the pH of the solutions was close to neutral (6.9–7.4). Throughout the whole preparation process, the flasks containing the dye solutions were kept wrapped in aluminium foil to prevent photodecomposition. Freshly prepared solutions were used for all experiments.

2.2.4. Confocal scanning laser microscopy (CSLM)

Confocal microscopy was performed using a LSM 410 invert system (Zeiss, Germany) attached to an inverted microscope (Axiovert 135M, Zeiss, Germany) fitted with a oil-immersion 40X/1.3 NA lens. A laser with a wavelength of 488 nm (in the majority of the recordings at 1/10 of its maximum power) was used to generate fluorescence. The confocal pinhole was set to 1 Airy unit giving an optical section thickness of approximately 500 nm. Amplification and blacklevel were adjusted for each sample to optimally use the dynamic range in the detectors. The data produced were stored and manipulated using the software provided by the microscope manufacturer. In most cases, both confocal laser scanning and transmitted light images were collected simultaneously. All experiments were repeated at least three times and several images of each of the samples were collected. Each of the micrographs presented here was chosen out of a set of 6 to 10 images.

Dye location in the starch granules along the temperature-induced gelatinization was followed *in situ* with the aid of a hot stage. The temperature was increased from ambient temperature (23 °C) to 95 °C at a heating rate of ca. 1.5 °C/min. Confocal-fluorescent and transmitted light micrographs were simultaneously collected along the whole temperature scan at intervals of 3–4 min. Readjustments of the amplification and blacklevel along the temperature scan were avoided whenever possible. All *in situ* heating experiments were repeated at least three times.

2.2.4.1. Sample preparation. One milliliter of amphiphilic dye solution was transferred into test tubes with screw caps. Starch (5 mg) was then added to the test tubes so a 0.5% w/v wb starch suspension was obtained. This suspension was kept under shaking for 30 min. Next, 20–50 μL of suspension were loaded into small "chambers" prepared on microscope slides. These "chambers" were created by mounting two cover slips (ca. 1.5 cm apart from each other) onto a common microscope slide and bridging them with another cover slip. Clear nail varnish was used to fix the cover slips into position as well as to seal open spaces. For the sample to be loaded, only a small slit was left open at either end of the space confined between the cover slips. Once the sample was loaded into the chamber, the small slits were sealed by applying several layers of nail varnish. The slides were subsequently dried in air for ca. 10 min before being transferred into the microscope stage for analysis. Whenever the influence of longer contact times on the staining pattern of the granules was investigated, the samples in the chambered slides were left, protected from light, at room temperature for 24 h.

3. Results and discussion

3.1. Complex formation ability of the dyes

The samples with the dyes in the presence of AM exhibited the characteristic endothermic transition attributed to the dissociation of AM-lipid complexes (see Table 2). Moreover, no thermal transitions were observed for any of the two dyes in the absence of AM. The dissociation of the AM-amphiphilic dye complexes was found to begin at temperatures of ~75 °C and ~103 °C for the short- and the long-chain dyes, respectively, while the dissociation enthalpies of the complexes were found to be of similar magnitude in both cases. Thus, it was concluded that the two dyes have the ability to form AM-dye complexes, which in turn exhibit similar thermal properties as the ones reported for complexes formed between AM and other short- and long-chain amphiphiles (Tufvesson, Wahlgren, & Eliasson, 2003a; Tufvesson et al., 2003b; Mira et al., 2006)

3.2. Staining patterns in ungelatinized starch granules: short contact times

As depicted in the scanning electron micrographs presented in Fig. 1, and in good agreement with the results from the particle size analysis, normal and waxy wheat starch exhibit similar features regarding granule size and shape. The familiar lenticular-shaped, big (>10 μm) A-granules and the spherical, small (<10 μm) B-granules can be identified in the micrographs of both the normal and the waxy wheat starches. Indentations, usually attributed to the influence of adjacent B-granules during biosynthesis in the endosperm (Evers, 1979), can be recognized on some of the A-granules

Fig. 2 shows confocal laser scanning micrographs of ungelatinized normal and waxy wheat starch in solutions of C_{12} and C_{16}Fluor dyes after short (<1 h) and long contact times (24 h). In these images, the fluorescence signal intensity is shown in a green colorscale.

It is observed that after short contact times most of the granules of normal wheat starch in the presence of

Table 2
Characteristic temperatures [onset ($T_{O\,CX}$), peak ($T_{PEAK\,CX}$)] and enthalpies (ΔH_{CX}) of the dissociation transition of the AM-amphiphilic dye complex in samples of amphiphilic dyes, AM and 0.1 M NaOH (0.2:1:3)

Dye	$T_{O\,CX}$ (°C)	$T_{PEAK\,CX}$ (°C)	ΔH_{CX} (J/g)
C_{12}Fluor	75.4 ± 2.7^{a}	85.0 ± 2.2^{a}	2.1 ± 0.5^{a}
C_{16}Fluor	102.8 ± 0.5^{b}	109.7 ± 0.8^{b}	3.2 ± 0.3^{a}

Reported values are means ± standard deviation of triplicates. Values within the same column followed by the same letter are not significantly different ($p \leqslant 0.05$).

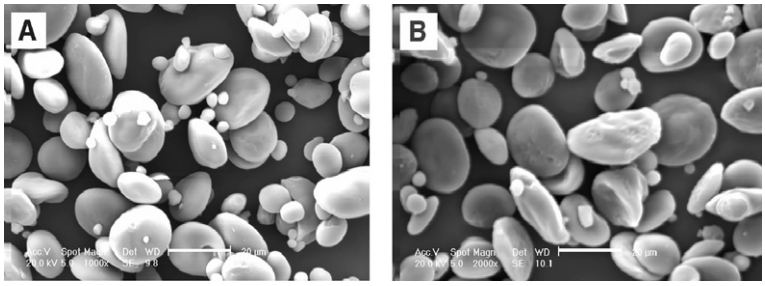


Fig. 1. Scanning electron micrographs of the (A) normal and (B) waxy wheat prime starches used in this study (scale bar = 20 μm).

C_{12}Fluor exhibit a fluorescent region which is confined to the outer regions of the granules. A few granules are found to exhibit patches of intense fluorescence or even appear to be fluorescent throughout. Conventional transmitted light micrographs (images not shown) confirmed that these corresponded to mechanically damaged areas or granules. In the presence of C_{16}Fluor, normal wheat starch granules show a similar staining pattern to the one observed in the presence of C_{12}Fluor, where the fluorescence is concentrated towards the granule surface. Thus, in normal wheat starch the location of the short- and long-chain dyes is restricted to the outer regions of the granules, both dyes being apparently unable to penetrate (within the time frame and the dye concentrations used in these experiments) other regions of the granule matrix.

The waxy wheat starch granules exhibited staining patterns resembling the ones found in normal wheat starch after short contact times in the presence of the amphiphilic dyes. However, the outer "fluorescence shell" found in waxy starches in the presence of both dyes was brighter and thicker than the one observed in the normal wheat starch granules. Thus, the probes were not only present in greater amounts, but were also able to diffuse further into the granule matrix of the waxy starch, which in turn resulted in many of the smaller granules being stained throughout. Moreover, the staining patterns of the short- versus the long-chain dye differed in the thickness of the fluorescence shell. Compared to C_{16}Fluor, the shorter chain dye appeared to have penetrated further into the matrix of the granules.

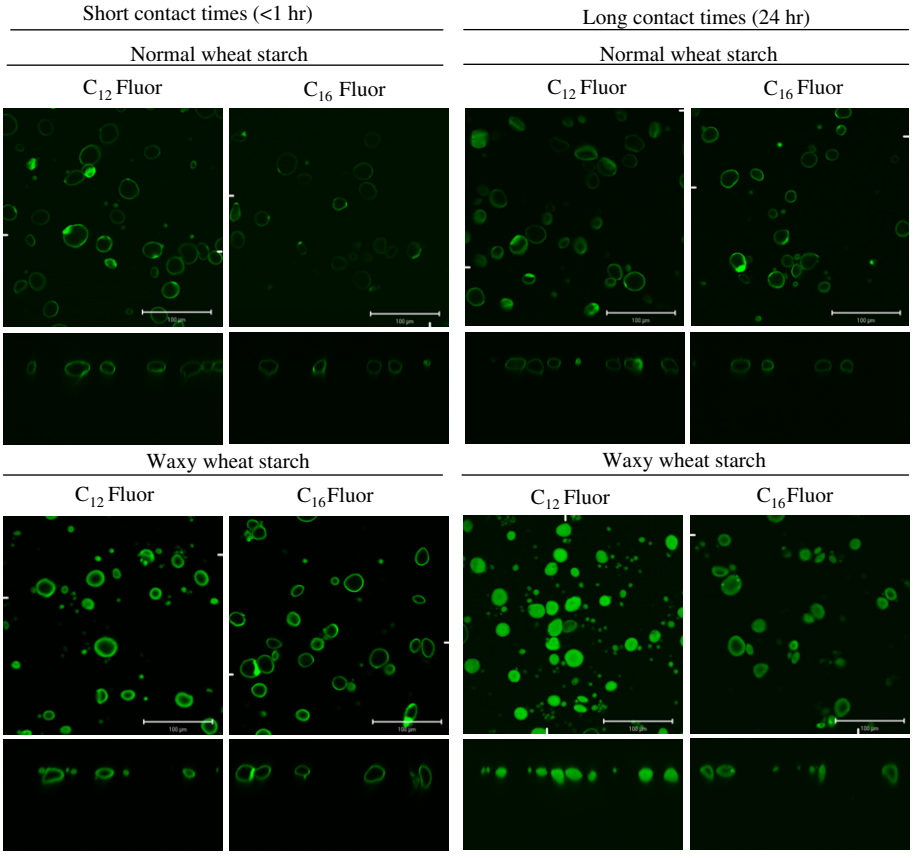


Fig. 2. Staining patterns of C_{12}Fluor and C_{16}Fluor dyes in ungelatinized normal and waxy wheat starch granules after short and long contact times. Smaller pictures at the bottom of each image depict a perpendicular section plane taken along the line that connects the two marks along the perimeter of each image (fluorescence signal intensity shown in a green colorscale, scale bar = 100 μm).

3.3. Staining patterns in ungelatinized starch granules: long contact times

After 24 h, the differences between the staining patterns of the dyes (short- and long-chain) in the two types of starches become apparent. After such contact times, the short-chain dye is observed to have diffused further into the matrix of the granules of normal wheat starch, whereas for the longer chain dye very little change is observed with respect to the staining pattern observed after short contact times. Changes in the staining patterns in waxy wheat starch granules after 24 h are much more dramatic. Waxy wheat starch granules in the presence of C_{12}Fluor appear brightly fluorescent throughout. Staining of these granules is complete and uniform which even allows for the identification of the granule growth rings (Fig. 3). In the presence of C_{16}Fluor, most of the waxy starch granules are found to be evenly stained by the dye. However, a non-fluorescent core could still be observed in some of the granules.

Radial channels, which have been otherwise clearly visualized through the use of various fluorescent probes in cereal starch granules (Huber & BeMiller, 1999) were not observed with the probes used here. Thus, if channels were present, the diffusion of the amphiphilic dyes through the granules did not take place preferentially through them but rather evenly through the whole granule matrix instead. Moreover, the findings regarding the staining patterns in ungelatinized starch granules after short (<1 h) and long contact times (24 h) have two main implications. In the first place, the differences found between the staining pattern of a given dye in normal and waxy wheat starches indicate that the diffusion of the dyes through the matrix of the waxy starch granules is much less restricted than in normal starch. Secondly, the differences found regarding the staining patterns of the two dyes for a given type of starch suggest that, compared to the shorter-chain dye, the diffusion of the longer-chain dye through the matrix of the starch granules is more restrained and, in consequence, slower.

The influence of structural differences between different starches on the penetration patterns of fluorescently labelled molecules has become evident in other studies (Gray & BeMiller, 2001). Differences in the granule organization and composition of different starches have also been long-known to play a vital role in determining their ability to absorb different reagents, ranging from anionic dyes (Zografi & Mattocks, 1963) to amphiphilic molecules as monoglycerides (Van Lonkhuysen & Blankestijn, 1974). The results found in the present study seem to be consistent with early findings on the sorption of anionic dyes by various corn starches (Zografi & Mattocks, 1963), which revealed that the extent of sorption was related to their ratios of AMP to AM, an increase in sorbed amount occurring with an increase in the AMP content of the starches. Waxy starches are known to have not only much higher AMP contents, but also lower amounts of internal lipids and proteins than non-waxy varieties (Morrison, 1995; Vasanthan & Hoover, 1991). Combined, these features result in a more loosely bound internal granule structure, which upon heating swells in an unrestricted fashion and at lower temperatures (Grant et al., 2001; Tester & Morrison, 1990). In itself, the loose nature of the internal structure of the waxy starch granules could very well account for the ease with which the amphiphilic dyes are observed to penetrate through the matrix of these granules.

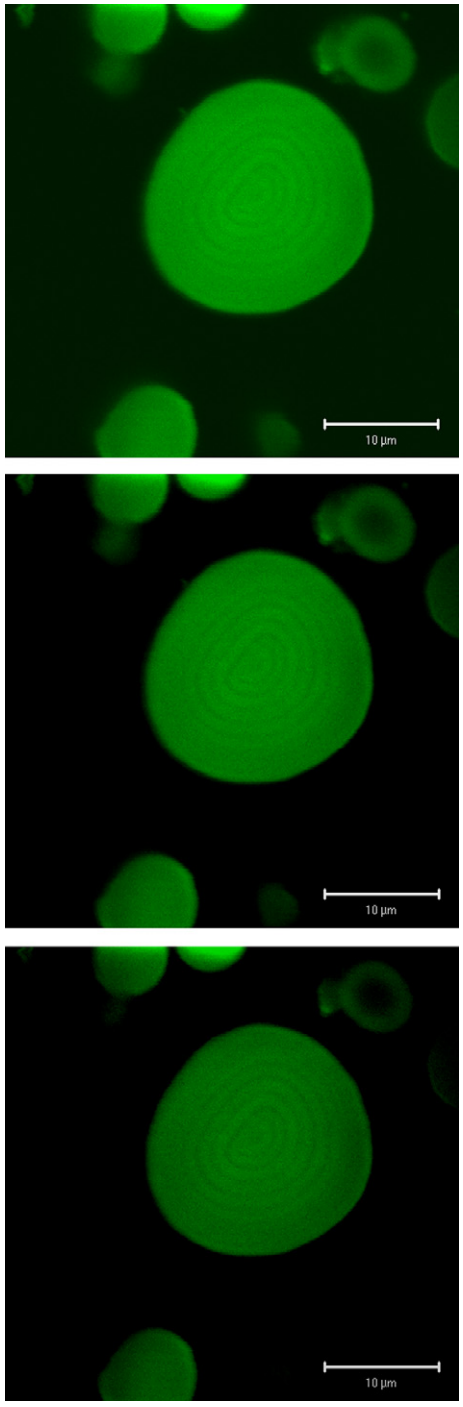


Fig. 3. High magnification micrographs (CLSM and transmitted light images) of an ungelatinized waxy wheat starch granule in the presence of C_{12}Fluor after long contact times (24 h). In the CLSM images (left) the fluorescence signal intensity is shown in a green colorscale (scale bar = 10 µm).

Beyond the driving force provided by the existence of a concentration gradient through the granule, interactions of chemical nature are expected to provide an important driving force for the penetration of reagents into the granules. It has been established using starch columns that only molecules no larger 800–1000 Da or with a hydrodynamic radius < 0.6 nm can penetrate granules (Brown & French, 1977; Planchot, Roger, & Colonna, 2000). However, despite these physical barriers, polysaccharides as large as FITC-Dextran ($M_W = 2{,}000{,}000$) have been found to be able to penetrate granule matrices (Villwock, 1999), whereas corresponding unsubstituted polysaccharides did not, suggesting that chemical properties provide driving forces that should also be considered. Thus, the occurrence of specific, chain-length-influenced interactions between the amphiphilic dyes and some of the starch granule components [for example the association with starch polysaccharides (AM and AMP)], may be partly responsible for the more restrained diffusion of the longer chain dye into the starch granules.

3.4. Staining patterns during starch gelatinization

Past their gelatinization temperature, starch granules in excess water lose their native semi-crystalline order and swell irreversibly several fold. At the same time AM (if present) is preferentially solubilized. Fig. 4 shows a series of higher magnification micrographs depicting a group of normal wheat starch granules as they underwent such changes in the presence of the C_{12}Fluor and C_{16}Fluor dyes. Transmitted light micrographs recorded along the temperature scan provided a good overview of the morphological changes that granules incurred as the gelatinization proceeded. In these sequences of images it is possible to recognize the typical and well-documented structural changes associated with the gelatinization of wheat starch granules in excess water (Bowler, Williams, & Angold, 1980; Eliasson, 1985).

The low solution concentration of the dye results in turn a in very low dye to starch ratio [0.6×10^{-5} mol/g starch (i.e., ~0.23% w/w)]. At levels of surfactant addition approximately twice as high as this one, surfactants (both food and non-food grade) have been reported to induce small (2–3 °C), yet significant changes in the pasting temperature (i.e., affect the granule swelling/AM-leaching properties) of wheat starch as determined by means of viscometric techniques (Mira, Eliasson, & Persson, 2005; Nierle & El Baya, 1990). In consequence, even at the low dye to starch ratio used in the present investigation the amphiphilic dyes may have the ability to alter, albeit not to a great extent, the swelling/AM-leaching properties of starch upon gelatinization. It will suffice for the purpose of this study to simply assume that such effect will be small. Thus, in the analysis of the micrographs, all the attention will be focused on the identification of any apparent differences in location/penetration patterns of the two dyes into the starch granules.

As revealed by the confocal laser scanning micrographs, along the restricted swelling stage of the granules (up to

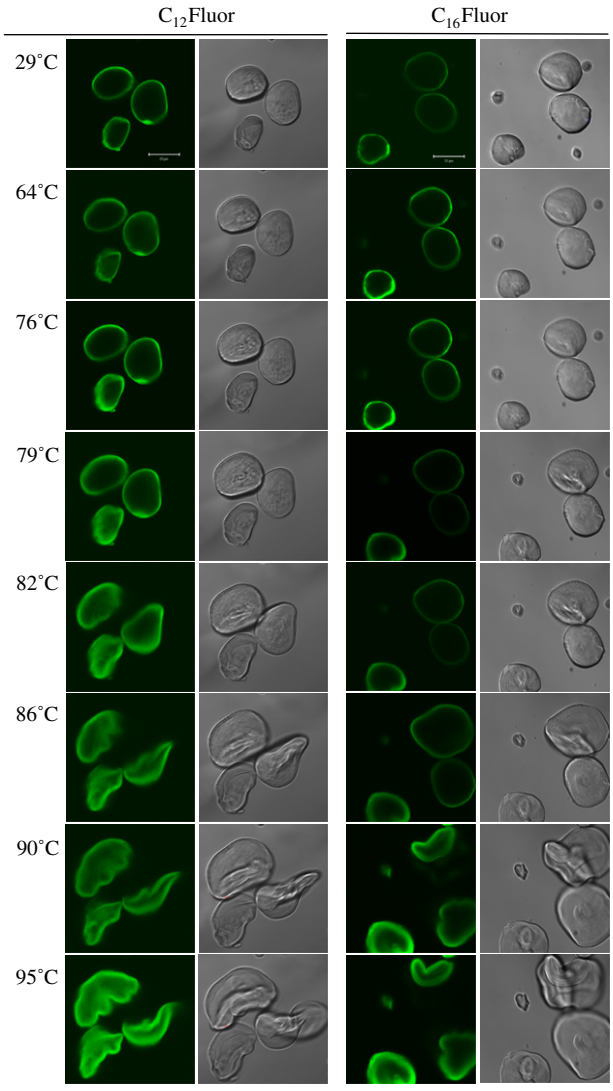


Fig. 4. *In situ* gelatinization of normal wheat starch in the presence of short- and long chain amphililic dyes (C_{12}Fluor and C_{16}Fluor, respectively). In the CLSM images (left) the fluorescence signal intensity is shown in a green colorscale (scale bar = 20 μm).

79 °C in Fig. 4) no extensive changes in the staining patterns of the dyes took place. Thus, both dyes tended to remain concentrated in the outer regions of the granules. However, as the temperature increased, the short-chain dye was observed to diffuse somewhat further into the granule matrix. In contrast, no noticeable changes in the staining pattern of the long-chain dye were observable within this temperature range. Along the extensive swelling stage of the granules (from 82 °C in Fig. 4), the diffusion of the short-chain dye into the granule matrix progressed rapidly and profusely. On the contrary, the long-chain dye remained confined to the outermost regions of the granules and a more extensive diffusion took place only after the granules had reached a high degree of swelling (90 °C in Fig. 4). Thus, it seems that as the granules gelatinize (i.e., the molecular order is lost and the internal structure of the granule becomes looser), the diffusion of the dye can proceed in a quicker and less restrained manner. Yet, the extent to which

the dye diffused into the granule matrix seemed to be controlled by the structure (i.e., chain length) of the dye.

Similarly to most linear-chain amphiphilic molecules, the two dyes used in this investigation were found to have the ability to form complexes with AM. Upon heating in excess of water (particularly after the internal order of the granules has begun to disassemble), amphiphilic molecules would form complexes with amyloglucans in the starch granules. In consequence, the possibility of a fraction of the amphiphilic dyes existing in the form of AM and/or AMP complexes is plausible. Moreover, the formation and characteristics of AM-dye complexes may partly account for the different diffusion behaviour of the dyes upon heating. Complexation with AM could restrict the mobility of the dye, which in turn could only diffuse freely once it is no longer complexed to AM. Thus, upon heating, profuse diffusion of the fraction of complexed dyes through the starch granules could only take place above the temperature at which the complexes start to dissociate, i.e., above ~75 °C for the short-chain dye and ~100 °C for the long-chain one (see $T_{O\,CX}$ in Table 2).

It is well-established that as the starch granules gelatinize, AM leaches out simultaneously as the granules swell. Furthermore, in the presence of amphiphilic molecules the AM could leach out alone or in association with these. The micrographs in Fig. 4 do not provide any conclusive evidence in this respect. Moreover, regardless of whether that is the case or not, the results presented here clearly indicate that as the starch gelatinization proceeds, more and more of the amphiphilic dye diffuses into the matrix of the granules.

The experimental setup chosen to carry out the *in situ* observations upon gelatinization of starch proved to be unsuitable for the investigation of waxy wheat starch samples. The rapid and unrestricted swelling characteristic of waxy starches made these samples highly sensitive to small, localized temperature gradients within the chambered microscope slide. Off-field waxy starch granules would invariably gelatinize first, thus creating local zones of gelatinized granules. The existence of such zones further hindered the attainment of an even heat and water distribution through the chamber, thus making the situation worse. Such an uneven gelatinization through the sample, made it impossible to find a field truly representative of any given temperature along the temperature scan. In consequence, the staining patterns of the amphiphilic dyes in waxy wheat starch granules are only analyzed on a "before" and "after-gelatinization" basis.

3.5. Staining patterns in gelatinized starch granules

At the final stage of the temperature scan (95 °C in Fig. 4 and lower magnification micrographs in Fig. 5), the gelatinized granules of normal wheat starch in the presence of both dyes exhibited a rather thick region of intense fluorescence towards the edge of the granules. On the other hand, the inner region the of granules exhibited different staining patterns depending on the type of dye present under gelatinization. The interior of granules gelatinized in the presence of the short-chain dye tended to look uniformly stained. In contrast, the innermost regions of granules gelatinized in the presence of the long-chain dye appeared either unstained or weakly fluorescent, implying that the long-chain dye had not been able to penetrate as far into the granule matrix as the short-chain dye.

The right hand images in Fig. 5 depict low magnification micrographs of gelatinized waxy wheat starch granules (i.e., at the end of a temperature scan from room temperature to 95 °C) in the presence of short- and long-chain amphiphilic dyes. Compared to normal wheat starch granules, gelatinized waxy starch granules are found to exhibit rather flattened and more distorted shapes. This finding is consistent with results from other studies which have found that upon gelatinization in the absence of mechanical shear waxy starches loose their integrity much faster and to a greater extent (complete disintegration) than non-waxy varieties (Van de Velde, Van Riel, & Tromp, 2002).

As observed in Fig. 5, the micrographs of gelatinised waxy wheat starch granules exhibited brightly stained granules, granules with brightly fluorescent inner cores and faintly stained granules where the dyes are concentrated

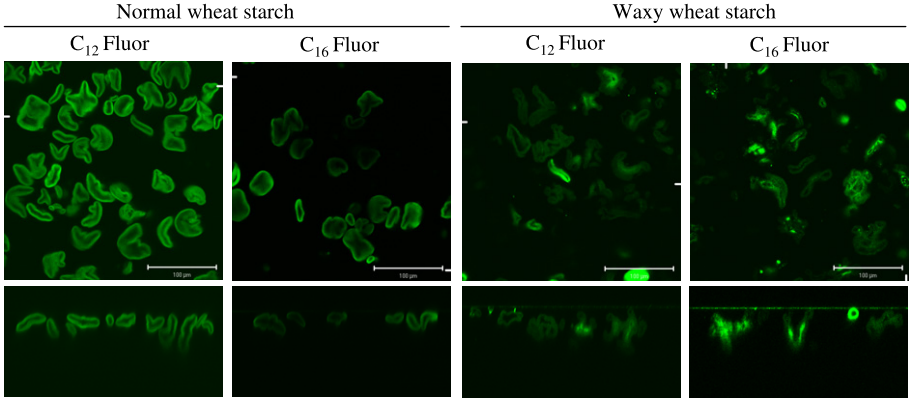


Fig. 5. Staining patterns of short- and long-chain amphililic dyes (C_{12}Fluor and C_{16}Fluor) in gelatinized normal and waxy wheat starch granules. Smaller pictures at the bottom of each image depict a perpendicular section plane taken along the line that connects the two marks along the perimeter of each image (fluorescence signal intensity shown in a green colorscale, scale bar = 100 μm).

towards the outer regions. Moreover, in contrast to what was found in normal wheat starch granules, no apparent differences can be recognized between the staining patterns of the two dyes. Thus, it seems that in this case (perhaps due to the absence of AM and the more loosely bound structure of waxy starches) the structural differences of the dye do not play such a central role in determining the final location of the dyes in gelatinized starch granules.

A remarkable, common feature regarding the staining pattern of gelatinized normal and waxy wheat starch granules is the tendency of the dyes to accumulate in the outer layers of the gelatinized granules. Interestingly enough, similar staining patterns have also been observed in gelatinized cereal and tuber starch granules stained with a water-soluble, anionic dye (zafranin) (Van de Velde et al., 2002). Thus, the accumulation of C_{12} and C_{16}Fluor in the outer layer of gelatinized starch granules should not be entirely attributed to the amphiphilic nature of the dyes. Light microscopy observations (smears and cryosections) of iodine-stained, gelatinized wheat starch suspensions (Langton & Hermansson, 1989; Richardson et al., 2003) seem to indicate that the outer layers of gelatinized starch granules consist mainly of AMP. Studies carried out on a series of gelatinized starches, revealed that protein (presumably granule bound starch synthase, GBSS) is concentrated in such outer regions as well (Han & Hamaker, 2002a). Consequently, the amphiphilic dye accumulated in these zones could be thought to consist of AMP and/or protein-associated dye. In addition, the presence of protein has also been thought to be partly responsible for the maintenance of the integrity of starch ghosts (gelatinized starch granule envelopes after the majority of internal starch polymers have been released) and remnants (Han & Hamaker, 2002a). Thus, it could be further hypothesized that the accumulation of dye in the outer layers of the gelatinized granule could also be the result of physical entrapment (due the presence of protein cross-links).

4. Conclusions

The two amphiphilic dyes used in this study were found to have a tendency to penetrate wheat starch granules suspended in aqueous solution. The penetration patterns were however found to depend on the contact time, the type of starch and the chain-length of the dye. The penetration of amphiphilic dyes through the matrix of the starch granules proved to be less restricted in waxy than in normal wheat starch, presumably due to the more loose bound internal structure of waxy starches. Moreover, for a given type of starch, the diffusion of the longer-chain dye through the granule matrix seemed to be more constrained than the one of the shorter-chain one.

In situ observations of normal wheat starch granules gelatinized in excess water in the presence of the two amphiphilic dyes revealed that up to ca. 79 °C the diffusion of both dyes through the granule matrix was rather restricted. However, even within this temperature range the diffusion of the short-chain dye was found to proceed in a less restrained fashion. The ability of some amphiphilic molecules to penetrate further and more quickly into the granule matrix would result in higher local amphiphile/starch ratios, which may affect their overall availability to interact with the AM and/or AMP fraction in starch as well as with proteins and other starch granule constituents as starch granules gelatinize. Beyond their ability to compl2007ex AM and/or AMP, and the effect the properties of such complexes may have on the swelling/AM-leaching processes, the different penetration ability of short- and long-chain amphiphiles through starch granule matrices may contribute to some extent to the distinctive effect that some short- and long-chain amphiphilic molecules have been reported to have on the swelling/AM-leaching properties of wheat starch.

After the onset of the extensive swelling state of the granules (ca. 80 °C), the diffusion of both dyes tends to proceed in a less restrained fashion. Yet, the shorter chain dye diffuses in more profusely and at lower temperatures than the longer chain one. Differences in the thermal stability (dissociation temperature) of the complexes formed between the dyes and AM may partly account for the different penetration patterns of the two dyes during starch gelatinization.

In gelatinized starch granules, the concentration of the amphiphilic dyes in the outer envelope of the granule was found to be significantly higher than in the rest of it. The amphiphilic dye accumulated in these zones is believed to consist mainly of AMP and/or protein-associated dye, although the possibility of such accumulation being the result of some sort of physical entrapment (due the presence of protein crosslinks) cannot be ruled out. In it self, the accumulation of amphiphilic molecules in the envelopes of the gelatinized granules can be expected to have important consequences for the properties of the gelatinized granules, and may partly account for the effect of surfactants on the rheological behaviour of starch pastes.

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