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Starch/carrageenan/milk proteins interactions studied using multiple staining and Confocal Laser Scanning Microscopy

A. Matignon[a,b,c,d], G. Moulin[a,b,c], P. Barey[e], M. Desprairies[e], S. Mauduit[e], J.M. Sieffermann[a,b,c], C. Michon[a,b,c,*]

[a] AgroParisTech, UMR1145 Ingénierie Procédés Aliment, F-91300 Massy, France
[b] INRA, UMR1145 Ingénierie Procédés Aliment, F-91300 Massy, France
[c] CNAM, UMR1145 Ingénierie Procédés Aliment, 1 avenue des Olympiades, F-75002 Paris, France
[d] Ferrandi, L'école française de gastronomie, F-75006 Paris, France
[e] Cargill, Usine de Baupte, F-50500 Baupte, France

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ABSTRACT

This study focused on the effects of the interactions between modified waxy maize starch, kappa carrageenan and skim milk on the microstructure of their mixed systems using Confocal Laser Scanning Microscopy (CLSM). A multiple staining of the components was set up with a view to improving starch covalent staining. In starch/carrageenan pasted mixtures, carrageenan was found to adsorb on and penetrate slightly into the starch granules, whereas no interactions were observed between starch and milk proteins. In ternary mixtures, interactions between starch granules and carrageenan were no longer observed, even when milk proteins were added after starch swelling in the carrageenan solution, thus showing preferential interactions between carrageenan/milk proteins in comparison to carrageenan/starch granules. Modifying the blending order of the components led to microstructure differences depending on several parameters such as starch/carrageenan interactions, carrageenan/milk proteins network structure, level of starch granules disruption and amylopectin contribution to the microstructure.

1. Introduction

Starch is used in a wide range of food products. In the 2–6 wt% concentration domain – for which water is in excess during processing - it is said to thicken the product's texture. A typical use of starch at a relatively low concentration (2–4 wt%) is in neutral dairy desserts: starch is combined with two other components, carrageenan and milk proteins, the three of them contributing to the final product structure.

Starch is composed of more-or-less-porous semi-crystalline granules containing packed amylose and amylopectin. The properties of the starch granules particularly depend on their amylose/amylopectin ratios, and on their architectural organization (Baldwin, 2001; Doublier, 1990; Gallant, Bouchet, & Baldwin, 1997; Park, Xu, & Seetharaman, 2011). When heated in the presence of excess water, above a characteristic temperature known as gelatinization temperature, the granules swell irreversibly letting molecular components (mostly amylose) seep out. If a mechanical treatment is applied, granules can be disrupted: then a majority of starch polymers are released but their envelopes – the starch granule ghosts – remain (Atkin, Abeysekera, & Robards, 1998). The level of disruption depends mainly on the nature and level of starch modifications and determines, for a given thermo-mechanical treatment, the granules' states after gelatinization, e.g. swollen starch granules still containing macromolecules, starch granule ghosts (empty granules), or their remnants (pieces of the envelopes) and starch macromolecular components freely dispersed in the medium. The structural properties of the resulting paste and its ability to gel under cooling depend strongly on the state of the granule after gelatinization.

Carrageenans are a family of linear polymeric sulphated galactans. Kappa carrageenan is composed of one sulphated group per disaccharide and is a gelling carrageenan. It undergoes a coil to helix transition depending on both the temperature and the ionic environment (Piculell, 1995).

Milk is composed of about 3.6 wt% protein, 80% of which are in the form of casein micelles and 20% in the form of whey proteins. Upon heating, at temperatures higher than 60 °C, whey proteins can denature and aggregate, mainly through hydrophobic

* Corresponding author at: AgroParisTech, 1 avenue des Olympiades, F-91300 Massy, France. Tel.: +33 1 69 93 51 27; fax: +33 1 69 93 50 05.
E-mail address: camille.michon@agroparistech.fr (C. Michon).

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interactions. These phenomena highly depend on temperature, time, composition and pH (Corredig & Dalgleish, 1999).

Starch, kappa carrageenan and milk proteins (especially casein micelles) are known to interact with one another. For a decade, their mixture microstructures often have been characterized by Confocal Laser Scanning Microscopy (CLSM). CLSM allows an optical section of a single focal plane through a three-dimensional specimen to be obtained (Dürrenberger, Handschin, Conde-Petit, & Escher, 2001; van de Velde, Weinbreck, Edelman, van der Linden, & Tromp, 2003). The contrast is obtained by differences in fluorescence. When signal collection from various probes emitting light at different wavelengths is possible, two or three components can be stained (Dürrenberger et al., 2001). The position of one ingredient relatively to the others in the mixture's microstructure can be determined and can lead to a better understanding of the interactions between components.

When mixed with milk, carrageenans mostly interact with casein micelles. Interactions between carrageenan/casein micelles have been widely detailed (Garnier et al., 2003; Langendorff et al., 2000). They lead to the formation of mixed carrageenan/casein micelles aggregates through local associations between kappa casein and carrageenan sulphated groups. However, a phase separation also occurs between the bridged casein micelle aggregates and the rest of the carrageenan solution which forms a continuous phase able to gel if the temperature decreases. Gelation, if performed quickly enough, freezes the macroscopic phase separation. Mixture microstructures were found to be highly dependent on the components ratios (Michon et al., 2003).

Hydrocolloids or milk influence the texture of starch-containing media, indicating possible interactions. However their nature is still not fully understood (Appelqvist & Debet, 1997; BeMiller, 2011; Considine et al., 2010). Either total or partial exclusion of the hydrocolloids and milk proteins by starch granules during pasting are detailed (Alloncle & Doublier, 1989; Matser & Steeneken, 1997). Still, specific interactions between starch granules and/or starch macromolecular components with carrageenan or milk proteins, mainly casein micelles, are also surmised (BeMiller, 2011; Considine et al., 2010). However, literature is not consistent about the mechanisms of these interactions. Microstructure studies, using CLSM, confirmed the exclusion effect (Aguilera & Baffico, 1997; Espinosa-Dzib, Ramirez-Gilly, & Tecante, 2012; Kett et al., 2012; Nagano, Tamaki, & Funami, 2008) but also pinpointed it (Kett et al., 2012; Savary, Handschin, Conde-Petit, Cayot, & Doublier, 2008; van de Velde et al., 2003): penetration and/or adsorption of carrageenan or milk proteins on/in starch granules (raw or swollen) were highlighted.

The study of binary mixtures permits to highlight more precisely the behavior of components two by two and leads to sequential understanding of more complex mixtures. Nevertheless, if a third one is added, the coupled interactions can be modified. When comparing CLSM images of carrageenan/starch mixtures with and without proteins, preferential interactions between carrageenan/casein micelles in comparison to carrageenan/starch granules were hypothesized but not confirmed (Espinosa-Dzib et al., 2012). The study of ternary mixtures implies numerous interactions. To gain a good understanding the impact of each component (modifying their ratios or process schemes) on the structure building should be hierarchized (Espinosa-Dzib et al., 2012; Nunes, Raymundo, & Sousa, 2006; Tarrega & Costell, 2006; Verbeken, Bael, Thas, & Dewettinck, 2006). During the process starch, through its exclusion effect, was found to have a major impact on the product's structure when determining the ratio of carrageenan/milk proteins in the continuous phase (Verbeken et al., 2006). In the final product's microstructure starch is, whatever its state, described as an inert filler: it is dispersed in the carrageenan/milk proteins network (Arltoft, Madsen, & Ipsen, 2008; Espinosa-Dzib et al., 2012; Nunes et al., 2006). Nevertheless, in the microstructure of ternary mixtures the role and positioning of starch is little highlighted.

Table 1
Excitation wavelengths and emission maxima of the different fluorescent dyes.

Fluorescent dyes	Excitation wavelengths (nm)	Emission maximum (nm)
APTS	488	520
RITC	543	620
Alexa 633	633	680

In mixtures, starch is mainly stained by protein-selective probes such as rhodamine B, FITC or safranin (Nagano et al., 2008; van de Velde, van Riel, & Tromp, 2002) effectively limiting specific starch localization when proteins are present. In this case, starch localization is mostly determined by negative staining (black part of the obtained images) (Espinosa-Dzib et al., 2012; Nagano et al., 2008; Vu Dang, Loisel, Desrumaux, & Doublier, 2009) or side-experiments (Arltoft et al., 2008; Nunes et al., 2006). This can hinder understanding the state of the starch in the medium but also the type of interactions involved. In more fundamental studies about starch structure, a covalent dye is used for starch: 8 amino-1,3,6-pyrenetrisulfonic acid (APTS) (Blennow et al., 2003). APTS stains starch granules by reductive amination of glucose (O'Shea, Samuel, Konik, & Morell, 1998). Recently the structure of starch granules during gelatinization was followed using this staining method (Chen et al., 2011). However, disruption of stained waxy maize starch granules was observed at low temperatures (~65 °C). Staining seemed to weaken the starch's structure. Were this shortcoming to be harnessed, this dye could be used to covalently stain starch granules before using them in mixtures, enhance starch localization and highlight its structural role in complex mixtures.

The main aim of this work was to study the effects of the interactions between modified waxy maize starch with carrageenan and skim milk on the microstructure of their mixed systems using CLSM. Our strategy was first to improve the covalent starch staining method using APTS in order to limit the disruption of stained starch granules during pasting. In a second time, the microstructures of mixtures containing starch with carrageenan or/and milk proteins were observed using multiple staining and CLSM. The impacts of both the order of blending and the state of starch on the microstructure obtained were also studied.

2. Materials and methods

2.1. Materials

Stabilized and cross-linked waxy maize starch (acetylated adipate distarch, C*Tex 06205), commercial carrageenan (Satiagel) and skim milk powder (low heat) were kindly provided by Cargill (Baupte). The starch was comprised of at least 99% amylopectin and of maximum 0.4 wt% endogenous proteins. The carrageenan sample was composed of a mix of kappa (73%), iota (24%) and lambda (3%) chains, for a molecular weight of 630 kDa.

Aminofluorophore, 8-amino-1,3,6-pyrenetrisulfonic acid (APTS), rhodamine B-isothiocyanate Celite (RITC) and Alexa 633 were purchased at Sigma Aldrich. 3-(4-CarboxyBenzoyl)Quinoline-2-CarboxAldehyde (CBQCA) was purchased at Invitrogen.

2.2. Choice of staining

To ensure the localization of one ingredient relatively to the others, different factors were taken into account. First, in order to limit superimposition effects, the separation of the different absorption maxima and emission spectra of the probes was defined by a distance of 20–30 nm between the emission and excitation wavelengths of each dye (Table 1). Then parameters such as the

specificity of each dye/component couple, or the correspondence of wavelengths between dyes and CLSM lasers were evaluated.

2.3. Staining of the products

Starch granules were stained with APTS using the procedure of Blennow et al. (2003) with modifications. The molecular probe was dissolved in 15% acetic acid which resulted in an acidified stained starch (pH of ~3.0). In literature, APTS is mainly used to observe raw starch granules (Blennow et al., 2003; O'Shea et al., 1998). In this study, the starch was thermo-mechanically treated. The procedure also included a neutralization of the stained samples, the efficiency of which will be discussed later on. A wash in a 1 M NaOH (1 μl) aqueous solution was performed, followed by a five-time wash with distilled water (1 ml each time). The resulting samples had a pH of approx. 8. In order to minimize granule disruption during pasting, a stabilized and cross-linked waxy maize starch was used.

Carrageenans were covalently stained with RITC using the procedure of Núñez-Santiago et al. (2011): after blending the reactants, k-carrageenan-RITC was precipitated with 95% (v/v) ethanol, filtered, washed, dried overnight at 30 °C, dissolved in water, dialyzed (3 days), freeze-dried and stored at 4 °C. The staining was assessed through determination of the intrinsic viscosity (MCR 301 rheometer, Anton Paar, Austria) while progressively diluting a 1 wt% k-carrageenan-RITC in a 0.1 Mol NaCl aqueous solution at 60 °C. The corresponding values were 6.9 dl g^{-1} (non-stained sample) and 6.4 dl g^{-1} (stained sample) for a λ coefficient of Huggins equation of respectively 0.27 and 0.32. This shows carrageenan labeling induces a slight hydrolysis phenomenon. However, since the carrageenan kept its gelling ability, interactions with other ingredients should not be much modified.

Proteins were stained with Alexa 633 that links selectively and covalently to primary amines through their succinimidyl ester group. 1 mg of Alexa was dissolved in 2 ml of dimethylformamide. 100 μL of phase were then poured in Eppendorfs, dried in an N2-enriched atmosphere and kept at −20 °C until use. Before use, 100 μL of dimethylformamide were added to one dried batch of Alexa, and 2 μL were then expanded in the whole surface of coverslips that were dried at 50 °C.

2.4. Preparation of the mixtures

Two fixed percentages of components, typical of neutral dairy desserts, were chosen: 0.17 wt% for carrageenan and 2 wt% for starch. The preparation processes of all the products were as follows. The carrageenan solutions were prepared by first dispersing the carrageenan powder in a 0.1 M NaCl aqueous solution then heating at 70 °C under stirring (500 rpm, 30 min). The skim milk was reconstituted (10.4 wt%) 24 h beforehand, and the resulting powder dispersed in distilled water under stirring (500 rpm). The starch powder was dispersed under stirring in its pasting medium, hydrated at 50 °C under stirring (500 rpm, 30 min) before thermo-mechanical treatment. Each studied mixture (Table 2) had a volume equivalent to 2 ml. Every thermo-mechanical treatment was performed under stirring (tiny magnetized bar) in a water bath at 90 °C: from 50 to 80 °C in 1 min. The different mixtures were then placed between a preheated (50 °C) slide (concave) and a coverslip. When the mixtures included milk, a coverslip with Alexa was used: Alexa stained the milk proteins by diffusion. Mixtures were observed at room temperature, 2 h after preparation.

2.5. Observation by CLSM

The samples were examined on an inverted Confocal Laser Scanning Microscope (CLSM) TCS SP2 AOBS (Leica, Germany) equipped with UV–vis sources and a motorized focal plane with a ×65 objective. The microscope had three lasers: two helium/neon lasers, used at excitation wavelengths of 633 and 543 nm respectively, and one argon laser used at an excitation wavelength of 488 nm. A spectral-filtering system allowed the selection of specific wavelength intervals: the reception wavelength zones could thus be optimized. For APTS, light was detected in an interval ranging from 500 to 530 nm, for RITC, from 580 to 620 nm and for Alexa, from 700 to 730 nm (Fig. 1). The sizes of the images were 512/512 pixels. During image acquisition, each line was scanned 8 times and averaged to reduce noise. Care was taken to avoid the areas near the sides or close to the top of the microscope slides.

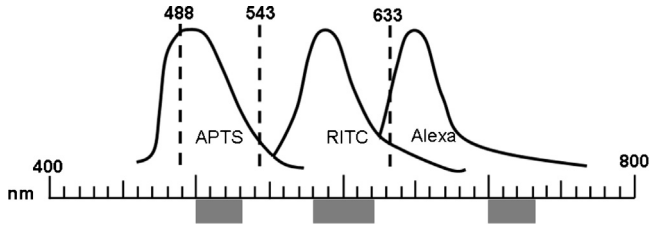

Fig. 1. Emission spectra of the three chosen dyes, excitation wavelengths of each laser (dotted line) and measurement wavelength zones (gray rectangle).

3. Results

3.1. Starch staining (Fig. 2)

Raw starch stained with APTS showed high fluorescence (Fig. 2a) even if the staining by APTS reductive amination is known to be less efficient for amylopectin than for amylose (Blennow et al., 2003; Chen et al., 2011). Chen et al. (2011) showed, however, that APTS could properly stain raw waxy maize starch, which is confirmed here. A truncated shape and high granule size heterogeneity (average ~ 15 μm) were observed, all results well-known in literature for waxy maize starches (van de Velde et al., 2002).

Starch staining was, following the protocol proposed by Blennow et al. (2003), performed in an acidic medium. APTS solution was dissolved in acetic acid. Thus, when the stained starch powder was suspended in a 0.1 Mol NaCl aqueous solution, pH turned acidic. Acidity can lead to the hydrolysis of amylose and amylopectin, especially when heating the suspension (Hirashima, Takahashi, & Nishinari, 2005), making neutralizating the stained starch compulsory: neutralization was launched at the end of the staining protocol, before washing. It did not impact the high fluorescence obtained on raw stained starch (Fig. 2b). The slight difference observed was only due to the use of different laser intensities.

Upon heating a stained starch suspension, starch granules swelled and only their peripheries remained fluorescent (Fig. 2a′ and 2b′). Without stained starch neutralization (Fig. 2a′), bright remnants of starch granule ghosts were observed. With the neutralization step at the end of the staining process (Fig. 2b′), shells of dense material – either intact granules or ghosts – and remnants of starch granule ghosts were observed. Neutralizing the stained starch led to less disruption of the starch granules during pasting, and thus limited the hydrolysis of the stained starch during the thermo-mechanical treatment.

During staining, starch granules were most certainly 'weakened' by the acidic treatment. Nevertheless, the rinsing step with 1 M NaOH aqueous solution added in this study permitted to limit starch granule disruption. This method was used for all the mixtures, and its results are shown below. It is, to our knowledge, the first time that covalent stained starch has been used in the study of complex medium microstructures.

Table 2
Details of the different single, binary and ternary studied systems.

Mixture type	Pasting medium	Starch			RITC-carrageenan	Milk proteins – Alexa stained	Observed suspension	Fig.
		Not stained	APTS stained					
			Without neutralization	With neutralization				
Single	0.1 M NaCl aqueous solution		X				Raw	2a
			X				Pasted	2a′
				X			Raw	2b
				X			Pasted	2b′
Binary	Carrageenan solution			X	X		Pasted	3a–d
	Skim milk			X		X	Pasted	3e–h and 4
Ternary	Carrageenan/skim milk			X	X	X	Pasted	5 and 7c, e
		X			X	X	Pasted	7a
	Carrageenan solution			X	X	X	After milk addition	6 and 7d, f
		X			X	X	After milk addition	7b

3.2. Binary mixtures

3.2.1. Starch pasted in a carrageenan solution (Fig. 3)

Fig. 3a–d shows images of APTS-covalently stained starch pasted in an RITC-covalently stained carrageenan solution. Fig. 3a and b shows the fluorescent zones due to APTS (green signal) and RITC (magenta signal) respectively. In Fig. 3a, the shells of dense material that were observed correspond to starch granules. Fig. 3b is almost identical but with starch granules stained in magenta corresponding to the RITC-covalently linked with carrageenan chains. The carrageenan seemed to be localized on the starch granules' surfaces. The overlaid image (Fig. 3c) highlights the starch/carrageenan colocalization spots (white color): almost all starch granules are magenta/white, meaning that carrageenan is localized mostly at the periphery of a granule. In literature, the same results were obtained in mixtures of starch with RITC-carrageenan (Espinosa-Dzib et al., 2012) and FITC-Gellan (van de Velde et al., 2003) but Savary et al. (2008) found no colocalization at

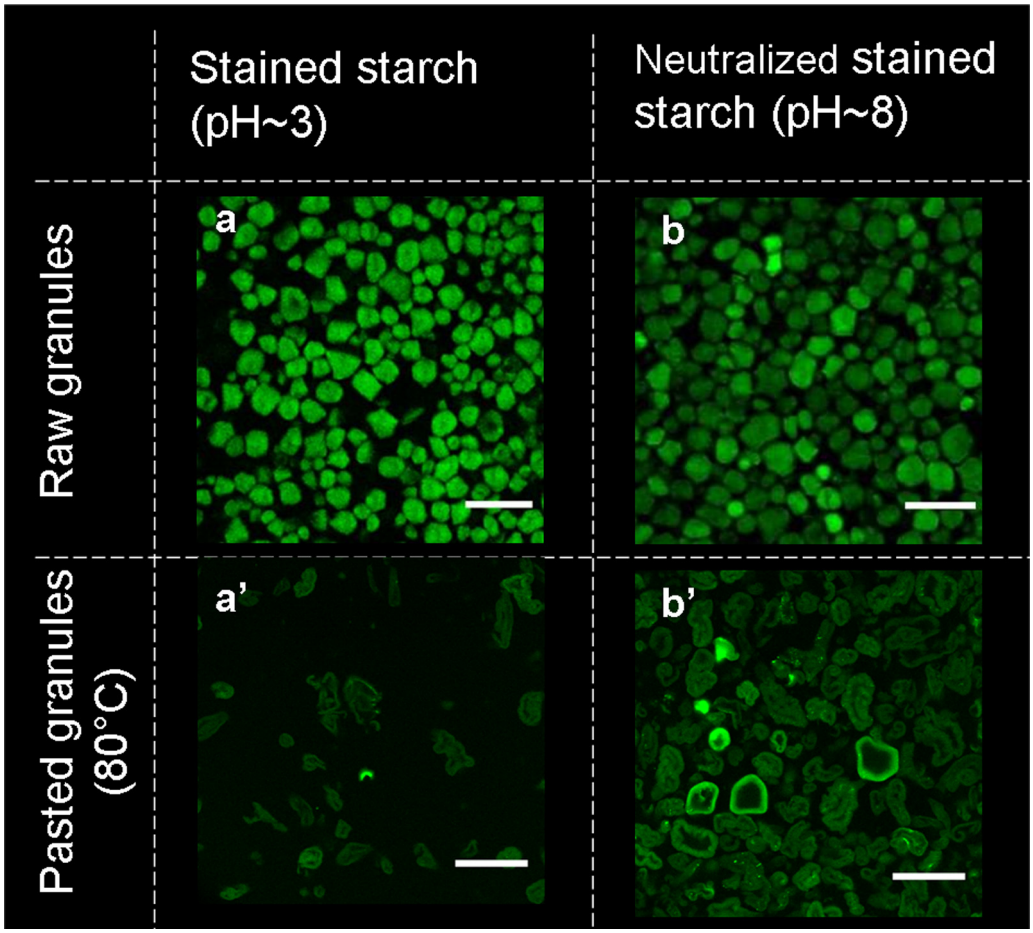

Fig. 2. Dispersions of 2 wt% stained starch (in green) in a 0.1 Mol NaCl aqueous solution (a–b) raw and (a′–b′) pasted. (a–a′) 'regular' stained starch following Blennow's (2003) method and (b–b′) same method but with a neutralization at the end of staining protocol. Bars = 50 μm.

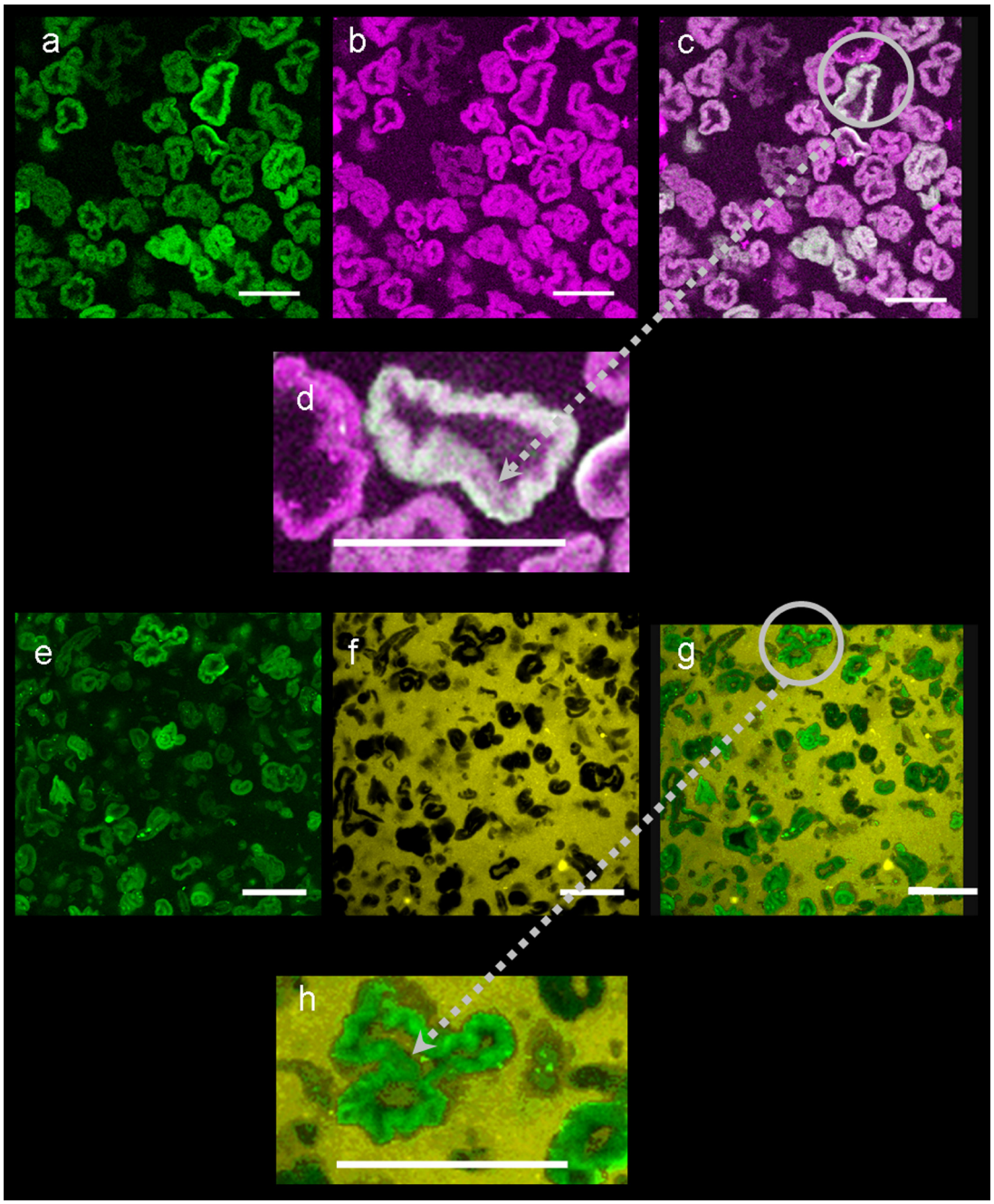

Fig. 3. Optical sections of double stained mixtures of (a–d) 2 wt% starch pasted in a 0.17 wt% carrageenan solution and (e–h) 2 wt% starch pasted in milk. (a, e) APTS signal in green (stained starch); (b) RITC signal in magenta (stained carrageenan); (f) Alexa signal in yellow (stained milk proteins); with the binary overlay images: (c, d) APTS and RITC signals in green and magenta (respectively stained starch and carrageenan) and (g, h) APTS and Alexa signals in green and yellow (respectively stained starch and milk proteins). Bars = 50 μm.

all in RITC-starch/FITC-carrageenan mixtures. RITC (or rhodamine B) and FITC are frequently used to stain starch granules by diffusion (Hans Tromp, van de Velde, van Riel, & Paques, 2001; Nagano et al., 2008; van de Velde et al., 2002, 2003). In order to check that such carrageenan positioning on the starch granule was not due to an interaction of starch with RITC (covalently linked with carrageenan chains), mixtures of starch and commercial rhodamine B-neutral dextran (average M_w 70,000) from Invitrogen (Carlsbad, CA, USA) were launched. No colocalization was observed. Thus, the colocalization of carrageenan on the starch granules was confirmed to be due to peculiar carrageenan characteristics only. The carrageenan chains seemed to be mostly adsorbed on starch granules (Fig. 3c). Still, a little more magenta was observed inside the granules (Fig. 3d), meaning that a few carrageenan chains may partly penetrate inside the starch granules.

3.2.2. *Starch pasted in a skim milk solution (Figs. 3 and 4)*

Fig. 3e–h shows images of APTS covalently stained starch pasted in reconstituted skim milk stained afterwards by Alexa dye diffusion. The fluorescent zones due to APTS (green signal) and Alexa (yellow signal) respectively are observed in Fig. 3e and f. In Fig. 3e, entire and disrupted starch granules could be well-identified. In comparison with Figs. 2b and 3a, a larger proportion of remnants was observed. Gelatinization of starch in a milk medium seemed

to enhance starch granule disruption in comparison to starch alone or starch/carrageenan mixtures. This was confirmed on unstained systems.

In Fig. 3f, corresponding to milk protein localization, a continuous yellow cover was observed in which black holes were dispersed. The milk proteins were localized in the continuous phase and starch granules or remnants were dispersed in it. The overlaid image (Fig. 3g) shows that the two signals were well-separated. Starch granules were embedded in a continuous protein phase. Looking carefully at the starch granules, two types were distinguished: empty ones (all black) and 'yellow-filled' ones. The latter seem to be filled with the continuous protein phase (Fig. 3h). Protein trapping inside the starch granules may be due to either aqueous phase trapping in cavities, the convoluted swollen starch granules folding (Espinosa-Dzib et al., 2012; Vu Dang et al., 2009) or to proteins penetrating in starch granules during swelling. A scan in the z direction was performed allowing a filled starch granule to be observed with a high level of magnification (Fig. 4). A slight yellowish veil was observed between the starch granule's folded extremities (Fig. 4, gray arrow). Thus, it seems that when proteins are found inside starch granules, it is mostly due to trapping inside convoluted starch granules.

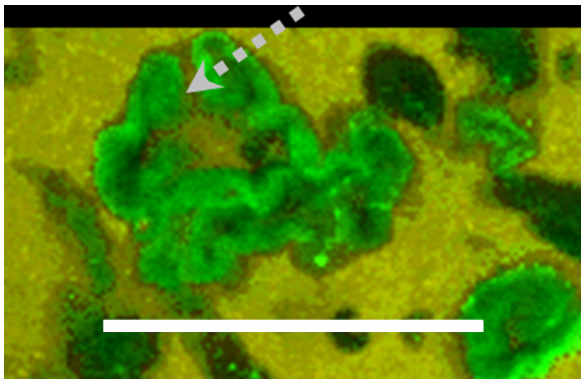

Fig. 4. Cross-section and zoom in on a part of the overlay image of a stained starch (2 wt%) and milk mixture, same image as (h) in Fig. 3 but deeper inside the sample (+9 μm). Bars = 50 μm.

3.3. Ternary mixtures

3.3.1. Starch pasted in a skim milk/carrageenan mixture (Fig. 5)

Images of covalently stained starch pasted in a skim milk/carrageenan mixture are reported in Fig. 5. Fig. 5a–c shows

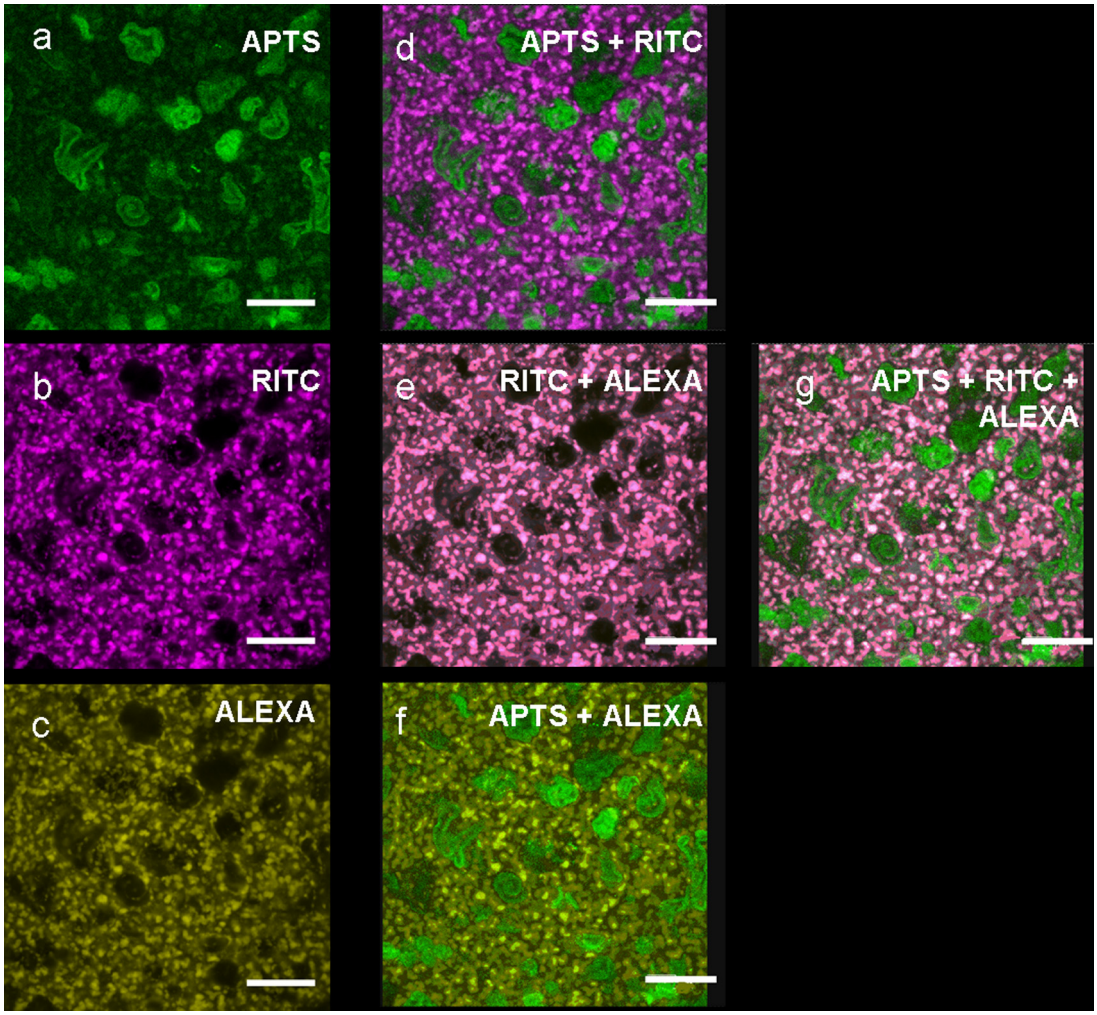

Fig. 5. Optical sections of a 2 wt% starch pasted in a skim milk-carrageenan (0.17 wt%) mixed system. (a) APTS signal in green (stained starch); (b) RITC signal in magenta (stained carrageenan); (c) Alexa signal in yellow (stained milk proteins); with the binary overlay images: (d) APTS and RITC signals in green and magenta (respectively stained starch and carrageenan); (e) RITC and Alexa signals in magenta and yellow (respectively stained carrageenan and milk proteins); (f) APTS and Alexa signals in green and yellow (respectively stained starch and milk proteins) and the ternary overlay image (g) APTS, RITC and Alexa signals in green, magenta and yellow (respectively stained starch, carrageenan and milk proteins). Bars = 50 μm.

the fluorescent zones due to APTS, RITC and Alexa corresponding to starch (green), carrageenan (purple) and milk proteins (yellow), respectively. Fig. 5d–f shows the overlaid images of the signals two by two: APTS/RITC, RITC/Alexa and APTS/Alexa. Finally, Fig. 5g shows the overlaid images of the three signals APTS/RITC/Alexa. Several differences can be observed in comparison with binary mixtures.

In addition to swollen APTS-stained starch granules, ghosts and remnants, another 'starch' material was detected all over the plane in the form of discontinuous 'clouds' of thin bright green material (Fig. 5a). They could be amylopectin macromolecular chains liberated after starch granule disruption. In skim milk/starch mixtures, despite a high level of starch disruption, no clouds were observed (Fig. 3e). Their great visibility in ternary mixtures meant that the amylopectin macromolecular chains were more concentrated and thus grouped. Phase separation between amylopectin, milk proteins and carrageenan could result in such grouping. In presence of only milk proteins no 'phase separation' was detected, and the presence of carrageenan, a high-molecular-weight sulfated polysaccharide, seemed thus to enhance this phase separation.

No more colocalization between carrageenan and starch granules was observed (Fig. 5a, b, and d). However, a colocalization between carrageenan and milk proteins was observed (Fig. 5b, c, and e). Preferential interactions between carrageenan/casein micelles seemed to occur in comparison to carrageenan/starch ones. Connected aggregates of carrageenan/casein micelles (lumps) are clearly observable. This result is consistent with the formation of two interpenetrated carrageenan/casein micelles and carrageenan networks. The fine interpenetration of the two networks certainly hindered the observation of the carrageenan-alone network (perfect colocalization of RITC and Alexa signals).

The overlaid images of the three signals (Fig. 5g) show a colocalization (white color) on the top of the carrageenan/casein micelles aggregates. Amylopectin macromolecular chains seem thus to be part of them. Looking closely at Fig. 5e and f, carrageenan/casein micelles aggregates seem to wrap amylopectin 'clouds' up despite phase separation. This wrapping could be due to mixture gelation. The other starch components, such as swollen granules, ghosts and their remnants were dispersed in the amylopectin/carrageenan/casein micelles interpenetrated networks. As in part 3.2.2 few 'filled' starch granules were observed. They corresponded, as was the case above, to folded or disrupted starch granules. In conclusion, starch is localized in the form of more-or-less-disrupted granules dispersed in a "continuous phase" made

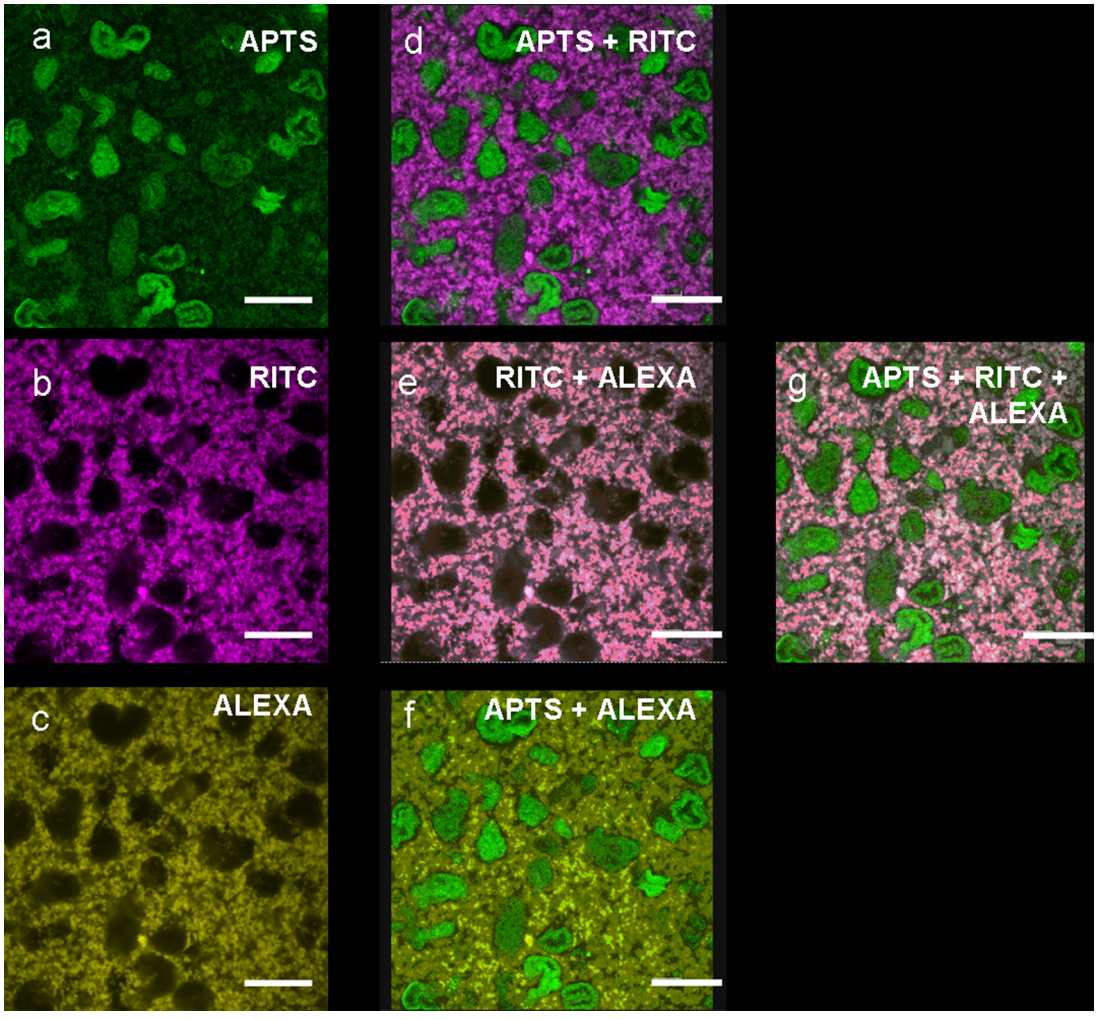

Fig. 6. Optical sections of 2 wt% starch pasted in a 0.17 wt% carrageenan solution and skim milk added at the end of the pasting. (a) APTS signal in green (stained starch); (b) RITC signal in magenta (stained carrageenan); (c) Alexa signal in yellow (stained milk proteins); with the binary overlay images: (d) APTS and RITC signals in green and magenta (respectively stained starch and carrageenan); (e) RITC and Alexa signals in magenta and yellow (respectively stained carrageenan and milk proteins); (f) APTS and Alexa signals in green and yellow (respectively stained starch and milk proteins) and the ternary overlay image (g) APTS, RITC and Alexa signals in green, magenta and yellow (respectively stained starch, carrageenan and milk proteins). Bars = 50 μm.

of amylopectin clouds trapped in a coupled carrageenan/casein micelles network.

3.3.2. Starch pasted in a carrageenan solution followed by an addition of skim milk solution (Fig. 6)

In order to confirm the preferential interaction of carrageenan with milk proteins, starch was first pasted in a carrageenan solution in order to associate it with carrageenan chains (Fig. 3b–d). Milk was then added to the suspensions. Observations of the mixtures' microstructures were made before and after adding milk. Before addition of milk, starch granules were identical to those described in part 3.2.1 (Fig. 3c) with carrageenan chains adsorbed all around the granule. However, after addition of milk (Fig. 6), no more colocalization of APTS/RITC signals was observed (Fig. 6d) meaning that carrageenan chains were removed from the starch granule surfaces. They interacted with casein micelles in the continuous phase (Fig. 6e).

The obtained microstructures differed in several ways in comparison with the previous ternary mixtures (starch swelled in a skim milk/carrageenan mixture described in part 3.3.1). Thin 'clouds' of amylopectin macromolecular chains were still observable but their structures were thinner and the starch granules seemed less disrupted (Fig. 6a). Moreover, even if the overlaid images of the three signals (Fig. 6g) show the same type of component organization and distribution, thinner and more numerous carrageenan/casein micelles aggregates were seen, resulting in a denser network (Fig. 6d). Still, no more 'filled' granules were observed and a black ring surrounding the starch granules could be noticed. Looking more closely at the 'black ring' on RITC and Alexa signals (Fig. 6b and c), a small fluorescence can be seen meaning that there is a smaller concentration in carrageenan and in protein at the periphery of the granules than in the rest of the continuous phase.

3.3.3. Comparison with ternary mixtures obtained with unstained starch

The impact of starch staining on the microstructures obtained for both ternary mixtures was evaluated. Ternary mixtures were launched with unstained starch. The overlaid images of their RITC/Alexa signals are presented in Fig. 7a and b. To make the comparison easier, the overlaid images of the RITC/Alexa (Fig. 7c and d) and APTS/RITC/Alexa (Fig. 7e and f) signals of the two mixtures obtained with stained starch were added in Fig. 7. The starch granules were bigger in size ('black holes') in unstained starch mixtures than in stained starch mixtures (Fig. 7a vs. c and b vs. d). This result is in good accordance with the weakening effect of starch staining. However in the case of stained starch, starch granules were still observable. All the microstructures obtained showed the same type of component organization and distribution. The impact of the blending order of the components on the microstructure obtained was similar using unstained starch or stained starch (Fig. 7a vs. b and c vs. d): starch pasting in a carrageenan solution followed by milk addition led to less starch granule disruption (bigger black holes) and a denser carrageenan/casein micelles network. Starch staining did not modify the type of interactions between the components. It permitted the nature and composition of the 'black holes' to be determined and to localize amylopectin macromolecular chains in the mixtures as discussed above.

4. Discussion

In binary mixtures, on the one hand, carrageenans were found to adsorb on and slightly penetrate into starch granules when

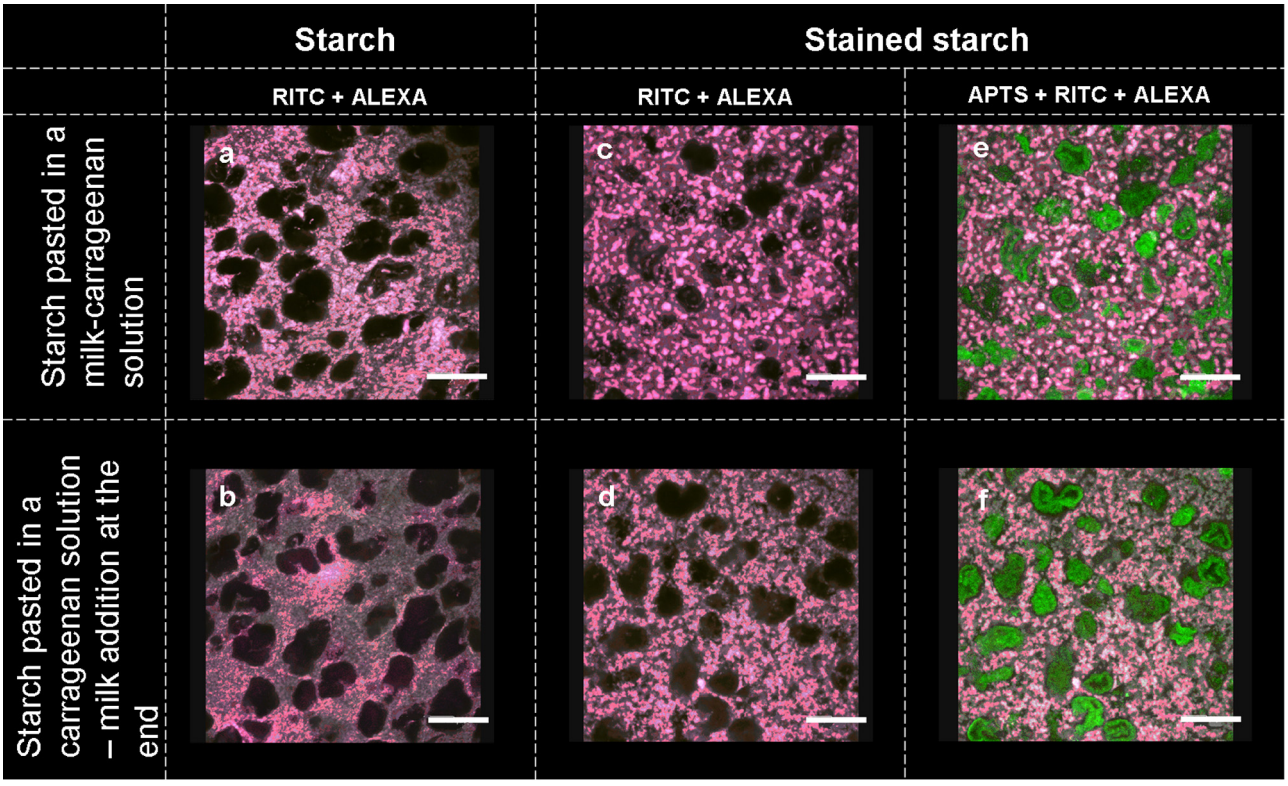

Fig. 7. Optical sections of 2 wt% starch pasted in (a, c, e) skim milk–carrageenan (0.17 wt%) mixed system and in (b, d, f) 0.17 wt% carrageenan solution and skim milk added at the end of the pasting. (c–f) Stained starch and (a, b) unstained starch. (a–d) the binary overlay images of RITC and Alexa signals in magenta and yellow (respectively stained carrageenan and milk proteins) and (e, f) the ternary overlay images of APTS, RITC and Alexa signals in green, magenta and yellow (respectively stained starch, carrageenan and milk proteins). Bars = 50 μm.

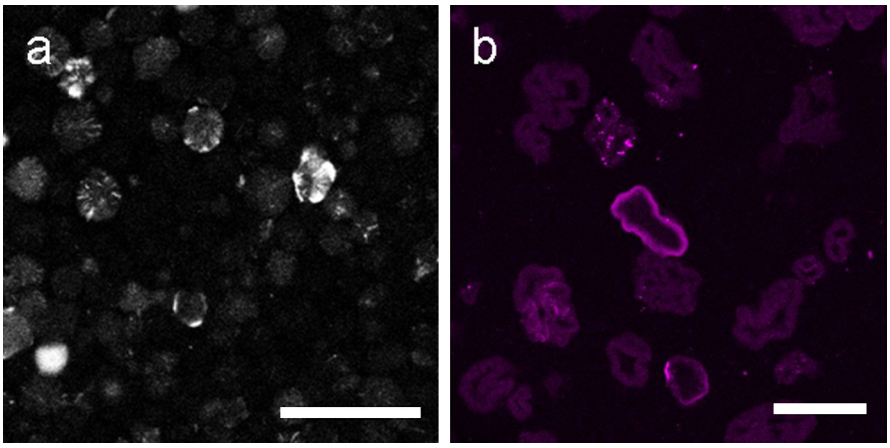

Fig. 8. (a) Raw starch granules stained with CBQCA and (b) Optical sections of 2 wt% starch pasted in stained carrageenan (0.17 wt%) solution, RITC signals in magenta. Bars = 50 μm.

they swelled in a carrageenan solution. On the other hand, no interactions were observed between milk proteins and starch granules either when starch granules swelled in milk or in a carrageenan/milk mixture.

In literature, starch granule surfaces are described as a peculiar material in comparison to the starch granule interior. A granule would be covered with parts of amylopectin macromolecules (Park et al., 2011), composed of a high proportion of endogenous proteins (Atkin et al., 1998; Baldwin, 2001) and riddled with surface pores connected or not to its internal part through channels (Gallant et al., 1997; Huber & Be Miller, 2000). The presence and positioning of endogenous proteins on the starch used in this work were checked with Han's method (Han, Benmoussa, Gray, BeMiller, & Hamaker, 2005; Han and Hamaker, 2002) (Fig. 8a). As observed by those authors, the endogenous proteins were found to be heterogeneously distributed and mostly located on the starch granule surfaces and on the starch pores or channels (bright voids inside starch granules). The natures of the surface endogenous proteins of waxy maize starch depend on their localization. The exterior surface would be composed of zein, a highly hydrophobic and uncharged protein, and the voids or channels composed of proteins of other types the nature of which remain hitherto not well-known (Han et al., 2005; Mu-Forster & Wasserman, 1998).

Carrageenan is a sulfated polysaccharide. Entanglements with amylopectin or intermolecular interactions with zein cannot explain the adsorption of carrageenan chains all around the starch granule. The hypothesis most likely to explain the observed interaction would be a superficial trapping of carrageenan chains by starch granules by means of their pores during swelling (Be Miller, 2011). Nevertheless, a close observation of an image of binary mixture of starch pasted in a carrageenan-RITC solution (Fig. 8b – RITC signal) highlighted brighter fluorescent zones with the same irregular size and non-homogeneous repartition around the starch granules as observed for proteins (Fig. 8a). Carrageenan chains would be more concentrated where starch endogenous proteins are located. Intermolecular interactions between carrageenan and starch endogenous proteins would thus also be at stake, but their precise nature and positioning are not known.

In the meantime, after swelling, no proteins were observed inside the granules or at their periphery, confirming the hypothesis of an exclusion of milk proteins by starch granules during gelatinization proposed by Matser and Steeneken (1997). However, pasting starch in milk in our conditions seemed to enhance starch granule disruption in comparison to pasting in water or in a carrageenan solution. This result is not well-understood and, to our knowledge, is yet to be described in literature. It would tend to prove the existence of interactions between starch and milk proteins that would result in a weakening of starch granules. The denatured whey proteins (at temperatures above 70 °C) expose both SH groups and a hydrophobic core, which could interact with the highly hydrophobic zein through hydrophobic interactions or disulphide links (Appelqvist & Debet, 1997). Those interactions could lead to a modification of starch granule surfaces and to an enhancement of the disruptive effects of thermo-mechanical treatment during swelling.

In ternary mixtures, changing the blending order of the components led to a change in the interactions and thus their microstructures. When starch was pasted in a skim milk/carrageenan mixture, classical carrageenan/casein micelles interactions took place, leading to a mixed continuous network but no more interactions between carrageenan/starch granule surfaces. When starch was pasted in a carrageenan solution and milk proteins added afterwards, a 'desorption' of carrageenan from starch granules was witnessed, and it entered in the carrageenan/casein micelles continuous network. The black ring surrounding starch granules in the obtained microstructure (Fig. 6g) may be due to this carrageenan displacement from starch granule to carrageenan/casein micelles coupled network caused by skim milk addition and preferential associative interactions of carrageenan with casein micelles. The black ring is made of a less-concentrated carrageenan/casein micelles phase. Carrageenan/starch granules interactions would only take place in absence of casein micelles. They are possible when carrageenan chains are either in coil or helix conformation, which is consistent with a recent study (Huc et al., 2013). These interactions are reversible. The penetration, if it occurs, is only superficial. These interactions led to less starch granule disruption in the final microstructure – a result consistent with the hypothesis set in literature of a protective role of carrageenan for starch granules (Appelqvist, Brown, Goff, Lane, & Norton, 1996; Tye, 1988). Starch/carrageenan interactions and starch/milk proteins interactions would thus have opposite effects on the starch granules.

Different blending orders led to different carrageenan/casein micelles coupled networks structures (Fig. 7c and e vs. d and f). Those observations were confirmed on mixtures containing unstained starch (Fig. 7a and b), they could be based on different phenomena. In the case of a classic process, skim milk and carrageenan are mixed at 50 °C and kept 30 min at this temperature for starch hydration. Associative and segregative interactions between carrageenan and casein micelles take place immediately after carrageenan chains are solvated. Then, during starch pasting, starch starts swelling at ~65 °C. Granules absorb water,

leading to a decrease in water volume outside starch granules and, consequently, to an increase in carrageenan and milk proteins concentrations in the continuous phase, but it also helps maintaining their relative proportion. In comparison, when starch is pasted in a carrageenan solution, it was shown recently (Huc et al., 2013) that the carrageenan concentration in the continuous phase at the end of the pasting was almost equal to the initial one due to carrageenan/starch granules interactions. The different carrageenan/casein micelles ratio could explain the different morphology of their microstructures (Garnier et al., 2003; Michon et al., 2003; Sedlmeyer & Kulozik, 2007).

These differences in network microstructures could also rely on the level of starch disruption. The different ternary mixtures obtained with unstained or stained starch granules, but also with the modification of the blending order, led to different levels of starch granule disruption. On Fig. 7, four levels of disruption are observable: Fig. 7b, a, d and c from least to most disrupted. It seemed that, the more disrupted the starch granules, the bigger the aggregates of carrageenan/casein micelles. Starch disruption diminishes volume reduction but also leads to amylopectin macromolecular chains leakage. The observation of 'clouds' of amylopectin was surmised to be caused by a phase separation between amylopectin and carrageenan/casein micelles network. A higher concentration of amylopectin in the continuous phase could enhance the phase separation and thus promote the formation of bigger aggregates. Depending on its state – granules or macromolecular chains – starch can play different roles in the building of the product's structure. The starch granules, whatever their state, would act as inactive fillers in the continuous medium (Arltoft et al., 2008; Espinosa-Dzib et al., 2012; Nunes et al., 2006) and the macromomecular amylopectin chains would contribute to its network structuring.

5. Conclusion

Starch covalent staining was improved, leading to less granule disruption during swelling. It permitted to observe more precisely starch material in the resulting microstructure and to sort out new hypotheses on the role of starch in the building of the product's structure. It could also allow the mixtures' microstructure evolutions during pasting to be followed. Observing the building of the product's structure in dynamic conditions would be possible and help improve our understanding of the interactions.

The different binary and ternary systems microstructures obtained using multiple staining and CLSM permitted to highlight the interactions between starch, carrageenan and casein micelles. This study permitted to point out:

- No observable interactions between starch and milk proteins, even if some may exist leading to an increase in granule fragility.
- Interactions between starch and carrageenan which would be due to carrageenan/endogenous starch proteins interactions. They are reversible.
- A competition between carrageenan/endogenous starch proteins interactions and carrageenan/casein micelles interactions. When carrageenan, starch and casein micelles are together, carrageenan/casein micelles interactions are formed preferentially.
- Different roles in the building of the product's structure were observed and hypothesized for starch granules and amylopectin macromolecular chains.

Changing the blending order of the components was found to be an interesting tool to study more precisely binary interactions, but also ternary ones: it modifies the characteristics of both the starch granules and the continuous phase. With one formula, different microstructures could be obtained. It would be interesting to see if introducing carrageenan and milk in a different order during the process could lead to different sensory properties.

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