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Interaction of modified celluloses and pectins with gluten proteins

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ABSTRACT

Physical and chemical techniques were applied to characterize the type of interaction between hydrocolloids and the gluten network in wheat dough, with and without NaCl. Modified celluloses (microcrystalline cellulose, MCC; carboxymethylcellulose, CMC, hydroxypropylmethylcelluloses, HPMC) and pectins of low (LMP) and high (HMP) degree of methylation were utilized as hydrocolloids to interact with gluten proteins. Modified celluloses were employed at 1.5% (flour basis) and pectins at 2.0% (flour basis). By microscopy (SEM and CLSM), it could be observed that NaCl induced a more marked cross-linking and orientation of gluten network. On the other hand, the addition of hydrocolloids led to more open matrices. Molecular mobility was evaluated by ^{1}H-NMR assays and significant effects of NaCl addition and hydrocolloid type were found on relaxation times (T_2). In presence of salt, significantly higher relaxation times were observed when modified celluloses were added. Hydrocolloid addition strongly affected the secondary conformation of proteins as studied by FT-Raman. In absence of NaCl, control and MCC samples exhibited the higher α-helix conformation percentage (indicating a more ordered and compact structure), followed by dough with HMP, HPMCs, LMP and CMC. In general, doughs with modified celluloses and NaCl showed a decrease of α-helix conformation. CMC dough showed the smallest percentage of α-helix conformation, and the highest contributions of more unfolded structures. Doughs with pectins and NaCl showed similar percentages of α-helix to control one but an increase of random coil structure was observed. Electrophoresis assays confirmed that the presence of certain hydrocolloids (CMC) during gluten formation could affect protein interaction promoting subunits lability from the matrix.

1. Introduction

Hydrocolloids from different sources have been increasingly studied as breadmaking improvers to evaluate their effect on dough and bread characteristics as well as product acceptability and preservation. Among those hydrocolloids commonly used as food additives, modified celluloses and pectins have been mainly applied in beverages, desserts and dairy products. An interesting aspect of these hydrocolloids is that they are obtained by chemical modification of native polysaccharides from plants and their structure (particularly in the case of celluloses) can be tailor-made in order to obtain derivatives with different physicochemical properties. Besides, they are obtained from relatively economical, renewable sources. In spite of their extended use as food additives, their application in breadmaking is less extended respect to other hydrocolloids and bread improvers.

Several studies about the influence of hydrocolloids on wheat dough behavior and bread quality have included some modified celluloses – mainly HPMC – and pectins – particularly the high methoxylated one – (Armero & Collar, 1998; Bárcenas, De la O-Keller, & Rosell, 2009; Bárcenas & Rosell, 2005, 2007; Bollaín & Collar, 2004; Collar, Andreu, Martínez, & Armero, 1999; Correa, Pérez, & Ferrero, 2012; Guarda, Rosell, Benedito, & Gallotto, 2004; Ribotta, Ausar, Beltramo, & León, 2005; Rosell, Rojas, & Benedito de Barber, 2001). However, there is yet scarce information about their effect on wheat dough microstructure. A better knowledge about the changes induced by these hydrocolloids on gluten network could help to understand their diverse effects on dough behavior and bread quality. A comparative analysis among different modified celluloses and pectins could also contribute to associate the type of chemical structure with their efficiency as product improver.

In the case of modified celluloses, different substitutions on cellulose backbone as the presence of anionic groups in the case of carboxymethylcellulose (CMC), or a certain degree of hydrophobicity in the case of hydroxypropylmethylcelluloses (HPMC) can lead to macromolecules with very diverse properties compared to

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native cellulose. Degree of methoxylation in pectins has also influence on their physicochemical properties; high methoxyl pectins (HMP) do not exhibit gelling properties in the presence of calcium ions as low methoxyl ones (LMP) do (Glicksman, 1982).

In previous works, the effects of different types of modified celluloses and pectins on the rheological characteristics of wheat dough were analyzed and the influence of the type and level of gum could be assessed. Pectins diminished dough stability and rendered softer but more cohesive doughs. Particularly, more viscous doughs were obtained with high methoxyl pectins (Correa et al., 2012). On the other hand, the effect of modified celluloses depended not only on their intrinsic structure but also on the presence or absence of salt in dough. CMC decreased dough stability in dough without salt and HPMCs affected it in dough with salt, probably by the promotion of hydrophobic interactions. Modified celluloses softened doughs and the combination of hydrocolloids and salt led to more cohesive and less resilient systems (Correa, Añón, Pérez, & Ferrero, 2010). These results indicate that the type of modification in the case of celluloses and the degree of methoxylation in the case of pectins are influencing the interaction with dough components.

Different techniques can be applied to study the interaction of hydrocolloids with dough components, particularly with gluten proteins, at a microstructural level. The use of different microscopic techniques is a very useful approach to dough microstructure allowing a general overview of the matrix characteristics (Baier-Schenk et al., 2005; Jeckle & Becker, 2011; Li, Dobraszczyk, & Wilde, 2004; Peighambardoust, van der Goot, van Vliet, Hamer, & Boom, 2006). Techniques as NMR relaxation assays (T_2) has been applied to study water mobility in dough and bread (Engelsen, Jensen, Pedersen, Norgaard, & Munck, 2001; Esselink et al., 2003; Leung, Magnuson, & Bruinsma, 1979; Linlaud, Ferrer, Puppo, & Ferrero, 2011; Lopez-Da-Silva, Santos, Freitas, Brites, & Gil, 2007).

The characteristic viscoelastic properties of wheat dough are the result of gluten development. Under mechanical action and in the presence of water, gluten proteins undergo hydration, unfolding, orientation and there is also an interchange between sulfhydryl (S—H) and disulfide (S—S) bonds (Campos, Steffe, & Ng, 1997; Shewry, Popineau, Lafiandra, & Belton, 2001). Thus, the interactions among these proteins and hydrocolloids can have a strong influence on the secondary structure of proteins as can be inferred from FT-Raman spectra and on the lability of subunits from the gluten matrix as seen by SDS-PAGE (Linlaud et al., 2011).

The objectives of this study were (a) to apply physical and chemical techniques to characterize the interaction between modified celluloses and pectins and the gluten proteins at a microstructural level and (b) to compare the effect of these hydrocolloids and relate it to their chemical structure.

2. Materials and methods

2.1. Materials

Commercial wheat flour was used for dough preparation (Type 000, Código Alimentario Argentino, 2012). Flour composition was: protein 11.4% (Kjeldahl factor = 5.7), moisture 14.2%, lipids 1.4% and ash 0.68%. Wet and dry gluten values were 31.5 ± 1.3 (g gluten/100 g flour) and 11.0 ± 0.3 (g gluten/100 g flour), respectively. The alveographic parameters of flour were: $P = 96$ mm H_2O, $L = 93$ mm and $W = 326 \cdot 10^{-4}$ J. This type of flour is suitable for breadmaking. Two types of food grade hydrocolloids were employed: modified celluloses and pectins. The modified celluloses used were: microcrystalline cellulose (MCC) (FMC Biopolymer, Philadelphia) which was copolymerizated with 12% of carboxymethylcellulose, carboxymethylcellulose (CMC) (Latinoquímica Amtex S.A., Argentina) with a degree of substitution of 0.9% and two different types of hydroxypropylmethylcellulose (HPMC) (Dow Chemical Company, USA) with a different degree of methoxyl and hydroxypropyl substitution: HPMC F 4M with 29.3% of methoxyl groups and 6.0% of hydroxypropyl groups and HPMC F 50 with 28.6% of methoxyl groups and 5.4% of hydroxypropyl groups. Also, two types of citrus pectins (CP Kelco, USA) were used: Genu Pectin 8001 (a low methoxyl pectin — LMP) and Genu Pectin 105 (a high methoxyl pectin — HMP). Purity of both pectins was 57% (w/w) and the degree of esterification was 67% for HMP and 43% for LMP. The degree of amidation for LMP was 16% (Correa et al., 2012). Distilled water and commercial salt (NaCl) were used to prepare dough.

2.2. Methods

2.2.1. Dough preparation

Dough was formulated with flour (100 g), without and with NaCl (2 g), and water according to farinographic absorption. Modified celluloses were employed at 1.5% (flour basis) and pectins at 2.0% (flour basis); dough without hydrocolloids was used as control. Hydrocolloids levels were the maximum ones among those used in previous works (Correa et al., 2010, 2012).

Dough samples were prepared without yeast in a microfarinograph Brabender (Duisburg, Germany). For each blend the farinographic development time was used as the mixing time. Each dough formulation was prepared in duplicate.

2.2.2. Scanning electron microscopy (SEM)

Micrographs of control and samples with hydrocolloids were taken. Small portions of dough were cut, fixed in 10% glutaraldehyde and sequentially embedded in acetone solutions of increasing concentration to ensure full dehydration. Samples were dried at the critical point and coated with gold particles. A scanning electron microscope (JEOL 35 CF, Japan) was employed with magnifications of 500×, 3000×, 5000×. For comparisons among samples, 500× was selected. Nine representative fields were obtained for each formulation.

2.2.3. Confocal scanning laser microscopy (CSLM)

2.2.3.1. Sample preparation. A mixture of rhodamine B (0.001%) and fluorescein isothiocyanate (FITC) (0.01%) in distilled water was used for non-covalent labeling. The fluorescent contrast depends of dye affinity and accessibility to the different components. A small portion of dough was cut and then spread on a glass slide with a rolling pin; immediately it was imbibed with the dye solution. The sample was let to rest for an hour within a closed recipient and in darkness, and then the specimen was washed with distilled water and covered with a glass cover slip. Dough samples did not show autofluorescence.

2.2.3.2. Confocal microscopy system. A LEICA TCS SP5 (Mannheim, Germany) inverted microscope equipped with Ar and HeNe laser was used. The excitation wavelengths were 488 nm (FITC) and 568 nm (rhodamine B) and the emission wavelengths were 518 nm (FITC) and 625 nm (rhodamine B). Images were acquired using a 20× HCX PL APO CS water immersion objective and with 1024 × 1024 pixel resolution in a constant z-position. Ten photographs (5 by each replicate) with the same magnification were obtained from representative fields. Softwares Leica Application Suite Advanced Fluorescence (LAS AF), version 2.2.1. build 4842 and Image J 1.43u were employed in the image analysis. Each micrograph was RGB color split and then was corrected by shading applying FFT filtering (Walter, 2003). The corrected image was subjected to an automatic thresholding and converted in a binary image as described by Peighambardoust et al. (2006).

From binary images, protein matrix value (%) and fractal dimension (FD) were calculated. The protein matrix value was

defined as the ratio between the area occupied by the protein network and the total area. FD is related with the pattern complexity of the image: a higher fractal dimension corresponds to a more complex pattern. To calculate it, the software counts the number of boxes of an increasing size needed to cover a one pixel binary object boundary and the FD is obtained as the negative of the slope from the plot "log(count)" as a function of "log(size)".

2.2.4. Nuclear magnetic resonance

Spin–spin relaxation times (T_2) were measured using a RMN equipment (Minispec, Bruker, Germany). Each dough formulation was prepared in duplicate. A total of 12 measurements for each type of formulation were accomplished.

Measurements were performed at room temperature by applying the Carr–Purcel–Meiboon–Gill (CPMG) pulse sequence with an interpulse spacing of 200 μs. The signal decay was fitted with an exponential equation (Eq. (1)).

$$I = \sum_{i=1}^{n} y_i \exp\left(\frac{-x}{T_{2i}}\right) \quad (1)$$

where I represents protons signal intensity, proportional to water quantity in the sample, T_{2i} corresponds to the relaxation time of protons i present in dough and y_i is the signal intensity of protons in T_{2i} state. To obtain the parameters that gave the best fit to Equation (1), a nonlinear regression at Origin 7.0 software was made.

2.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of protein fractions obtained by extraction with 50% propanol and 0.1 M acetic acid were performed according to the modified sequence of Osborne and proteins obtained under denaturing conditions. The protein extractions were made from freeze-dried dough.

2.2.5.1. Sample preparation

2.2.5.1.1. Sequential extraction of proteins. The solvents used were 50% 1-propanol to obtain the gliadins fraction and 0.1 M acetic acid to extract the glutenins fraction, after the separation of albumin and globulin. Three grams of lyophilized dough were weighed and 15 ml of extraction solvent was added. The dispersion was stirred for 30 min at room temperature and centrifuged at 12.096 × g for 15 min at 4 °C. This procedure was performed twice with each solvent.

2.2.5.1.2. Extraction under reducing conditions. A sequential extraction of gliadin and glutenin fractions was performed from lyophilized dough according to the technique described by Singh, Shepherd, and Cornish (1991) and modified by Nieto-Taladriz, Ruiz, Martinez, Vazquez and Carrillo (1997). The extraction was done from 200 mg of lyophilized dough in a thermostatic bath at 65 °C.

To carry out the electrophoresis equal volume of sample buffer (0.37 M Tris, A glycerol 25% w/v SDS, 4.0% w/v bromophenol blue 0.1% w/v.) was added to both protein fractions.

2.2.5.2. Electrophoretic runs. Runs were carried out in stacking and separating gels containing 40 g/l and 120 g/l acrylamide, respectively. The following continuous buffer system was used: 0.375 M Tris–HCl, pH 8.8, 1 g/l SDS for the separating gel; 0.025 M Tris–HCl, 0.192 M glycine and 1 g/l SDS, pH 8.3 for the running buffer, and 0.125 M Tris–HCl, pH 6.8, 200 ml/l glycerol, 10 g/l SDS, and 0.5 g/l bromophenol blue as sample buffer.

The protein molecular mass standards used under not reducing conditions were: phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa) (GE Healthcare, UK). Under reducing conditions the molecular standards used were: myosin (212 kDa), α2-macroglobulin (170), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa) (Pharmacia, Biotech, USA).

Gels were fixed and stained with Coomassie Brilliant Blue Stain. Gels images were analyzed with Sigma Gel version 1.0 (Jandel Scientific, USA) in order to determine the molecular masses of the polypeptides. Electrophoretic runs were repeated at least twice.

2.2.6. FT-Raman spectroscopy

The secondary structure of gluten proteins present in the dough was study by Fourier-Transform Raman spectroscopy (FT-Raman). Dough samples were prepared as described in 2.2.1. and then were lyophilized and pulverized. It is worth to mention that starch is present in great proportion in the studied mixtures but no characteristic starch bands appeared in the Raman spectrum in the region assigned to the proteins Amide I band (Dupuy & Laureyns, 2002); for this reason the analysis could be performed without interferences.

Raman spectra were collected on a Bruker IFS 113 FT-IR spectrophotometer (Bruker Optics, Germany) provided with the NIR Raman attachment equipped with an Nd:YAG laser at 1064 nm laser. Frequency calibration of the instrument was undertaken using the sulfur line at 217 cm^{-1}. Spectra were recorded at room temperature with a laser power of 500 mW, and spectral resolution of 6 cm^{-1}. Each spectrum was obtained after collecting and averaging 1000 scans in order to obtain high signal-to-noise ratio spectra. FT-Raman spectra were plotted as intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}). Because of the inhomogeneity of the dough samples, all the spectra were vector normalized on the basis of the starch band at 480 cm^{-1}. The curve-fitting procedure was performed by stepwise iterative adjustment. Shape and position of Raman bands were determined using the second derivative of the original spectra and assuming an initial mixed Lorentzian–Gaussian line-shape functions with full width band at half-height (FWHH) of 6–8 cm^{-1}. In Amide I region, a straight baseline passing through the ordinates at 1700 and 1600 cm^{-1} was adjusted in order to calculate this band intensity. To calculate the secondary structure components, this region was truncated and fitted. The resulting fitted curve was analyzed taking into account the band assignment for the secondary structure previously reported in the literature (Carey, 1982; Herrero, 2008; Ngarize, Herman, Adams, & Howell, 2004; Tu, 1982). The assigned structures were antiparallel β-sheet: 1675–1695 cm^{-1}, β-turns: 1666–1673 cm^{-1}, α-helix: 1650–1658 cm^{-1}, random coil: 1637–1645 cm^{-1}, solvated helix: 1625–1637 cm^{-1}, parallel β-sheet: 1613–1625 cm^{-1}. In order to calculate the percentage contribution of the different types of conformations to the area of all the components, bands assigned to a given conformation were summed and divided by the total Amide I area. The obtained number was taken as the proportion of the polypeptide chain in the corresponding conformation. Baseline corrections, normalization, derivation, curve fitting and area calculation were carried out by means of Grams/32 (Galactic Industries Corporation, USA) software and OPUS 4.0 (Bruker Optics, Germany). All analyses were performed on two independent experiments, and the results were reported as averages of these replicates. Before spectra were analyzed the raw data were subjected to a quality test using Opus software.

2.2.7. Statistical analysis

The software Statgraphics plus for Windows 5.1 was used to perform ANOVA and to compare means among samples. Bonferroni's multiple comparison procedure was applied at 95% confidence level.

3. Results and discussion

3.1. Microscopic analysis of gluten matrix

Figs. 1 and 2 show representative images of dough microstructure obtained with SEM and CLSM techniques for samples without and with NaCl and hydrocolloids. Comparing both techniques, in general, the modifications due to NaCl or hydrocolloid addition were more easily observed with CLSM than with SEM.

SEM micrographs showed that control samples without and with NaCl (Figs. 1A and 2A respectively) exhibited areas with gluten films and gluten filaments. Gluten films were able to wrap starch granules. However the effect of NaCl addition was evident through the formation of a more orientated filamentous structure, with higher crosslinking. By CLSM it could be confirmed that control with NaCl (Fig. 2B) exhibited gluten filaments with a more orientated and crosslinked arrangement leading to a less open network

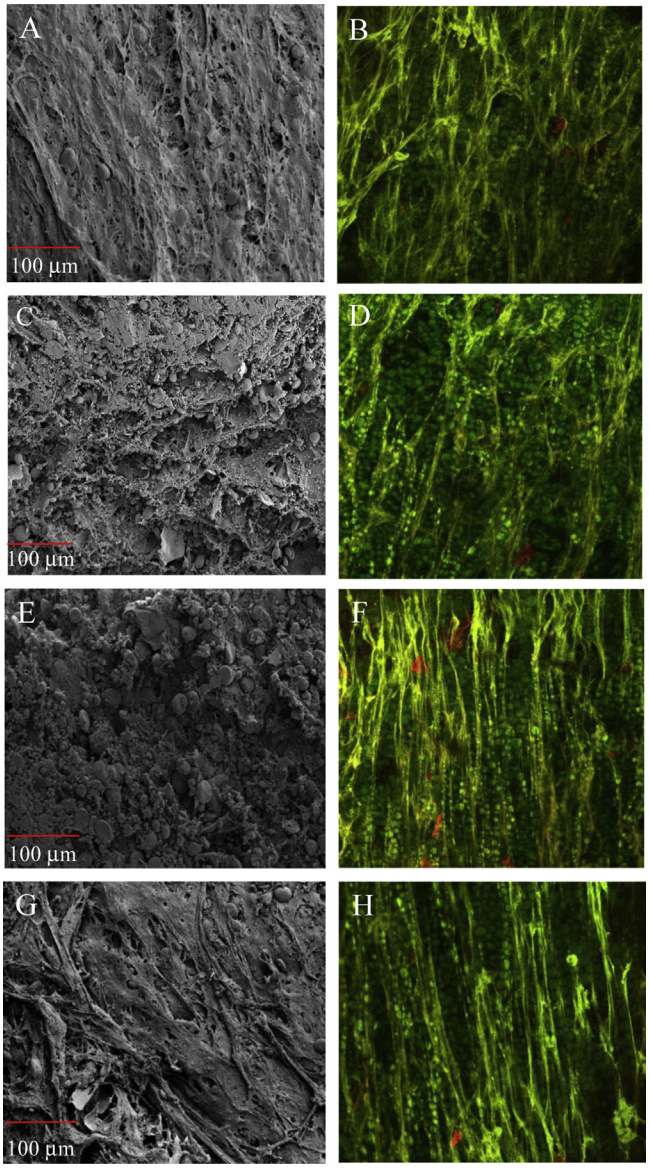

Fig. 1. SEM and CLSM of dough without NaCl. A, B) Control; C, D) with LMP; E, F) with CMC; G, H) with HPMC F 4M. Red spots correspond to rhodamine B crystals that remained after the wash. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

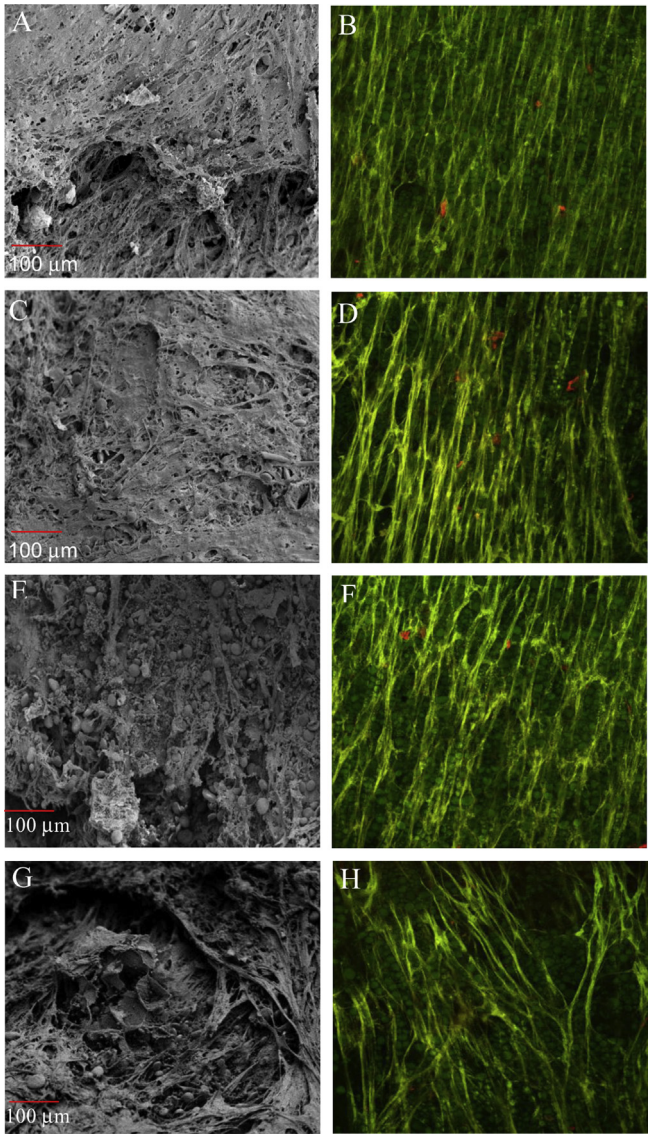

Fig. 2. SEM and CLSM of dough with NaCl. A, B) Control; C, D) with LMP; E, F) with CMC; G, H) with HPMC F 4M. Red spots correspond to rhodamine B crystals that remained after the wash. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than the sample without NaCl (Fig. 1B). The presence of NaCl could promote hydrophobic interactions between polypeptide chains leading to a more compact network.

Samples with LMP and without NaCl exhibited a disaggregated gluten network as seen by SEM and CLSM (Fig. 1C and D). Pectins are negatively charged polymers at dough pH and they can establish electrostatic interactions with gluten proteins also inducing a repulsive effect between contiguous chains. Chang, McLandsborough, & McClements (2011, 2012) have demonstrated the ability of pectins to form complexes with a cationic charged polymer (ε-polylysine). When these complexes remained positively or negatively charged, repulsive effects inhibited further aggregation. In the present case, disaggregation of gluten network could be indicating this repulsive effect. As it was observed in the case of control dough, when salt was added, a more regular and orientated network was obtained (Fig. 2C and D). NaCl addition could be shielding polymer charges and also promoting hydrophobic interactions, thus leading to a more crosslinked network. HMP

samples (results not shown) exhibited markedly aligned and crosslinked filaments and in this case the effect of NaCl addition was not so pronounced as for LMP. The more accentuated effect of LMP respect to HMP could be due to the higher negative charge density of this pectin (Chang et al., 2012).

MCC samples without and with salt showed similar structural characteristics compared to their respective control samples (results not shown). This type of modified cellulose is obtained by hydrolytic cleavage of native cellulose fibers. Acid degrades the amorphous zones of native cellulose releasing crystalline particles (Glicksman, 1982). So, MCC particles would act as fillers in dough matrix which could explain the scarce effect on network microstructure.

Samples with CMC and without NaCl exhibited a highly filamentous and oriented gluten network with few entanglements (Fig. 1E and F). Otherwise, the addition of NaCl led to a more crosslinked matrix (Fig. 2E and F). Respect to control, CMC samples exhibited a more open matrix. CMC is negatively charged so, in absence of salt, it could also establish electrostatic interactions with gluten proteins also inducing a repulsive effect between contiguous chains. A less crosslinked network than control, with a marked orientation, could be indicating this repulsive effect. As in the case of pectins, salt addition would shield CMC charges and could promote hydrophobic interactions with an increase on crosslinking behavior.

In general, HPMCs led to a more filamentous structure than MCC and CMC. The addition of NaCl in dough with HPMCs led to marked structural differences. Dough sample with HPMC F 4M without NaCl showed a gluten network with grouped and aligned filaments resulting in a more open and orientated network in relation to control (Fig. 1G and H). When NaCl was added, a matrix with thicker filaments was obtained. Otherwise, doughs with HPMC F 50 and without NaCl led to a highly open protein network very different from control sample (results not shown). Both HPMCs can establish hydrophobic interactions with proteins due to their type of substitution (hydroxyalkyl groups). NaCl addition, favoring hydrophobic interactions, would increase this effect, leading to thicker filaments and more open spaces.

These network characteristics are partially reflected in the parameters PMV and FD shown in Table 1. In general, higher PMV and FD values were obtained in samples with NaCl addition, in coincidence with more extended, crosslinked and complex networks. Among samples without salt and celluloses, the addition of HPMC F 50 led to a significantly lower PMV value respect to control which is in accordance with the highly open protein networks observed for HPMCs by CSLM. Although no significant differences ($p > 0.05$) were found in the other cases a slight tendency to obtain lower values of these parameters was found. When HMP was added without NaCl significant higher values of PMV and FD than LMP and control samples were obtained, which is in agreement with the type of matrix (markedly aligned and crosslinked filaments). On the other hand, in doughs with NaCl significantly lower values of PMV and FD than control were obtained only for samples with HPMC F 4M. This fact is in agreement with the more open network with thicker filaments that was observed in this case. It is appropriate to indicate that high PMV values are not necessarily a measure of dough quality since Peighambardoust et al. (2006) found high PMV for both unprocessed and overmixed dough.

Microstructural characteristics of this gluten network could be related with rheological behavior of dough samples, previously reported (Correa et al., 2010). Relating microscopic results with farinographic ones, those formulations exhibiting a more crosslinked structure showed in general a better tolerance to the overmixing action and also a higher stability and a lower softening. For example, addition of CMC in absence of NaCl reduced dough stability (less crosslinking behavior, Fig. 1F) but stability was not affected when salt was present (more crosslinking behavior, Fig. 2F). On the other hand, open matrices could be related with more viscous and softer doughs (like doughs with HPMC F 4M and NaCl).

3.2. Matrix mobility by ^{1}H-NMR

Data was fitted with exponential models of one and two terms. The best fit was found with the one term model, indicating that only one population of protons could be identified. Leung, Steinberg, Wei, and Nelson (1976) reported the mobility of water in doughs from different wheats; they found two distinct fractions: mobile water (with longer relaxation times) and immobile water (with shorter relaxation times). In this case, only one population could be distinguished. In Table 2, relaxation times (T_2) for dough without and with NaCl and different hydrocolloids are shown. Significant effects of NaCl addition and hydrocolloid type were found ($p < 0.05$). In presence of salt, significantly higher relaxation times were observed when modified celluloses were added. In dough without salt, HPMC F 4M increased relaxation time respect to control and HPMC F 50 significantly decreased it. In the case of pectins, a decrease in relaxation time was observed in dough with HMP with and without salt. A decrease in relaxation time is related to a diminished mobility of the matrix (a more rigid matrix) while an increase reveals major mobility.

Esselink et al. (2003) found that kneading for longer times rendered a gluten network with a threadlike structure with higher relaxation values. The highest mobility of this structure was attributed to the release of water due to the partial disruption of the gluten network. They also found that relaxation times were decreased (lower water mobility) after the molding step, involving

Table 1
Protein matrix value percentage (PMV) and fractal dimension of micrographs obtained by confocal microscopy.

Sample	Without NaCl		With NaCl	
	PMV	FD	PMV	FD
Control	25.8 ± 1.5^{b}	1.62 ± 0.02^{b}	27.6 ± 2.7^{bc}	1.64 ± 0.03^{b}
MCC	22.9 ± 0.5^{ab}	1.59 ± 0.01^{ab}	29.3 ± 1.0^{bc}	1.66 ± 0.01^{b}
CMC	22.6 ± 3.4^{ab}	1.58 ± 0.04^{ab}	29.6 ± 2.3^{c}	1.66 ± 0.02^{b}
HPMC F 4M	22.5 ± 3.1^{ab}	1.58 ± 0.03^{ab}	19.5 ± 2.3^{a}	1.55 ± 0.03^{a}
HPMC F 50	17.1 ± 6.4^{a}	1.54 ± 0.06^{a}	25.6 ± 3.5^{b}	1.62 ± 0.04^{b}
Control	25.8 ± 1.5^{a}	1.62 ± 0.02^{a}	27.6 ± 2.7^{a}	1.64 ± 0.03^{a}
LMP	22.7 ± 4.6^{a}	1.59 ± 0.05^{a}	26.0 ± 2.5^{a}	1.62 ± 0.03^{a}
HMP	30.9 ± 0.03^{b}	1.68 ± 0.03^{b}	26.2 ± 3.6^{a}	1.63 ± 0.03^{a}

±SD. Within each column and for each type of hydrocolloid (modified cellulose or pectin) different letters indicate significant differences among samples and/or respect to control ($p < 0.05$).

Table 2
Relaxation times (T_2) for dough with different hydrocolloids.

Sample	Without NaCl	With NaCl
	T_2 (ms)	T_2 (ms)
Control	11.7 ± 0.3^{b}	11.2 ± 0.2^{a}
MCC	12.2 ± 0.5^{b}	12.6 ± 0.1^{c}
CMC	12.2 ± 0.1^{bc}	11.9 ± 0.3^{b}
HPMC F 4M	12.7 ± 0.4^{c}	13.1 ± 0.1^{cd}
HPMC F 50	10.9 ± 0.2^{a}	13.3 ± 0.2^{d}
Control	11.7 ± 0.3^{b}	11.2 ± 0.2^{b}
LMP	11.5 ± 0.2^{b}	11.3 ± 0.2^{b}
HMP	10.3 ± 0.1^{a}	10.6 ± 0.1^{a}

±SD. Within each column and for each type of hydrocolloid (modified cellulose or pectin) different letters indicate significant differences among samples and/or respect to control ($p < 0.05$).

gluten stretching and orientation, which led to a more ordered, less mobile network. Lusse and Arnold (1998) studied the relaxation phenomenon in polysaccharide-aqueous systems and they attributed the observed differences to reorientation processes with respect to the binding site and to the mobility of the polymer backbone. Solutions with a stiff polymer backbone exhibited larger relaxation rates (lower relaxation times, so lower mobility) than those of flexible polymers.

The results of this work suggest that less flexibility and mobility cannot be related to a more oriented and crosslinked structure, since networks with different degree of crosslinking led to similar relaxation times.

3.3. SDS-PAGE

Fig. 3A and B show the typical electrophoretic profiles obtained by SDS-PAGE of extracts in 50% 1-propanol obtained from lyophilized dough without and with NaCl, respectively. All the extracts analyzed of the lyophilized dough without NaCl showed the same number of bands and in a similar position (Fig. 3A). The profiles showed the presence of very faint bands of 68, 61, 56 and 48.8 kDa that correspond to ω-gliadins, followed by other polypeptides species of 41, 37, 35, 32 kDa which could be assigned to α, β-and γ-gliadins and LMW-GS and bands of 26, 17 and 15 kDa that would correspond to albumin and globulins. In some cases where hydrocolloids were added to dough (HPMC F 50, CMC and MCC) diffuse high molecular weight aggregates were observed at the top of the gel. These aggregates could correspond to gliadins associations. Dough samples with NaCl (Fig. 3B) also showed a similar electrophoretic profile. These profiles showed polypeptide species corresponding to aggregates (106, 98, 93 and 88 kDa), ω-gliadins (52, 47 and 42 kDa), gliadin and/or subunits of LMW-GS (bands between 40 and 37 kDa) and albumin and globulins (bands between 21 and 15 kDa). The intensity of the bands corresponding to the profile of the CMC dough is greater than that shown by the control, whereas in the case of HPMC F 4M dough a lower intensity of some bands was detected, respect to control.

These results suggest that the propanol lability of the protein subunits from the gluten network is dependent on the presence or absence of NaCl. In all doughs without NaCl a greater proportion of lighter polypeptide species were extracted while in doughs with NaCl the intensified bands of high molecular mass are indicating the presence of protein aggregates formed by gliadins, at least partially stabilized by disulfide bonds. We cannot rule out – for the experimental conditions used in the electrophoresis runs – other types of interactions, such as electrostatic and hydrophobic interactions in the aggregates present in the doughs. Besides, lability seems influenced by the type of hydrocolloid. A faint profile like in the one case of dough with HPMC F 4M and salt, indicates a difficult extraction of protein subunits with propanol, which in principle implies a strong association of prolamins with the gluten network. On the other hand, the presence of CMC and salt would facilitate extraction suggesting the presence of interactions sensitive to the alcohol.

Fig. 3C shows the SDS-PAGE corresponding to the soluble protein fraction in 0.1 M acetic acid obtained from freeze-dried dough without NaCl. In all samples were observed protein aggregates with molecular mass greater than 97 kDa besides gliadins (56–33 kDa),

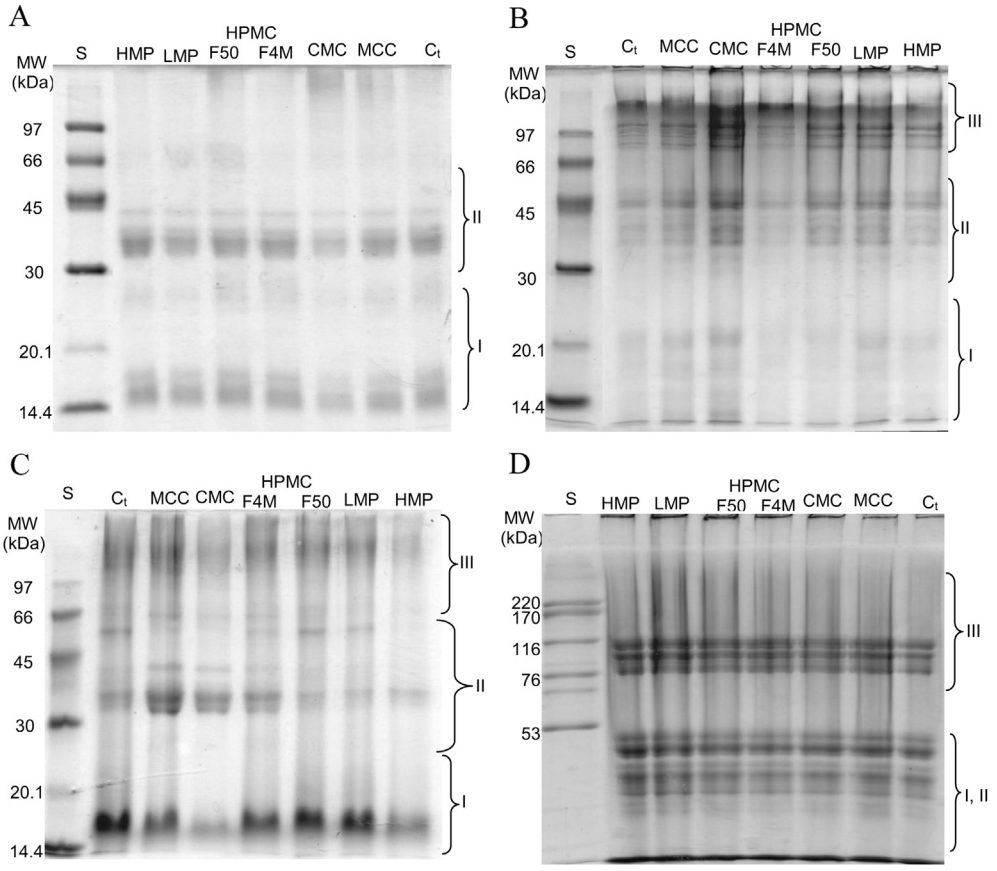

Fig. 3. SDS-PAGE of extracts obtained with 50% 1-propanol from dough without (A) and with NaCl (B), with 0.1 M acetic acid from dough without NaCl (C) and with 50% 1-propanol + Tris–HCl 0.08 M from dough without NaCl under reducing conditions (DTT) (D). I = albumin and globulins; II = gliadins and LMW-GS; III = HMW-GS and HMW-GS aggregates; S = molecular mass standard; C_t = control.

albumins and globulins (masses below 25 kDa). Burnouf and Bietz (1989) found that with the use of dilute solutions of acetic acid albumin, globulins and gliadins are extracted together with HMW-GS and LMW-GS.

The presence of hydrocolloids resulted in changes in the electrophoretic profile. The control and MCC doughs showed an increased intensity of bands which would indicate a greater extraction of polypeptide species. On the other side CMC and HMP doughs showed lower band intensities of the high molecular mass species (HMW-GS subunits and aggregates HMW-GS), while the HMP dough also showed very faint bands in the area of gliadins and LMW. It is also noted qualitative differences between the profiles of the different samples assayed. Extracts of HPMC F 4M, HPMC F 50 and LMP doughs showed the 56 kDa band (very weak in the case of HPMC F 4M), but this band is not present in MCC, CMC and HMP dough profiles. On the other hand, in the lanes corresponding to MCC, HPMC F 4M and CMC doughs could be observed (although very weakly with CMC) bands at 65 kDa and 41 kDa that are virtually absent in the other samples. These results would suggest different lability of the network protein of doughs in the presence of such hydrocolloids regarding the extraction conditions. The most prominent cases are those of the samples with CMC and HMP addition. The presence of hydrocolloids in the matrix did not increase the intensity of the bands corresponding to HMW-GS or HMW-GS aggregates respect to the control sample (and in the lanes of the samples with CMC and HMP intensity was decreased); this would indicate there was no depolymerization of the gluten leading to an increased amount of soluble aggregates.

SDS-PAGE electrophoresis of extracts with 0.1 M acetic acid from the dough with NaCl showed a band profile similar to extracts from dough without NaCl. However, the presence of salt diminished intensity of different bands (results not shown).

The electrophoretic profiles of extracts obtained from doughs without NaCl under reducing conditions after extraction with 50% 1-propanol (Fig. 3D) showed the presence of HMW aggregates (134, 122 and 108 kDa), HMW subunits (99, 93 kDa), LMW-GS subunits (52, 47 and 44 kDa), gliadins of lower molecular mass (39, 35 kDa), albumins and globulins (25, 20 and 18 kDa). All samples presented similar bands besides protein aggregates with molecular mass of 220 kDa that did not enter in the separating gel. The electrophoretic profiles corresponding to the extracts of doughs with NaCl were similar to those found in the doughs without NaCl (results not shown). In this case, reducing conditions have promoted the same degree of protein lability regardless the presence of other factors like hydrocolloids and NaCl. Reducing conditions used in these electrophoretic runs allowed the destabilization of aggregates confirming the earlier assumption made regarding the stabilization of the extracted aggregates by disulfide bonds.

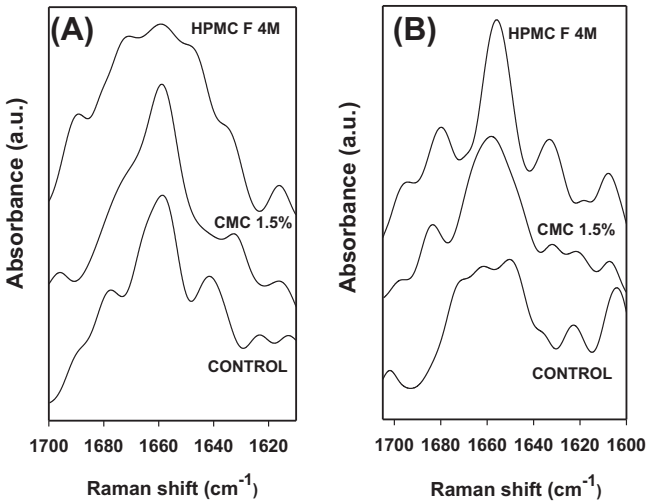

Fig. 4. FT-Raman spectrum for control, CMC and HPMC F 4M samples. A) dough without NaCl; B) dough with NaCl.

3.4. FT-Raman spectroscopy

FT-Raman spectra of proteins show several bands due to different vibrations of the peptide moiety. Amide I and amide III bands are the most frequently used to assign secondary structures to proteins. Amide I originates from C═O stretching vibration of the amide group while amide III comes from C–N stretching and N–H bending (Pelton & McLean, 2000). Even though glutamine side chains can contribute to amide I band, it has been demonstrated that this contribution does not exceed 10% for most proteins (Popineau, Bonenfant, Cornec, & Pezolet, 1994). In this work, the effect of hydrocolloids on protein secondary structure was evaluated through amide I region (centered around 1650–1660 cm^{-1}), since in this zone starch spectrum did not overlap with proteins bands.

Fig. 4A and B show amide I region of spectra for control, CMC and HPMC F 4M samples without and with NaCl, respectively. It can be seen that the addition of hydrocolloids and NaCl modified the spectrum profile respect to control. The spectrum shape indicates the existence of several overlapped bands. Bands in the region of 1675–1695 cm^{-1} may be attributed to antiparallel β-sheet while bands at 1613–1625 cm^{-1} are assigned to parallel β-sheet. The unsolvated and solvated α-helix conformations are associated to bands between 1650–1658 cm^{-1} and 1625–1637 cm^{-1}, respectively. Bands attributed to more disordered structures are in the region of 1666–1673 cm^{-1} (β-turns) and 1637–1645 cm^{-1} (random coil) (Herrero, 2008; Ngarize et al., 2004; Tu, 1982). Each polypeptide conformation could be related to a greater or lesser degree

Table 3
Relative proportion of different conformations in dough with hydrocolloids and without NaCl.

Samples without NaCl	Relative proportion (%)					
	β-sheet		β-turn	Random coil	α-helix	
	Antiparallel	Parallel			Unsolvated	Solvated
Control	14.19 ± 0.02	3.52 ± 0.06	13.25 ± 0.68	7.65 ± 0.06	52.61 ± 1.18	8.82 ± 0.06
MCC	26.35 ± 0.17	6.39 ± 0.04	0.88 ± 0.02	11.16 ± 0.26	48.09 ± 0.12	7.00 ± 0.02
CMC	6.84 ± 0.08	3.79 ± 0.11	40.65 ± 1.07	36.20 ± 0.41	10.69 ± 0.53	1.69 ± 0.05
HPMC F 4M	19.64 ± 0.22	23.42 ± 0.20	12.19 ± 0.21	13.10 ± 0.15	24.94 ± 0.25	6.70 ± 0.10
HPMC F 50	26.66 ± 0.09	9.76 ± 0.25	11.64 ± 0.18	5.87 ± 0.09	22.78 ± 0.09	23.24 ± 0.07
LMP	24.99 ± 0.13	21.45 ± 0.07	12.98 ± 0.10	17.91 ± 0.04	18.26 ± 0.07	4.44 ± 0.17
HMP	22.62 ± 0.03	4.20 ± 0.01	14.50 ± 0.19	9.38 ± 0.19	35.66 ± 0.13	13.62 ± 0.27

Averages (two replicates from separate doughs) ± SE.

Table 4
Relative proportion of different conformations in dough with hydrocolloids and NaCl.

Samples with NaCl	Relative proportion (%)					
	β-sheet		β-turn	Random coil	α-helix	
	Antiparallel	Parallel			Unsolvated	Solvated
Control	28.4 ± 0.23	13.18 ± 0.02	1.20 ± 0.01	–	39.25 ± 0.07	18.37 ± 0.20
MCC	16.18 ± 0.18	33.26 ± 0.37	17.51 ± 0.23	15.46 ± 0.19	15.29 ± 0.20	2.26 ± 0.01
CMC	14.66 ± 0.01	15.09 ± 0.04	34.03 ± 0.18	27.58 ± 0.25	3.95 ± 0.06	4.67 ± 0.02
HPMC F 4M	11.99 ± 0.04	28.87 ± 0.57	11.19 ± 0.31	27.10 ± 0.48	16.94 ± 0.39	3.92 ± 0.03
HPMC F 50	14.91 ± 0.07	15.61 ± 0.09	37.80 ± 0.16	–	16.47 ± 0.10	15.22 ± 0.06
LMP	14.82 ± 0.27	10.29 ± 0.07	10.00 ± 0.19	6.75 ± 0.11	46.94 ± 0.14	11.07 ± 0.23
HMP	21.91 ± 0.02	14.06 ± 0.15	4.15 ± 0.03	5.27 ± 0.01	46.37 ± 0.12	8.27 ± 0.02

Averages (two replicates from separate doughs) ± SE.

of protein folding. Both α-helix conformations (solvated and unsolvated) are more compact structures and the random coil conformation is a less compact and more disordered one (Ferrer, Bosch, Yantorno, & Baran, 2008; Ferrer, Gómez, Añón, & Puppo, 2011). The effect of NaCl and hydrocolloids addition on gluten proteins folding could be evaluated by the changes in the relative proportion of each conformation.

Table 3 shows the relative proportions of different secondary structures in doughs with hydrocolloids and without NaCl. As it is evidenced by the higher α-helix conformation percentage, control and MCC samples showed the more compact and less unfolded protein structures followed by dough with HMP, HPMCs, LMP and CMC.

CMC dough exhibited the lowest α-helix (unsolvated + solvated) percentage and an increased amount of more unfolded conformations: random coil and β-turn, being this effect more pronounced in the presence of salt.

In general, HPMC F 4M and LMP doughs showed similar proportion of β-sheets and β-turn. However, HPMC F 4M sample exhibited a higher proportion of α-helix conformations than LMP. Finally, HPMC F 50 and HMP doughs exhibited lower percentage of α-helix structures than control and MCC samples but higher percentage than HPMC F 4M and LMP doughs. According to Belton et al. (1995), β-sheet and other extended structures are related to the higher chain mobility produced by an increasing hydration of glutenins. As protein is hydrated, protein–protein bonds are replaced by protein–water interactions and this would allow sufficient movement of sections in the polypeptide chain to form β-sheet structures. In the present work, the addition of hydrocolloids led to an increased proportion of β-sheet in most cases.

Table 4 shows the relative proportion of each type of secondary structure present in doughs with NaCl. Addition of NaCl in sample without hydrocolloid led to a diminution of α-helix and an increase of β-sheet (parallel and antiparallel) respect to control without NaCl, indicating a higher level of polypeptide unfolding when NaCl is added. Besides, a diminution of β-turn was observed. A similar effect of NaCl on β-sheet and β-turn conformations was observed by Ukai, Matsumura, and Urade (2008) in gluten proteins studied by FT-IR. As reported by Popineau et al. (1994), a higher relative content of β-sheet is related with longer glutenin polymers. Thus, this conformational change could be the cause of the higher stability of doughs with NaCl reported in a previous work (Correa et al., 2010).

In general, doughs with modified celluloses and NaCl showed a decrease of α-helix conformation respect to control dough with salt. As it was observed in the case of samples without gums, the percentage of α-helix was smaller in dough with NaCl respect to doughs without NaCl, indicating a higher contribution of less ordered structures in presence of NaCl. A less ordered structure could facilitate protein interaction (crosslinking behavior and aggregation). A notable increase of random coil structure was observed in HPMC F 4M sample when adding salt, which is the structure with a more open aspect and thicker filaments, and less protein lability.

As in the case of doughs without NaCl, CMC dough showed the smallest percentage of α-helix conformation, and the highest contributions of random coil and β-turn structure which is also indicative of polypeptide unfolding. The comparison between CMC dough without and with NaCl showed that the addition of NaCl led to a decrease of α-helix, random coil and β-turn conformations and an increase of β-sheet.

Doughs with pectins and NaCl showed a similar percentage of α-helix conformation respect to control with NaCl. Otherwise, doughs with pectins and NaCl exhibited higher percentages of α-helix conformation than the corresponding doughs without NaCl. These results are indicating that pectins do not cause the same degree of disorder on protein conformation than other hydrocolloids.

No trends are evident when trying to correlate the prevalence of certain conformations with the mobility of the matrix found by RMN assays. This would indicate that the particular distribution of conformations in each case is determining the flexibility of the chain. However, it is possible to relate the more disordered structures (promoted by NaCl and the presence of hydrocolloids) with more possibilities of interaction among proteins leading to more crosslinked structures and/or more aggregates.

4. Conclusions

Modified celluloses and pectins affect gluten network conformation. The interactions that these hydrocolloids can establish with gluten proteins are dependent on the type of hydrocolloid and NaCl addition. By microscopy, it was possible to analyze the type of gluten network obtained in each case. The employment of hydrocolloids, particularly modified celluloses, seemed to diminish the crosslinking behavior in doughs without NaCl. The type of substitution seems to be determining the type of gluten network obtained. In the case of anionic molecules like CMC or pectins, they could interact with gluten proteins through electrostatic forces leading to a less crosslinked structure. On the other hand, molecules with a certain hydrophobic character like HPMCs could establish hydrophobic interactions leading to aggregation and a thicker network. The addition of NaCl has a shield effect on molecular charges and also promotes hydrophobic unions thus changing the characteristics of gluten matrix. The addition of NaCl increased the crosslinking behavior and favored orientation of gluten filaments.

Through FT-Raman assays the effect of modified celluloses, pectins and NaCl on the secondary structure of proteins was established. In general, a decrease in α-helix conformation and an increase of less ordered structures were observed with the addition

of hydrocolloids. When NaCl was added a higher percentage of β-sheets were observed in most cases. Gluten network exhibited the highest unfolding in presence of CMC since the predominant structures were random coil and β-turn. The protein unfolding observed in the presence of hydrocolloids and NaCl could facilitate the establishment of more interactions among polypeptide chains, leading to a more pronounced crosslinking behavior or even aggregation or facilitate the protein extraction. The results of SDS-PAGE confirmed that there is a differential effect of hydrocolloids on dough lability depending on the presence or absence of NaCl. For example, the presence of CMC facilitates gliadins extractions and difficult glutenin one. The greater or lesser lability of gluten network would indicate that a weaker or stronger association among subunit proteins has been established. The RMN assays showed that in presence of NaCl all modified celluloses increased matrix mobility, whereas in absence of it different effects were obtained depending on the type of hydrocolloid.

These results are in accord with the changes in dough characteristics observed when these hydrocolloids are added. In general, modified celluloses and pectins addition softened dough, which could be related to more protein unfolding and matrix flexibility. However, the particular effect of each hydrocolloid can be modulated by the presence or absence of NaCl.

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