Study of the formation of soluble complexes of sodium caseinate and xanthan in solution

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Abstract The main objective of this work was to determine the optimum conditions for the formation of soluble complexes between sodium caseinate and xanthan in solution at neutral pH, in the presence of the NaCl. The study of the influence of the concentrations of these three substances showed that salt was the most influent factor. It worsens the thermodynamic incompatibility of the two biopolymers in solution, when they are present at large amounts. However, it contributes to soluble complexes formation, when sodium caseinate concentration is below 5.5%. In this case, gels with enhanced rheological properties were obtained. Infrared spectroscopy confirmed that the complexes formation within these gels involves hydrophobic interactions. On the other hand, dynamic light scattering revealed that dilution cause their dissociation. These soluble complexes are promising ingredients to ensure new texturing properties.

Keywords Soluble complexes · Hydrophobic interactions · Sodium caseinate · Xanthan · FTIR

Introduction

Biopolymers are among the most used natural substances to ensure functional properties in food industry (McClements et al. 2009; Piorkowski and McClements 2014). Several scientists (Bouyer et al. 2012; Lam and Nickerson 2013) were studied protein and polysaccharide mixtures in the aim to obtain new textures. The proteins are mainly used for their amphiphilic properties to stabilize emulsions. They have the ability to adsorb to the interfaces and reduce the interfacial tension, inducing sufficient strength to extend the emulsions lifetime (Singh 2011; Lam and Nickerson 2013). The polysaccharides essentially contribute to a viscosity increase stabilizing the emulsion by improving its rheological properties (Piorkowski and McClements 2014; Bouyer et al. 2012). The use of these two entities simultaneously within emulsions can lead to synergistic effect of stabilization, or else, it may generate undesired destabilizing mechanisms, like flocculation by bridging or by depletion (Surh et al. 2006). To overcome destabilizations phenomena, complexation of these two types of macromolecules before introduction in emulsions, appears as an interesting approach to functional properties improvement (Rodriguez Patino and Pilosof 2011). Complexes formed with protein and polysaccharides were widely treated in literature (Turgeon et al. 2007; Evans et al. 2013). They result of associative interactions, due to covalent bounding (conjugates), or electrostatic interactions of oppositely charged biopolymers (De Kruif et al. 2004). These complexes can be insoluble in water leading to the formation of precipitates or coacervates, or soluble forming a unique liquid phase. The complexation appear under specific conditions including pH, ionic strength, protein/polysaccharide ratio, however, the most frequently encountered case is the thermodynamic incompatibility

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leading to segregative phase separation of the biopolymers (Rodriguez Patino and Pilosof 2011; Schmitt and Turgeon 2011).

The choice of our study was based on sodium caseinate, NaCN, and xanthan, XTH. Sodium caseinate is a milk derived protein, known by its exceptional emulsifying properties, high flexibility, and wide use as stabilizer in food emulsions (Huck-Iriart et al. 2011). However, this protein is very sensitive to acid pH conditions (Takeuchi and Cunha 2008), and to the presence of some substances like salts (NaCl, CaCl₂,...) (Pitkowski et al. 2009), sugars (Belyakova et al. 2003) and polysaccharides (Ghorbani Gorji et al. 2014). Xanthan gum is a widely used polysaccharide in food and pharmaceutical formulations and has interesting rheological properties influencing texture and stabilization. Sodium caseinate–xanthan mixtures were early studied by several researchers in aqueous solutions and emulsions and they reported different cases of interactions in these systems. Hemar et al. (2001) have reported microscopic phase separation in aqueous systems containing sodium caseinate and xanthan gum at concentrations up to 1% of the latter. These authors attributed this thermodynamic incompatibility to the repulsion interactions between these two biopolymers negatively charged at neutral pH. By rheological approach, Moulai-Mostefa et al. (2014) and Hadjsadok et al. (2010) have studied the interactions between these two substances in aqueous and emulsion media and associated them to associative or segregative mechanisms. Kobori et al. (2009) have studied sodium caseinate–xanthan mixtures *without salt* at different pH values, and they reported hydrophobic interactions between sodium caseinate and xanthan gum at neutral pH, which would be weakly attractive due to the electrostatic repulsion between the two biopolymers, negatively charged at this pH.

The objective of this work is to determinate the optimal concentration ranges of the two biopolymers and the salt in solution, to obtain soluble complexes. The methodology is based on the use of the experimental design method. Rheological analyzes are investigated to determine the impact of these nano-objects on the texture of mixtures. Dynamic light scattering (DLS) studies are performed to evaluate the size and polydispersity of the complexes. Finally, infrared spectroscopy (FTIR) is carried out in order to determine the hydrophobic or electrostatic nature of the interactions between the two polymeric substances.

Materials and methods

Materials

Sodium Caseinate (containing 0.09 wt% calcium, 1.45 wt% sodium, 2.67 wt% phosphate, and 5.4 wt%

moisture) was obtained from ARMOR Protein (France), Pharmacopoeial grade xanthan gum (USP/NF) was purchased from Orkila (Algeria). All other chemicals were of analytical grade and were purchased from Sigma Chemical Co. (Germany).

All water used in the experiments was deionized water.

Methods

Preparation of NaCN and XTH mixtures

Stock solutions of XTH (5%, w/w) and NaCN (20%, w/w) were prepared by dispersing and stirring the appropriate amounts of biopolymers in deionized water, during 3 h at room temperature. Sodium azide (0.03%) was added to both of solutions to avoid microbial contamination. The stock solutions were remained overnight to ensure complete dissolution.

An ultracentrifugation at 24,000 rpm during 30 min was performed in the aim to remove insoluble residual particles.

The solutions of NaCN–XTH mixtures were prepared by mixing the appropriate amounts of stock solutions and NaCl, with stirring at room temperature, during 30 min. pH of mixtures was adjusted to 7 with 0.1 M NaOH and 0.1 M HCl solutions.

Rheological properties

The rheological properties were assessed using a Paar Physica Rheolab MC1 rheometer (Germany), provided with US200 software for the control of the equipment and data analysis. The analyses were performed using a validated method of earlier study (Hadjsadok et al. 2010). A shear test was performed by measuring the shear stress (σ) over a shear rate ($\dot{\gamma}$) from 1 to 1000 s^{−1} at 20 °C.

Flow curves, expressed in terms of $\sigma = f(\dot{\gamma})$ have the characteristic shape of non-newtonian fluid with shear-thinning behavior. The Casson model, writing: $\sigma^{1/2} = \sigma_0^{1/2} + (\eta_c \dot{\gamma})^{1/2}$, seems to be the most appropriate to adjust them, with a determination coefficient R² close to 1 for all experiments. Its characteristic parameters are the yield stress, σ_0 , and the limiting viscosity of Casson, η_c . Measurements were done in triplicate for each mixture, and average values and standard deviations were calculated. The values of standard deviation of both parameters were negligible compared to the mean values of measurements.

Experimental design

In order to study the concentrations effect of NaCN, XTH and salt (C_{NaCN}, C_{XTH}, C_{NaCl}) on the rheological behavior

of solutions mixtures, the Response Surface Methodology (RSM) was applied, a fine modelization study based on a second-order polynomial model. The experimental design used for this purpose is a central composite face-centered design, CCF, containing 17 experiments, three experiments in the center of the design for assessing the reproducibility. The reduced centered variables X_1, X_2, X_3 of the design, are enclosed between -1 and $+1$ and associated respectively with C_{NaCN} : [1–12%, w/w], C_{XTH} : [0–0.5% w/w] and C_{NaCl} : [1–100 mM].

The main response, representing the rheological properties of the solution mixtures, is the yield stress, σ_0 of Casson model. The other characteristic parameter, namely the Casson limiting viscosity, η_c was not retained, due to the poor quality of the fit obtained by RSM. Table 1 shows the experimental matrix of the experiments carried out and the corresponding σ_0 values.

The modelization was performed using STATISTICA® software (Statsoftware, v.8.0).

Dynamic light scattering (DLS)

The size distribution of dispersions of NaCN, XTH and their mixture was measured by DLS with a Zetasizer (Nano-ZS, Malvern Instruments, Worcestershire, UK), according to the method of Hadsjadok et al. (2008). The measurement determined the apparent hydrodynamic radius, R_h (nm), associated with a diffusion angle $\theta = 90^\circ$. The laser beam is polarized vertically at a wavelength $\lambda = 532$ nm. The

electrical current intensity autocorrelation function, $g_1(t)$, is measured at $\theta = 90^\circ$ which related to the relaxation time value (τ) by the equation: $g_1(t) = e^{-t/\tau}$. The diffusion coefficient, D , is subsequently deduced by the relation: $D = 1/(\tau q^2)$ with $q = 2.22 \times 10^{-2} \text{ nm}^{-1}$ for $\theta = 90^\circ$. Finally, the hydrodynamic radius value, R_h , is obtained by using the Stokes–Einstein relation: $D = KT/(6\pi\eta R_h)$. K is the Boltzman constant and η is the viscosity of the dispersant phase which is in our case water at 20°C .

The analyzed solutions, NaCN solution (1 wt% NaCN, 100 mM NaCl), XTH solution (0.5 wt% XTH, 100 mM NaCl), and optimized mixture (1 wt% NaCN, 0.5 wt% XTH, 100 mM NaCl) were diluted to 1/100 in 100 mM NaCl solution, then filtered using a $0.45 \mu\text{m}$ Millipore® filter, in order to eliminate turbidity, which can distort the measurement.

Measurements were performed in triplicate.

Infra-red spectroscopy (FTIR)

The FTIR spectra of NaCN, XTH (powders), and lyophilized mixtures were carried out using a Perkin Elmer spectrum two spectrophotometer (Perkin Elmer, Spain, S.L., Madrid, Spain), equipped with an ATR (attenuated total reflectance) sampling device containing diamond/ZnSe crystal. Samples (powders) were placed on the surface of the ATR crystal without any previous preparation. Measurements were performed at room temperature, at spectral resolution of 2 cm^{-1} over a range of $4000\text{--}400 \text{ cm}^{-1}$, using 32 scans. All measurements were carried out in triplicate.

Table 1 Experimental matrix (factor levels associated to their responses)

No.	C_{NaCN} (%)	C_{XTH} (%)	C_{NaCl} (%)	Yield stress (σ_0 , Pa)
1.	1	0	1	0.278
2.	12	0	1	0.736
3.	1	0.5	1	0.315
4.	12	0.5	1	0.029
5.	1	0	100	0.609
6.	12	0	100	0.455
7.	1	0.5	100	3.426
8.	12	0.5	100	0.241
9.	1	0.25	50.5	1.224
10.	12	0.25	50.5	0.308
11.	6.5	0	50.5	0.341
12.	6.5	0.5	50.5	1.904
13.	6.5	0.25	1	0.564
14.	6.5	0.25	100	0.207
15.	6.5	0.25	50.5	0.645
16.	6.5	0.25	50.5	0.653
17.	6.5	0.25	50.5	0.675

Results and discussion

Surface response modelization

The coefficients of the quadratic polynomial model, giving the response surfaces σ_0 as function of the three reduced centered variables, were calculated using the partial least square method (PLS). The quality of regression, traduced in terms of the determination coefficient, R^2 , was evaluated at 0.85. Thus, we obtain:

$$\sigma_0 = 0.72 - 0.33X_1 + 0.29X_2 + 0.25X_3 + 0.24X_2^2 - 0.23X_3^2 - 0.32X_1X_2 - 0.29X_1X_3 + 0.27X_2X_3$$

Effects of C_{NaCN} and C_{XTH} on yield stress σ_0 at different C_{NaCl}

The effect of biopolymers concentrations on the yield stress was investigated, at salt concentrations ranging from

1 to 100 mM, in order to investigate the impact of mixture composition on their structure.

Case of $C_{NaCl} = 1$ mM

Figure 1a shows the evolution of the iso- σ_0 contours as function of the simultaneous variation of biopolymers concentrations, at salt content of 1 mM. We observe two main gradients explaining the variation of σ_0 : the first one (red color), oriented upwards in the positive direction of σ_0 , suggesting that the use of moderate amounts of NaCN (<5.5 wt%) ensure that xanthan retains its texturing properties, having an important influence on the yield stress. In this region, the two biopolymers develop synergistic interactions strengthening the structure of mixtures. Kobori et al. (2009) have studied solution mixtures containing low biopolymers concentration [NaCN (0.1%) and XTH (0.1%)] at neutral pH, they did not observe phase separation and explained the reinforcement of structure, by associative hydrophobic interactions between the two species.

The second gradient (blue color), oriented upwards in negative direction of σ_0 , denoting the effect of using high protein concentrations, which inhibits the texturing power of xanthan on the system. On the contrary, σ_0 will drop by the addition of the least amount of this polysaccharide and may even lead to phase separation. This situation has already been observed, in the case where the salt is almost absent, by Moulai-Mostefa et al. (2014), Hadjsadok et al. (2010) and Hemar et al. (2001). Hemar et al. (2001) have observed phase separations by microscopy study, and attributed this phenomenon to thermodynamic incompatibility between NaCN and XTH macromolecules.

Case of $C_{NaCl} = 40$ mM

Figure 1b shows the evolution of the iso- σ_0 contours, when the salt content is increased to 40 mM. We can observe the persistence of the first gradient (red color) which remains almost oriented upwards, in the positive direction of σ_0 and C_{XTH} . It is noted that this region is more extended, including lower concentrations of xanthan around 0.18%. Also, the values of σ_0 , ranged between 0.8 and 1.6 Pa, are higher compared to those of 1 mM NaCl, which vary between 0.3 and 0.8 Pa. This highlights the significant effect of salt on the enhancement of the attractive interactions between the two biopolymers. Hadjsadok et al. (2008) showed, by DLS, that in the absence of salt, the sodium caseinate monomers had a size of 3 nm. With the increase of salt concentration to 40 mM, these monomers were associated together to form micelles about 7 nm size. This may explain the increase in σ_0 values, by the association of xanthan with NaCN micelles.

The second gradient (blue color), oriented in the negative direction of σ_0 , appears to be more inclined

Fig. 1 Iso- σ_0 contours of mixtures in solution, as a function of the simultaneous variation of X_1 (C_{NaCN}) and X_2 (C_{XTH}). **a** $C_{NaCl} = 1$ mM and $T = 20$ °C, **b** $C_{NaCl} = 40$ mM and $T = 20$ °C, **c** $C_{NaCl} = 100$ mM and $T = 20$ °C

towards the horizontal axis of NaCN. This gradient shows that the reduction of the yield stress is mainly associated with the use of large amounts of caseinate, and to a lesser extent, with high concentrations of xanthan. Overall, the addition of 40 mM NaCl favors the increase of the yield stress of the mixtures and makes it possible to extend the range of variation in concentration of the two biopolymers.

Case of $C_{NaCl} = 100$ mM

Figure 1c shows the evolution of the iso- σ_0 contours, when the NaCl concentration is increased to 100 mM. At high protein concentrations (>5.5 wt%), the gradient of σ_0 is parallel the C_{NaCN} axis, and increase with its decrease. This suggests that, the addition of xanthan to highly concentrated NaCN solutions generates unfavorable interactions, due to thermodynamic incompatibility, within the structure of mixture, causing phase separation. However, at lower NaCN concentrations (<5.5 wt%), both xanthan and NaCN contribute to important increase of the yield stress, associated with gel formation.

Formula optimization

The optimal mixture studied in this work will serve as emulsion stabilizer, it should provide appropriate rheological properties, in order to ensure the desirable resistance to flocculation and other underlying destabilization mechanisms. So, the optimal formula corresponds to the NaCN–XTH mixture with the highest σ_0 value leading to formation of homogenous consistent gels. The value was determined by numerical simulation of the response σ_0 , using a polynomial model. Owing to the model a maximum value, $\sigma_0 = 3.1$ Pa, was determined, for the solution containing: $C_{NaCN} = 1\%$, $C_{XTH} = 0.5\%$ and $C_{NaCl} = 100$ mM.

The optimum mixture appears as homogeneous turbid gel. This can even due to the cohabitation of the two biopolymers in solution without interaction, or to their attractive interaction generating complexes. In order to determine the nature of interactions involved in this system, the DLS and FTIR characterizations were studied.

Dynamic light scattering (DLS)

Figure 2 shows the particle size distribution of solutions of NaCN (1%), XTH (0.5%) and their mixture at the same concentrations. These solutions were diluted to 1/100 in a saline medium containing 100 mM NaCl. The particle size distribution of protein is bimodal. The fast mode is associated with the small population of apparent hydrodynamic radius, R_{h1} of about 12 ± 1 nm. This value is in dimension range of sub-micelles, as determined by Chu et al. (1995), Nash et al. (2002) and Hadjsadok et al. (2008), around 8.8, 10 and 11 nm respectively. The slow mode, associated with large population, having an apparent hydrodynamic radius, $R_{h2} = 100 \pm 5$ nm. Hadjsadok et al. (2008) have found a value around 60 nm and related it to the residual fat in the solution. Chu et al. (1995), Nash et al. (2002) and Dickinson et al. (2001) observed a size around 74, 80 and 100 nm, respectively, which attributed to NaCN micelles.

Fig. 2 Particle size distribution of the diluted solutions to 1/100 of NaCN (1%), XTH (0.5%) and the mixture ($C_{NaCN} = 1\%$, $C_{XTH} = 0.5\%$, 100 mM NaCl). $T = 20$ °C

Xanthan has a bimodal distribution, a fast mode associated with a population of $R_{ha1} = 17 \pm 3$ nm and a slow mode associated with population of $R_{ha2} = 112 \pm 9$ nm. This second population is the result of the aggregation of xanthan macromolecules which are in semi-dilute regime (Oliveira et al. 2013).

The size distribution of mixture exhibits two populations, one at about 15 ± 3 nm and the second at around 140 ± 13 nm. The first have a comparable size to that of the rapid mode of the two biopolymers, thus it may be attributed either to the individual macromolecules of XTH or to the sub-micelles of NaCN, or both of them. The second population has a slightly greater size compared to those of NaCN or XTH aggregates. The absence of a new entity (new mode) in the solution suggests the absence of interactions between the two species due to dilution effects. Indeed, even if there was formation of complexes in the initial mixture, these complexes probably undergo a dissociation following the dilution to 1/100. This assumption was further investigated by FTIR analysis. Nash et al. (2002) support the hypothesis of the absence of interactions, through DLS characterizations, of low concentrations of protein and polysaccharide ($<0.08\%$ wt/wt).

FTIR spectra

Figure 3 shows the superposition of the FTIR spectra of the optimized mixture and of the two biopolymers. We note that the asymmetric stretching band of the CH_2 was shifted to 2926 nm compared to its position in the individual polymers. This indicates an interpenetration of the chains of the two macromolecules interacting non-electrostatically (Herrero et al. 2011; Arrieta et al. 2013; Lii et al. 2002).

Moreover, the relative band to the amine is slightly displaced (Table 2). Furthermore, we observed a displacement of the carbonyl band of the amide located at 1635 cm^{-1} in sodium caseinate and at 1642 cm^{-1} in the mixture, the adjacent band related to the same group has shifted to higher wave number. Probably non-polar interactions play an essential role in complex formation. Also, the stretching vibration of the CO groups in the glucosic units shifted by 5 cm^{-1} .

In the spectral range between 4000 and 2000 cm^{-1} , the subtraction of the caseinate spectrum from that of the mixture (Fig. 4) revealed the envelope band of the complex. The interaction involves the hydroxyl band of the xanthan at 3376 cm^{-1} , and the amine band at 3280 cm^{-1} . These bands in the pure constituents are located respectively at 3341 and 3285 cm^{-1} . This proves that the spectrum of the mixture does not result from a simple addition of the respective spectra of the pure biopolymers. It is therefore concluded that complexes are formed through hydrophobic interactions.

Conclusion

Aqueous mixtures of sodium caseinate and xanthan were studied in the presence of salt (NaCl) at neutral pH. The objective was to determinate the optimum conditions of soluble complexes formation, using experimental design method. The rheological analysis of the mixtures showed a shear thinning behavior, according to Casson model. The exploration of RSM model of the Casson Yield stress, σ_0 , allows to establish the iso-response contours, and to determine the regions in which gels are obtained without phase separation. FTIR analysis of the optimized gel

Fig. 3 FTIR spectra of: **a** NaCN, **b** optimum mixture, **c** Xanthan

Table 2 FTIR spectral patterns of NaCN, XTH and optimum mixture and band assignments

$\nu\text{ (cm}^{-1}\text{)}$			
Complex	XTH	NaCN	Band assignment
	3341		ν_{OH} intramolecular Hydrogene bond
3282		3285	ν_{NH} intramolecular Hydrogene bond
2926	2935	2959	ν_{CH_2} asymmetric stretching
2874	2884	2874	ν_{CH_2} symmetric stretching
	1717		ν_{COOH}
	1600		$\nu_{\text{C=O}}$, δ_{OH}
1642		1635	$\nu_{\text{C=O}}$, δ_{OH}
1536		1517	$\nu_{\text{C=N}}$, δ_{NH}
	1370		ν_{COO^-}
1312		1312	δ_{NH}
	1251		δ_{CH}
1155	1150		ν_{CO} (glucose unit)
1060		1023	ν_{CO} , $\nu_{\text{C-C}}$, δ_{COOH}

Bold values indicate main groups involved in hydrophobic interactions

Fig. 4 FTIR differential spectrum of NaCN from that of complex

formula revealed the formation of complexes and the hydrophobic nature of the involved interactions. DLS investigation allowed us to conclude that these complexes are reversible and dissociate by dilution.

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