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The influence of *O*-formylation on the scale of starch macromolecules association in DMSO and water

Thomas Divers [a], Eric Balnois [a,*], Jean-Francois Feller [a], Jiri Spevacek [b], Yves Grohens [a]

[a] *Laboratoire Polymères, Propriétés aux Interfaces et Composites (L2PIC), Université de Bretagne-Sud, Rue de Saint-Maudé, BP 92 116, 56321 Lorient, France*

[b] *Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, 16206 Prague 6, Czech Republic*

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Abstract

There is a great challenge in finding treatments that may degrade the initial grain structure of native starch in order to get better mixing properties of the resulting degradation product with other polymers. Among the possible treatments, *O*-formylation has already been proposed but only little structural information on the resulting degradation products is available, certainly due to its heterogeneity. The present study aims to complement our knowledge on the structure of macromolecules following the chemical reaction by means of different techniques. At first, polarimetry reveals, on amylose and maltodextrin samples, that the helix content remains unchanged after esterification. Then, high resolution ^{1}H NMR measurements allow to determine the association parameter, *p*, and it was found that the fraction of associated helix, although strongly influenced by the nature of the solvent, is similar for native and starch formate. It was deduced that formic acid is not reactive on the less accessible macromolecules such as the aggregates preserved from the initial destructuration of starch during gelanitisation and following the thermal treatment, *i.e.* the larger molar weight samples. Finally, AFM observation corresponded well with the results obtained by the other techniques, since large aggregates, such as helix assemblies or amorphous and globular aggregates, were found to be unaffected by the formic acid treatment.

Keywords: Starch; *O*-Formylation; Helix association; Aggregation; NMR; AFM

1. Introduction

Research on biodegradable polymers has received increased attention in recent years because of their wide applications in environmental friendly packaging, biomedical materials, but also in various industrial applications such as in computers and mobile phones. The most popular and biodegradable polymers are aliphatic polyesters, such as polylactic acid (PLA), polycaprolactone (PCL), poly(butylene adipate terephthalate) (PBAT) and polyhydroxybutyrate (PHB). However, since these polymers are still expensive and do not respond to all the technical requirements for several applications, blends of these polymers with starch, which is a cheap abundant resource, lead to a huge number of papers and patents (Avella et al., 2000; Avérous, Moro, Dole, & Fringant, 2000; Bastioli, 1998; Koenig & Huang, 1995; Novamont, 1993, 1998, 2002; Martin & Avérous, 2001; Singh, Pandey, Rutot, Degée, & Dubois, 2003; Wu, 2003).

Starch is a naturally occurring polysaccharide present in many plants to store energy. It is mainly composed of two macromolecular α-glucans, an essentially linear one (amylose) and a highly branched one (amylopectin) (Buléon, Colonna, Planchot, & Ball, 1998). These two polymers arrange themselves into a complex structure comprising different levels of organization with alternating crystalline and amorphous regions (Gallant, Bouchet, & Baldwin, 1997), leading to the well-known 10–100 μm granule structure depending on the starch origin (Tester, Karkalas, & Qi, 2004). These resulting structures have recently been

* Corresponding author.
E-mail address: eric.balnois@univ-ubs.fr (E. Balnois).

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observed using microscopic techniques such as electron microscopy (Gallant, Bouchet, & Baldwin, 1997) and AFM (Baldwin, Adler, Davies, & Melia, 1998; Juszczak, Fortuna, & Krok, 2003; Ohtani, Yoshino, Hagiwara, & Maekawa, 2000). Their internal organization has also been explored using AFM (Ridout, Gunning, Parker, Wilson, & Morris, 2002) and scattering techniques (Donald, Kato, Perry, & Waigh, 2001) to reveal the existence of "blocklet" structure.

Unfortunately, there are strong limitations for developing starch-based materials due to its poor mechanical properties, to its water-sensitivity and important post processing variation of the properties (*e.g.* Avérous & Boquillon, 2004). Nano-scale dispersion of starch particles in the polymer is the actual challenge for achieving the designed properties which will make these blends competitives with classic polymers. It is now well-established that starch can not be used in its native form to develop a biodegradable blend with optimised properties and it is necessary to degrade the initial grain structure of native starch in order to get better mixing properties of the resulting degradation product with other polymers. This can be achieved through physical and chemical modification (Blaszczak, Valverde, & Fornal, 2005; Buchanan, Doane, Russell, & Kwolek, 1975; Fringant, Rinaudo, Foray, & Bardet, 1998; Heeres, Van Doren, Gottlieb, Bleeker, & Kellog, 1998; Kirby, 1986; Lewandowicz, Fornal, & Walkowski, 1997, 2000; Morton, Rutenberg, & Solarek, 1984; Mullen & Pacsu, 1942; Rinaudo & Fringant, 1997; Stolt, Oinonen, & Autio, 2001; Whistler & Hilbert, 1994; Wolff, Olds, & Hilbert, 1951).

Oxidation of starch can be, for example, obtained by reacting starch with a specified amount of sodium hypochlorite (NaOCl) under controlled temperature and pH (*e.g.* Kuakpetoon & Wang, 2001; Whistler, Linke, & Kazeniac, 1956). The reaction results in the oxidation of starch hydroxyl groups into carbonyl groups and then to carboxyl groups. These studies reported that starch structure highly influence the extent of the oxidation reaction. For example, Kuakpetoon and Wang (2001) showed that more amorphous starch structures, or more loosely packed crystalline structures, may provide more accessible reaction site for oxidizing agent in comparison with crystalline starch. It was also demonstrated that high amylose corn starches may also influence the extent of oxidation, by changing the packing of amylopectin double helices (Jenkins & Donald, 1995). Jenkins and Donald (1995) suggested that amylose might disrupt amylopectin double helical packing by, firstly a mechanism of co-crystallization between amylose and secondly the penetration of amylose macromolecules into the amorphous region of the cluster. As a consequence, the crystalline lamellar size increases. Other treatments were based on acidic reaction (hydrochloric acid hydrolysis) and yielded a degradation of the starch granules through the preferential hydrolysis of the amorphous regions (Dufresne & Cavaillé, 1998; Putaux, Molina-Boisseau, Momaur, & Dufresne, 2003). It was demonstrated in these studies that hydrochloric acid, within their experimental conditions, has no effect on the molecular packing of the double helices in the crystalline region and generates an insoluble residue made of polydisperse and more or less individual platelet nanocrystals, corresponding to the lamellae formed by the association of amylopectin side branches into parallel arrays of double helices. Formic acid is also another interesting reactant which promotes starch compatibility with synthetic polymers by decreasing its hydrophilic character (Aburto, Alric, & Borredon, 1999, 2005; Aburto et al., 1999; Chronakis, 1998; Divers, Pillin, Feller, Lévesque, & Grohens, 2004; Gottlieb, Caldwell, & Hixon, 1940; Pillin, Divers, Feller, & Grohens, 2005; Tarkow & Stamm, 1951; Wolff, Olds, & Hilbert, 1951, 1957). For example, Gottlieb et al. (1940) showed that esterification chiefly occurs on C-6 of the glucose unit, conducting to a monoformic ester with a degree of substitution (DS) equal to 1. These results are consistent with our previous studies in which the same regioselectivity was observed (Divers et al., 2004; Pillin et al., 2005). It was also reported, by Aburto et al. (1999, 2005), that the highly active formic acid leads to a destructuration of the semi-crystalline starch granule and dispersion of amylose and amylopectin macromolecules and reacts with some of the alcohol functions of these two polymers to produce their "formate esters", but their statements were not experimentally demonstrated. In another paper by the same authors, they reported that *O*-formylation reaction gelatinises starch and allows further esterification of free hydroxyl groups (Aburto et al., 1999). More recently, Divers et al. (2004) studied starch modification, destructuration and hydrolysis during *O*-formylation. They showed that acid formic acts both as an efficient reactant and as a powerful destructuring agent. Following the evolution of storage modulus values (G') by dynamic rheological measurements, they determined the optimal experimental conditions of acid formic concentration, reaction time and temperature in order to sufficiently destructure starch granules and to obtain a relatively high degree of substitution (DS), but also to limit the fragmentation/hydrolyis of the macromolecular starch chains. They observed a gelatinisation of starch after formic acid treatment which indicates a destructuration of starch up to a gel structure made of amylose and amylopectin macromolecules or aggregates. Nonetheless, the structure or nature of starch formate (individual macromolecules of amylose and amylopectin, homoaggregates or heteroaggregates of the same polymers, helices, helix associations) was not reported. Finally, the same authors observed the formation of fillers with nanometric dimensions following their acid formic reaction (Pillin et al., 2005).

From the above studies, it is clearly demonstrated that the structure of starch may strongly influence the efficiency of the chemical reagents. Up to now, the different steps involved in the chemical reaction of the complex and multiscale organisation of starch is not clear and the question of the influence of the chemical reaction on the resulting modified starch structure has not yet been addressed. The

aim of this paper is thus to gain an understanding on the exact structure of starch particles following formic acid treatment. Due to the highly heterogeneous structure of the native material and of its chemically modified compounds, the use of different but complementary techniques was necessary. Firstly, the structure of amylose, used as a model polysaccharide, in DMSO and in water, was investigated by means of polarimetry which is sensitive to the presence of helices. Secondly, NMR studies were performed and an original approach, based on a comparative determination of the association parameter, was conducted. Finally, the AFM technique was adapted to probe the structure of starch, before and after the destructuration process.

2. Experimental

2.1. Materials

Wheat starch (reference I59-113H10) was provided by ROQUETTE (France). It is roughly composed of 24% amylose, 75% amylopectin and 1% of minor components (0.3% lipid, 0.35% protein and 0.35% mineral). Its water content, measured by TGA, was found to be 13%. All these results were provided by technical data sheets.

Maltodextrin was used as a model system of starch for polarimetry measurements because of its better solubility in water in comparison with starch. Maltodextrins contains linear amylose and branched amylopectin degradation products from enzymic hydrolysis of starch (Chronakis, 1998) and are characterized by a dextrose equivalent (DE), which reflects the degree of hydrolysis (the DE of starch is 0 while the DE of glucose is 100). Maltodextrin with a DE of 6 were obtained from Sigma–Aldrich.

The different polysaccharides solutions, including native starch, modified starch, amylose (Sigma–Aldrich) and maltodextrin were prepared by dissolving the samples in distilled water (MilliQ-Plus) or in DMSO to a final concentration of 10–1000 mg L^{-1}. The solutions prepared in DMSO were boiled for 1 h and gently stirred during at least 24 h to ensure a complete dissolution (Han & Lim, 2004) The solutions prepared in water were heated at 80 °C for 4 h and gently stirred during at least 24 h, they were then kept in the fridge when not being used and were nonetheless discarded after 1.5 weeks.

2.2. Methods

2.2.1. Starch and maltodextrin esterification

Formic acid solution (FA) at 99% was used as received from Sigma–Aldrich to modify native starch, amylose and maltodextrin and used as follow: 50 g (0.31 mol) of dry native starch (maltodextrin) were introduced in a three-necked flask containing 250 mL of 99% formic acid (FA), which corresponds to 305 g (6.62 mol) of FA. The mixture was stirred at 20 °C for 6 h. At the end of the reaction, the solution was gently poured into methanol (1 L) and the precipitated polymer was filtered off and washed three times with methanol (3 × 300 mL) to remove FA in excess until a neutral pH was obtained. The samples were then dried in an oven at 50 °C for 24 h under vacuum. The yield of the reaction was 85% for starch and 80% for maltodextrin.

2.2.2. Determination of the degree of substitution

Before analysis, starch and maltodextrin formates were dried at 70 °C for 24 h. About 1 g of formate was introduced into a flask containing 100 mL of an ethanol–water solution (75/25 v/v). The suspension was stirred at 50 °C for 30 min, cooled at room temperature and 50 mL of a 0.5 mol L^{-1} KOH solution was added. After 72 h of stirring, the base excess was titrated with hydrochloric acid (0.5 mol L^{-1}) using phenolphthalein as indicator (Wurzburg, 1964). A blank analysis was carried out on native dried sample. The DS was calculated from Eqs. (1 and 2):

$$\%\ \text{acyl} = \frac{m_a}{m_e} \times 100 \quad i.e.$$

$$\%\ \text{acyl} = \frac{(V_b - V_a) \times 0.5 \times M_E}{m'_e} \times 100$$

$$\text{DS} = \frac{162 \times \%\ \text{acyl}}{(M_E \times 100) - [\%\ \text{acyl} \times (M_E - 1)]} \tag{2}$$

where m_a is the ester weight in formate (g), m_e is the sample weight, V_b is the HCl solution volume (L) for native sample, V_a is the HCl solution volume (L) for ester, M_E is the molecular weight of grafted ester group (g mol^{-1}) ($M_{CHO} = 29.04$ g mol^{-1}) and m'_e is dried formate weight.

The DS obtained were 1.25 for starch formate and 1.45 for maltodextrin formate. This difference can be explained by the complexity of starch architecture, as compared to maltodextrin architecture, which limited an effective reaction of formic acid on the hydroxyl groups due to steric effect.

2.2.3. Viscometric measurements

Reduced viscosities were determined measuring the flow time of a polymer solution of known concentration and the flow time of the pure solvent in a capillary viscometer at 25°C. The reduced viscosities of formates were measured at 0.50% concentration in a DMSO (dimethylsulfoxide): H_2O mixture, 90:10 (w/w). Flow times were measured with a Type I SCHOTT GERATE UBBELOHDE tube driven by a SCHOTT GERATE AVS 310 controller. The reduced viscosity was calculated according to the following equation:

$$\eta_{red} = \frac{1}{C}\frac{\eta - \eta_0}{\eta_0} = \frac{1}{C}\frac{t - t_0}{t_0} \tag{3}$$

where C is the polymer content (g mL^{-1}), t and t_0 are the flow times for the solution and the pure solvent, respectively.

Unfortunately, this method does not allow any quantitative determination of the molecular weight distribution of starch due to its highly heterogeneous structure. Even in

optimised sample solubility in aqueous solvents (in 0.001 N KOH), size exclusion chromatography experiments shows the occurrence of some aggregates which indeed demonstrates the difficulty to determine the molecular weight distribution of starch (Chen, Fringant, & Rinaudo, 1997).

The reduced viscosity of maltodextrin only slightly decreases after *O*-formylation (from 3.5 to 3.1 g/mL) which therefore indicates a limited hydrolysis of the polysaccharides chains. In comparison, the reduced viscosity of native starch is decreased by almost 40% (from 170 to 103 g/mL) after the formic acid treatment. Since starch and maltodextrin are chemically identical but structurally different, we hypothesized that this large decrease in viscosity for starch formate could be interpreted as a consequence of an important destructuration rather than a hydrolysis process.

2.2.4. Determination of rotation angles by polarimetry

Rotation angles were determined using a POLAX-2L polarimeter from ATAGO, in 200 mm long observation tubes. The wavelength of the measuring light was fixed at 589 nm, and the experiment was performed at 50 °C. Eq. (4) allows for the calculation of the relative optical rotation from the rotation angle values:

$$[\alpha]_{589\ \mathrm{nm}}^{50\ ^\circ\mathrm{C}} = \frac{\alpha}{l \times C} \tag{4}$$

Where $[\alpha]_{589\ \mathrm{nm}}^{50\ ^\circ\mathrm{C}}$ is the relative optical rotation, α is the measured angle of rotation, l is the length of the observation tube (dm) and C the concentration of the optically active matters in sample ($\mathrm{g\,mL^{-1}}$). This measurement allows to determine the concentration of helices present in the samples according to Eq. (5):

$$\chi = \frac{[\alpha]_\lambda^{\exp} - [\alpha]_\lambda^{\mathrm{coil}}}{[\alpha]_\lambda^{\chi=1} - [\alpha]_\lambda^{\mathrm{coil}}} \tag{5}$$

where χ is the helix content, $[\alpha]_\lambda^{\exp}$ is the measured angle of rotation, $[\alpha]_\lambda^{\mathrm{coil}}$ is the angle of rotation of a solution for which $\chi = 0$ and $[\alpha]_\lambda^{\chi=1}$ is the angle of rotation of a solution for which $\chi = 1$.

It must be noted that the objective of this experiment was not to obtain an exact value of the concentration of helices present in solution but to observe their variation as a function of the nature of the solvent and especially when the acid formic concentration increases.

2.2.5. High-resolution ^1H NMR measurements

High-resolution (HR) ^{1}H NMR spectra were measured with a Bruker Avance DPX 300 spectrometer at 300.1 MHz. Typical measurement conditions were as follows: pulse width 15.6 μs (90° pulse), relaxation delay 10 s, spectral width 5995 Hz, acquisition time 2.73 s, 8 scans. The absolute integrated intensities were determined with the spectrometer integration software with an accuracy of ±1% on solutions (gels) of the same concentration (w/v) and measured under identical instruments conditions. In all measurements, the temperature was maintained constant within ±0.2 °C using a B-VT 2000 temperature unit. The solutions were studied in 5 mm NMR tubes; sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and hexamethyldisiloxane (HMDS) were used as internal NMR standards for D_2O and dimethyl sulfoxide-d_6 solutions, respectively. Solutions were prepared at 340 K, previously to analysis and measurement conducted every 10 °C from 335 to 295 K. For each temperature, NMR tubes were left in the magnet around 20 min before measurement in order for to stabilize the temperature.

From these NMR measurements, an association parameter p, introduced by Spevacek (e.g. Spevacek, Hiller, Heltrich, & Joel, 1989 and references therein), was studied as a function of the temperature and calculated according to the following equation:

$$p = 1 - \frac{I}{I_0} \tag{6}$$

where I is the intensity detected for the considered polymer band and I_0 the intensity when no association occurs. P values are comprised between 0 and 1.

When polymer chains are self-associated, the dynamics of the chains will decrease. When the motion of the chains is reduced, the NMR peaks broaden so much that its intensity at a given chemical shift decreases and consequently, the p value, which is inversely proportional to I, increases. The p value can be used to compare the relative level of association (aggregates versus isolated entities that could either be individual macromolecules, single or multiple helices) which could exist in such heterogeneous samples. Indeed, at each temperature, I_0 was chosen for the sample with the highest integrated intensity, namely amylose in DMSO-d_6, and consequently its associated p was considered equal to zero even if it may not be the right value.

2.2.6. AFM imaging

Polysaccharide solutions were deposited onto freshly cleaved mica using the drop deposition. In the drop deposition method, a drop of the solution (5 μL) was pipetted and directly deposited onto the mica or a silicon wafer and allowed to evaporate under ambient conditions (23 °C and 60% of relative humidity) for at least 15 min prior to observation. The samples were placed into a Petri Dish under ambient conditions for at least 20 min. The advantages and limitations of this preparation method for the observation of biopolymers by AFM have already been discussed elsewhere (Balnois & Wilkinson, 2002; Wilkinson, Balnois, Leppard, & Buffle, 1999). The spin coating deposition method was also used in this study in order to evaluate the effect of the deposition method on the observed structure of the samples. In brief, the difference between the two deposition methods can be summarised as follow: on the one hand, the drop deposition will allow for the observation of all the species in solution, although almost inevitably, concentration gradients were created during the drying process, on the other hand, the spin coating is simply used to obtain a homogeneous thin film on the substrate surface with limited sample concentration effect.

AFM images were obtained under ambient conditions (23 °C and 60% of relative humidity) using light tapping mode AFM (TM-AFM) on a multimode scanning probe microscope (Nanoscope IIIa, Veeco). The ratio of the set-point amplitude to the free amplitude was maintained approximately at 0.9. Commercial RTESP AFM tips (Veeco), with typical resonance frequency between 300 and 400 KHz and with tip radius between 5–15 nm, were used. Height and length measurements of the deposited macromolecules were made using the section analysis software of the microscope (V5.12r3 by Digital Instruments).

3. Results and discussions

The results are presented and discussed according to the nature of the existing structure in starch, before and after modification, and are investigated to figure out the scale of the formic acid structural modification. Therefore, two main levels of organization were distinguished: (i) individual entities such as macromolecular chains or single helices and (ii) aggregates including helix associations (double helices or helix assemblies) and non-structured aggregates. It has to be mentioned that, due to the highly polydisperse nature of starch, some classic techniques are sometimes not adapted and model compounds such as maltodextrin, a lightly hydrolysed starch (DE = 6) and amylose, which represents roughly 24% of glucan macromolecules in wheat starch, were consequently used.

3.1. Comparison between double helix content and association

Angles of rotation were determined for, on the one hand, two solutions of amylose in DMSO:H_2O mixtures, 90:10 and 50:50 (w/w) (Fig. 1a), and, on the other hand, three solutions of maltodextrin in FA:H_2O mixtures, 0:100, 20:80 and 100:0 (w/w) following a reaction of 6 h (Fig. 1b). The first graph (Fig. 1a) relates that the amount of helices is higher in a mixture with increasing DMSO content. This result is consistent with the literature which reported a change in conformation from helix to coil with decreasing DMSO concentration (Cheetham & Tao, 1998; De Vasconcelos, Pereira, & Fonseca, 2001). The existence of this helix-coil transition was reported to occur for a DMSO:H_2O ratio of 67:33 (w/w). It further pointed out that polarimetry is well-suited to observe whether or not the helical structures have been modified upon the *O*-formylation reaction. Nonetheless, due to the poor solubility of starch in aqueous solvents, polarimetry experiments were not possible on starch and starch formate sample, but only possible on maltodextrin. As already discussed in the experimental part, this sample is commonly compared to starch. Fig. 1b presents the angle of rotation as a function of the maltodextrin Concentration for different FA:H_2O solution concentrations. This result indicates that the total helix content in the maltodextrin solution is not altered by increasing FA, within our experimental set-up. This means that even though FA induces maltodextrin hydrolysis, the amount of helical entities, determined by polarimetry, remains the same as it is in water. Moreover, this helix content is not modified, at least after six hours of *O*-formylation reaction. As maltodextrin and starch have similar structures, we assume that these results can be extrapolated to starch and consequently, based on these measurements, the structural modification of formic acid does not seem to be reactive on helical entities and may process on another scale of organisation of starch.

3.2. High-resolution ^1H NMR measurements

Figs. 2a and b represent the variations of *p* parameter for starch, modified starch and amylose, respectively in DMSO and water, as a function of the temperature. According to Spevacek et al. (1989), the *p* parameter is sensitive to the association of polymeric helix sequences within a sample. Therefore, *p* will be discussed as the fraction of helix assembly present in the sample in the liquid or gel state. This can be illustrated by looking at the *p* parameter of amylose, which is present as individual linear chains forming single helices in highly concentrated DMSO aqueous solutions. For amylose, *p* is found to be higher in water and this increase can probably be explained by the helix-coil transition that occurs in water (DMSO is known to stabilize amylose simple helix conformation) that may

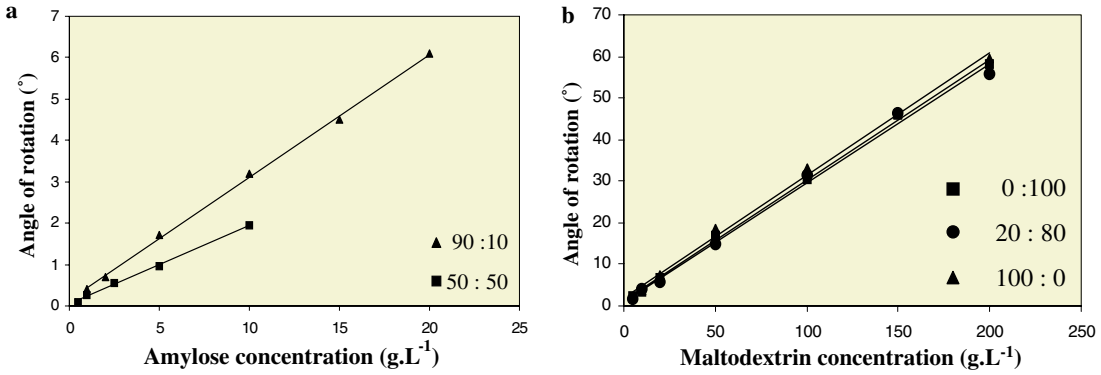

Fig. 1. Determination of relative angle of rotation – (a) amylose in DMSO:H_2O solutions (w/w), (b) maltodextrin in FA:H_2O solutions (w/w).

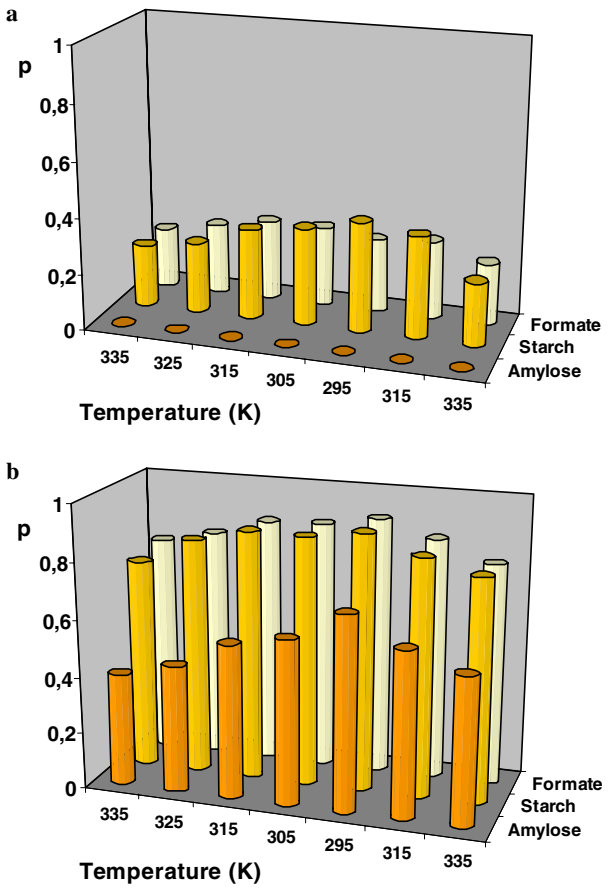

Fig. 2. Determination of p parameter for amylose, starch and starch formate – (a) in DMSO-d_6, (b) in D_2O.

generate aggregates between associated fractions of coils and/or helices, resulting in an increase of the p factor (Cheetham & Tao, 1998).

It is also interesting to note that p value is very low for amylose whatever the temperature whereas higher values are obtained for starch and starch formate, in DMSO and in water. This indicates that starch and starch formate are mainly present as aggregates, possibly amorphous ones or helix assemblies. Furthermore, the presence of amylopectin in starch might thus induce an increase in p value as compared to solely amylose because of highly organized sequences between these two polymers.

No significant difference in p value can be found between starch and modified starch ($p \sim 0.4$). It can therefore be concluded that, at the scale of helix assembly, a DS of 1.5 does not modify the starch structure. This result corresponds well with polarimetry measurements on maltodextrin that shows a non-variation of the helix content when FA concentration increases. We assume that this is due to the resistance of a well-ordered region, such as helix aggregates, to the acid diffusion and reaction.

If we now compare the p values between the two solvents, they are systematically found to be higher in water solution in comparison with DMSO-d_6 solution. This effect was also expected since DMSO is known to be a destructuring solvent for many polysaccharides and is consistent with the observation of Everett and Foster reporting that starch solubilization could be achieved in DMSO without structural degradation (Everett & Foster, 1959). For example, DMSO is also known to break up triple helix associations of schizophyllan into individual chains (e.g. Sato, Norisuye, & Fujita, 1981, 1983). In D_2O, amylose has the lower p value since it is known to have better affinity with water than amylopectin.

Finally, the effect of the temperature was also investigated and p was found to increase with decreasing temperature. This result is also consistent with the assumption that the level of helix association is dependent on temperature as it is favoured at lower temperatures. An effect of the temperature can also be observed for this polymer with an increase of p when decreasing the temperature. This is also consistent with the assumption of temperature dependency for the level of helix association which is favoured at lower temperatures. Starch and starch formate yield exactly the same p values and very slight temperature dependency in the studied range. Likewise, p is lower in D_2O for amylose than for starch and starch formate

The important information drawn from this graph are (i) the p values for amylose, starch and starch formate are lower in DMSO-d_6 than in D_2O, showing that the degree of association, possibly helix structures as revealed by polarimetry, is reduced according to the well-known solvatation properties of DMSO, and (ii) the similar values of p for both starch and starch formate in DMSO-d_6 ($p \cong 0.4$) and in D_2O ($p \cong 0.8$) highlights the fact that the degree of association of the locally organized polymeric chain segments is roughly the same. It can then be inferred that the helix associations present in starch, probably in crystalline regions, are strong enough to resist the formic acid treatment.

3.3. AFM imaging

Fig. 3 shows TM-AFM images of amylose, native starch and starch formate materials in DMSO or in water deposited on freshly cleaved mica by the drop deposition technique. It is worth mentioning that, for AFM, solutions were highly diluted (10–100 mg L^{-1}) in comparison with the NMR studies (10–100 g L^{-1}). Furthermore, in AFM the observed structures are adsorbed or deposited conformational structures on a surface that do not necessarily reflect a 3D solution conformation of the polysaccharides. Many effects may arise from the deposition process because of the competition of various driving forces such as surface interactions, capillary forces, or concentration gradients acting during the adsorption/evaporation process (*e.g.* Balnois & Wilkinson, 2002). However, in order to compare the obtained structures for the various polymers, two sample deposition techniques, the drop deposition and the spin coating methods, were tested and no major differences were observed within the samples, indicating that the structures imaged on mica were independent of the sample preparation used here.

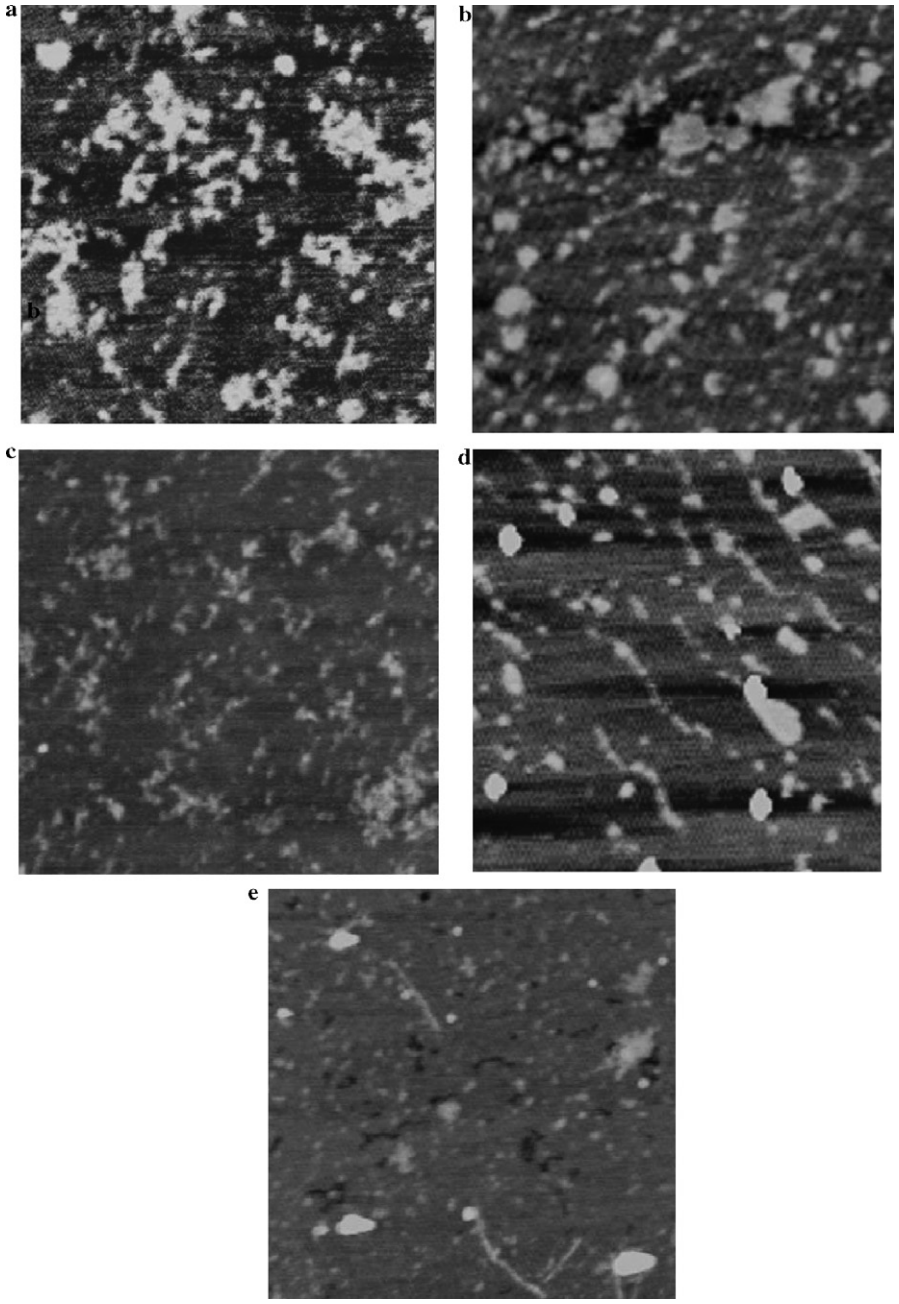

Fig. 3. TM-AFM images of (a) amylose in DMSO deposited on mica (scan size is 2×2 μm); (b) amylose in water deposited on mica (1×1 μm); (c) starch in DMSO deposited on mica (1×1 μm), (d) starch in water deposited on mica (1.2×1.2 μm) and (e) starch formate in water deposited on mica (1.2×1.2 μm).

Fig. 3a represents the AFM image of amylose in DMSO (10 mg L^{-1}) deposited on mica. The macromolecules appear as fairly elongated structures with typical height of 0.3–0.5 nm and typical length of 50–150 nm. The measured heights of the chains correspond well with previous studies (Gunning et al., 2003; McIntire & Brant, 1999). Furthermore, assuming a linear mass density of $\sim$1200 g mol^{-1} nm^{-1} and a length of 1.33 nm per monomer (Walton & Blackwell, 1973), the estimated molar weight of these entities would correspond to $\sim 5 \times 10^5$ g mol^{-1}, in good agreement with the expected molar mass of amylose. When amylose in water is deposited on mica, the polymers adopt a different conformation as revealed by Fig. 3b. In this case, quasi-spherical points can be observed, which are typical of a coil conformation of flexible polymers (*e.g.* Wilkinson et al., 1999). The spots have a large size distribution in contrast with the same sample in DMSO: this larger size distribution can be due to the formation of aggregates that can be formed on the mica surface during the evaporation process. Nonetheless, these results on amylose are consistent with the expected conformation of this polymer in DMSO and in water, *i.e.* a rather elongated

structure in DMSO, possibly a helix conformation and a transition to a coil in water.

Starch macromolecules, after dissolution in DMSO and deposited on mica (Fig. 3c) appear as a sample with mainly two types of entities: (i) large and branched fairly globular aggregates with typical height between 2 and 5 nm and lateral dimension from 100 up to 400 nm, and (ii) a fairly homogeneously distributed linear and elongated structure, with typical height and length of 0.4–0.6 and 50–150 nm, respectively. These second entities, similar in size and shape to amylose macromolecules observed in Fig. 3a, were sometimes found on mica as interconnected and associated chains forming a 2D gel-like structure, due to a non-homogeneous evaporation of the solvent on the surface, therefore creating locally high gradients of concentration. These gel structures were not observed when the sample were prepared by spin coating since this deposition technique is known to limit sample concentration effect on substrate in comparison with the drop deposition one. Nonetheless, it has to be pointed out that this gel structure was the only different structure found between the samples prepared by the two different sample preparation techniques. Since these fairly linear entities were attributed to amylose, we hypothesize that the second entity present on the image, *i.e.* the more condensed and globular aggregates, are aggregates of amylopectin. This large globular aspect could be related to the branching character of this macromolecule which could be responsible for a cluster model arrangement in which short chains, which may be multiply branched, are arranged in clusters along the main chains which are themselves linked together.

Fig. 3d shows an AFM image of starch dissolved in water, and deposited by the drop deposition method on freshly cleaved mica. The image clearly reveals that the sample is fairly heterogeneous, both in size and shape. Two main types of structures can be observed: long and rigid chains with typical heights of 0.6–1.0 nm and lengths >300 nm which are probably helix associations and large and non-structured aggregates. The major difference between the images obtained from the different solvent is the disappearance of the long and highly rigid structures (with $L > 300$ nm) that were found in water. This observation is consistent with the expected effect of DMSO that may de-structure helix associations or aggregates, corresponding well with NMR results.

The AFM images of starch samples following *O*-formylation and redissolved in water (Fig. 3e) display the presence of nearly the same entities as for starch in water, but in different proportion. Less elongated chains were found following the acidic reaction. Nonetheless, there is still some highly rigid rods (with $L > 300$ nm) that were attributed to helix association. This result is in good agreement with the NMR studies that indicate that the p factor was similar before and after the *O*-formylation. Some amorphous aggregates were also found with similar sizes as for native starch. The only observable difference on the structure after the acid reaction can be attributed to the conformation of the smaller entities that go from a fairly elongated structure before the *O*-formylation to a more coiled one. No major differences were found between the two starch samples when they are in DMSO (image not shown here): this could be explained by the fact that in DMSO, associated structures such as helix assemblies present in starch are dissociated.

The AFM was successfully used to identify the existing structures of starch before and after *O*-formylation reaction. Nonetheless, due to the highly heterogeneous structure of starch, one must be cautious in the interpretation of the result. The main observation deduced from the AFM experiments lies in the presence of rigid rods, both in starch and starch formate samples, therefore indicating that the acid reaction in this study was not efficient to destructure these entities. Furthermore, the large and globular aggregates, presumably amylopectin, are also found on both samples, also indicating that the reaction does not dissociate the high molar weight entities. These results are consistent with the polarimetry and NMR experiments which also indicate a non-destructuration of the aggregates, including helix association, in starch formate prepared within our experimental conditions.

4. Conclusion

The mechanism of *O*-formylation on starch has been investigated by means of complementary techniques and model polymer constitutive of starch. It was first shown by optical polarimetry that helix structures of maltodextrin are not degraded by the esterification reaction. The same phenomenon is supposed to occur with starch helix structure. The preservation of helix or helix associated structures was confirmed by NMR experiments on complex systems such as starch and starch formate which do not reveal any change in the p factor. On the other hand, the destructuring effect of DMSO on associated helices was clearly observed on the same complex system. Finally AFM images of the highly polydispersed starch and starch formate samples reveal that aggregated and fairly rigid structures, such as helix assemblies, are still present after the acid attack. This result was found in good agreement with the NMR and polarimetry experiments and underlines the fact that *O*-formylation reaction is highly dependant on the initial structure of the macromolecules.

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