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Stabilization of Whey Protein Isolate−Pectin Complexes by Heat

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Protein−polysaccharide complexes formed under electrostatic associative conditions could have interesting functional properties. However, their stability over a wide range of pH limits their widespread application. The aim of this work was to determine the time−temperature combination required to stabilize whey protein isolate (WPI) and pectin (LMP) complexes formed at pH 4.5 to a further pH adjustment to 7. The effect of storage for 28 days at 4 °C was also evaluated. Stability was confirmed by quantification of sugar and nitrogen in each phase after centrifugation. Three heat treatments were performed: 73 °C/5 min, 85 °C/15 min, and 90 °C/2 min. At 73 °C/5 min, adjustment to neutral pH led to disruption of WPI−LMP complexes. The most severe heating conditions (85 and 90 °C) allowed the stabilization of WPI−LMP complexes. Heated complexes (90 °C) could be preserved for up to 28 days of storage at 4 °C without affecting their stability.

KEYWORDS: Pectin; whey protein isolate; electrostatic complexes; heat treatment

INTRODUCTION

For many years, proteins have received great attention because of their key role in terms of the texture and structure of food. In addition, they are well recognized for their high nutritional value. Polysaccharides added to food systems affect functional properties (water retention, viscosity, gel-forming properties). Controlling the interactions between these two macromolecules is critical to developing new, high-potential ingredients with desirable functional properties. Under specific conditions, protein and polysaccharide are capable of forming electrostatic complexes (*1*−*5*). This association may result in an improvement of functional properties as compared with those of individual macromolecules (*2*, *4*, *6*). The electrostatic association between protein and polysaccharide depends on many factors, such as protein−polysaccharide ratio, ionic strength, pH, temperature, total solids, and charge density (*1*, *2*, *7*). This attraction involves positively charged local patches of proteins (mainly $-NH_3^+$ groups) interacting with the negative charges carried by carboxyl groups of polysaccharides. These macromolecules are linked together mainly by electrostatic interactions, and other types of interactions such as hydrogen bonds and hydrophobic interactions can also be involved. These interactions are strongly affected by pH and ionic strength of solvent (*1*, *3*, *5*). The stability of complexes is also largely dependent on structural characteristics such as size and conformation, nature of the interactions involved, and the number of interacting groups (*5*). Soluble protein−polysaccharide complexes generally occur when biopolymers carry opposite charges, i.e., at pH below or next to the isoelectric point (IEP) of proteins (*3*, *8*). Insoluble complexes can be formed below the IEP (*1*, *3*, *8*).

Some food applications of protein−polysaccharide complexes have been documented, for example, whey protein isolate and xanthan gum complexes as fat substitutes (*9*). Schmitt et al. (*10*) have developed whey protein isolate and gum arabic complexes. The improvement of foaming properties relative to those of individual biopolymers has justified their application in frozen sorbet (*10*). The widespread utilization of electrostatic complexes is limited due to the nature of interactions that are highly sensitive to pH and ionic strength (*11*, *12*). These types of complexes can be dissociated by changing the pH to 7. Consequently, the potential use of complexes to modulate the functional properties of food systems is limited because most food products have a neutral pH. The stability of complexes at higher ionic strength and pH can be achieved by partial or complete unfolding of protein upon heating (*5*, *13*, *14*). During the unfolding process, the protein structure will expose some charged groups, which are free for further reactions, and the lateral chain will remain more flexible. This improvement of flexibility will help to maximize interactions (*3*). The number of linkages, like hydrogen bonds and hydrophobic interactions, will also increase (*5*). Few studies have investigated the impact of heat treatment on the resulting interactions between complexed biopolymers (*6*, *15*−*17*). Heating protein−polysaccharide complexes above the denaturation temperature of the protein was sufficient to stabilize them to further pH adjustments between 3 and 7. For complex formation, all of these studies used β-lactoglobulin as the protein source at low concentration ($< 0.5\%$). Previous work in our laboratory (unpublished results) has shown that concentration is a critical parameter

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to consider in developing a process to stabilize protein−polysaccharide complexes by heat. For similar protein−polysaccharide ratios, increasing protein content modified their behavior by favoring protein−protein interactions. Under these conditions, it is more difficult to obtain stable complexes.

In North America, polysaccharides are often added as stabilizing agents in yogurt formulation to reduce technological problems such as syneresis (*18*). However, as these technological problems are not fully resolved, we propose the use of stabilized protein−polysaccharide complexes as functional ingredients in yogurt. The potential use of electrostatic complexes is justified because the formation of complexes between protein and polysaccharide may improve functional properties as compared to those of the individual macromolecules. The application of protein−polysaccharide complexes in yogurt has never been studied. However, before using electrostatic complexes in various food products, they have to remain stable at pH 7. Their addition in yogurt would likely be done at the standardization step in milk (pH 6.7−6.8).

Whey protein isolate (WPI)−low methoxyl pectin (LMP) complexes were previously developed by our team (*19*). WPI−LMP complexes were formed between pH 5 and pH 6. Preliminary results have shown that WPI−LMP complexes formed at pH 5 were dissociated after heat treatment at 90 °C for 2 min (*20*). During the heat treatment, in WPI−LMP complexes formed at pH 5, protein−protein bonds were favored to the detriment of protein−polysaccharide interactions. At this pH, close to the IEP of whey protein (5.2−5.4), repulsive electrostatic protein−protein interactions are minimized, allowing protein aggregation. Below the IEP of whey protein (pH 4.5), the solubility could be improved because proteins carry positive charges, resulting in maximum electrostatic interactions with the anionic polysaccharide (*21*). Protein−polysaccharide interactions would be favored over protein−protein interactions upon heating. The purpose of this work was to investigate the effect of heat treatment on the stability of WPI−LMP complexes formed at pH 4.5 (below the IEP of the proteins) upon pH adjustment to 7. The effect of storage on the physical and microbiological stability of the WPI−LMP complexes was also determined in order to facilitate their further utilization as stabilizing agents in yogurt formulation.

MATERIALS AND METHODS

Materials. Low methoxyl pectin (LMP) powder was obtained from CPKelco (Lille Skensved, Denmark). This polysaccharide, with 30% esterification and 19% amidated groups, was extracted from citrus peel. LMP powder contained 3% nitrogen. Whey protein isolate (WPI) was provided by Davisco Foods International (Bipro, Le Sieur, MN). The WPI was constituted of 98% total solid, 90% protein, and 2% ash. NaOH (USP grade), lactic acid (food grade), and microbiological media (BD Difco) were purchased from Fisher Scientific (Ottawa, Canada). Sodium azide was obtained from Sigma Chemical Co. (St. Louis, MO).

Complex Formation. A solution containing 1.5% (w/w) LMP powder was prepared in distilled water under magnetic agitation. The LMP solution was heated at 85 °C for 1 min to ensure complete dissolution. In parallel, a solution containing 16% (w/w) WPI was prepared in distilled water. The WPI solution was gently mixed under magnetic stirring for 2 h at room temperature. Both solutions were stored overnight at 4 °C under gentle magnetic agitation. After storage, solutions were used at room temperature. The pH of the LMP solution was first adjusted to 5.0 ± 0.05 with 0.5 N NaOH, and then 0.1 N NaOH was used to increase the pH to 5.5. The WPI solution was adjusted to pH 6.5 with 10% (v/v) lactic acid. The WPI and LMP solutions were weighed and mixed together in distilled water to reach a final concentration of 2% WPI and 0.5% pectin (ratio of 4:1). The mixture was left at room temperature for 2 h under gentle magnetic agitation. Complex formation (WPI−LMP complexes) was achieved by acidifying mixtures to pH 4.5 with lactic acid (10%) and leaving them at room temperature for 2 h under gentle magnetic agitation. One part of the WPI−LMP complexes was immediately stored at 4 °C under magnetic agitation until used (COM25). Another part of the WPI−LMP complexes was adjusted to pH 7 (dissociated complexes) with 0.5 N NaOH and then stored at 4 °C under magnetic agitation. The remainder was heat treated as described in the following section.

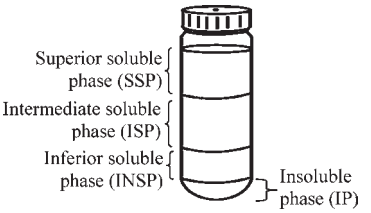

Figure 1. Schematic representation of each phase in WPI−LMP complexes formed at pH 4.5 (COM25) after centrifugation.

Heat Treatment. In an attempt to stabilize WPI−LMP complexes, three heat treatments were tested: 73 °C/5 min (COM73), 85 °C/15 min (COM85), and 90 °C/2 min (COM90). The last two heat treatments should allow irreversible unfolding of whey proteins (*22*). The same heat treatments were applied to 2% WPI solutions (WPI73, WPI85, and WPI90) and 0.5% LMP solutions (LMP73, LMP85, and LMP90). Heat treatments were performed in a water bath, and the temperature in the sample was monitored with a thermocouple. The temperature of water was adjusted to 3 °C above the targeted temperature. For instance, to reach 90 °C, the temperature of the water was adjusted to 93 °C. It took 5 min for samples to reach the desired temperature. After heating, the solutions were cooled to room temperature by immersion in an ice bath (15 min). COM25, the unheated WPI−LMP complexes, was used as control.

Stability Test. The stability of WPI−LMP complexes after heat treatment and pH adjustment to 7 was ascertained by centrifugation techniques. This method was based on the distribution of whey proteins (expressed as total nitrogen (TN) content) and pectin (expressed as total sugar (TS) content) in the various phases obtained after centrifugation at 42700*g* for 1 h 30 min and at 25 °C (Beckman Coulter, Model J2-21, Mississauga, Canada). The stability of the WPI−LMP complexes was evaluated by measuring the weight of each phase, quantifying TN and TS, and calculating the TS to TN ratio in each phase. Centrifugation conditions were chosen to ensure separation of complexes at the bottom of the tube, with noninteracting protein and polysaccharide remaining in the supernatant. For instance, centrifugation of the control condition (COM25 at pH 4.5) produced four phases from top to bottom of the tube: a superior soluble phase (SSP), an intermediate soluble phase (ISP), an inferior soluble phase (INSP), and an insoluble phase (IP), as shown in **Figure 1**. Phases were carefully separated with a disposable 5 mL pipet and then weighed. Mixtures were centrifuged in duplicate, and each corresponding phase was mixed together to ensure sufficient volume for further analysis. TS and TN contents were quantified in each phase and also in the uncentrifuged WPI−LMP complexes. The concentration of TS and TN in the IP phase (pellet) was obtained by difference. The theoretical TS to TN ratio for WPI (2%)−LMP (0.5%) complexes was 1.6 (0.486% TS and 0.303% TN). We hypothesized that stable WPI−LMP complexes (presence) after heat treatment and pH adjustment to 7 should have the same TS to TN ratio as the control (COM25 at pH 4.5) in the INSP and IP. The assumption is that the macromolecular complexes formed by WPI and LMP interactions should be larger than native WPI and LMP (without complex formation) and, consequently, the WPI−LMP complexes should be found in the INSP and IP after centrifugation.

Effect of Storage. The effect of storage on the stability of WPI−LMP complexes was studied to facilitate their utilization as potential new stabilizing agents in yogurt at the laboratory scale and for their further use in the food industry. The stability of WPI−LMP complexes, as described above, the evolution of total mesophilic aerobic flora, and the pH were measured at 1, 14, and 28 days of storage. The enumeration of the total mesophilic aerobic flora was performed to evaluate the microbiological quality of this ingredient during storage. Four mixtures were studied: COM25 at pH 4.5, COM25 at pH 4.5 with the addition of 0.01% (w/w) sodium azide as preservative, COM90 at pH 4.5, and COM90 adjusted to pH 7 after the heat treatment.

Table 1. Weight Proportion of Each Phase after Centrifugation of WPI−LMP Complexes (COM) and WPI or LMP Solutions at pH 4.5 or pH Adjusted to 7 after Heat Treatment

		weight proportion[b] (%)			
mixture[a]	pH	SSP	ISP	INSP	IP
COM25	4.5	34.7 i	32.7 b	20.3 b	12.0 b
	7	98.8 a	NP[c]	NP	1.17 g
COM73	4.5	88.4 d	NP	NP	11.6 b
	7	27.7 j	70.8 a	NP	1.52 g
COM85	4.5	59.5 f	22.3 c	8.2 c	10.1 c
	7	39.8 h	22.0 c	21.7 b	16.5 a
COM90	4.5	65.6 e	14.0 d	10.6 c	9.75 c
	7	45.3 g	NP	52.0 a	2.77 f
WPI25	4.5	98.8 a	NP	NP	1.21 g
	7	97.2 ab	NP	NP	2.75 f
WPI73	4.5	96.7 ab	NP	NP	3.27 f
	7	97.3 ab	NP	NP	2.73 f
WPI85	4.5	92.7 c	NP	NP	7.30 d
	7	93.6 bc	NP	NP	6.39 e
WPI90	4.5	92.9 c	NP	NP	7.09 d
	7	96.9 ab	NP	NP	3.08 f
LMP25	4.5	98.9 a	NP	NP	1.09 g
	7	96.7 ab	NP	NP	3.28 f
LMP73	4.5	98.6 a	NP	NP	1.37 g
	7	98.8 a	NP	NP	1.20 g
LMP85	4.5	99.0 a	NP	NP	0.98 g
	7	98.7 a	NP	NP	1.26 g
LMP90	4.5	99.0 a	NP	NP	1.01 g
	7	97.2 ab	NP	NP	2.79 f

[a] COM, WPI, LMP = the number refers to the heat treatment: 25 (no heat treatment), 73 (73 °C/5 min), 85 (85 °C/15 min), and 90 (90 °C/2 min). [b] SSP = superior soluble phase; ISP = intermediate soluble phase; INSP = inferior soluble phase; IP = insoluble phase. [c] NP = no phase was observed. a−j = means with the same superscript in the same column did not differ at $P < 0.05$.

All solutions were maintained at 4 °C under constant magnetic agitation for 28 days.

Analytical Methods. Nitrogen was quantified by the Kjeldahl method (*23*). Sugar was determined by the colorimetric method of Dubois et al. (*24*) at 490 nm using an EL-808 spectrometer from Bio-Tek Instruments Inc. (Winooski, VT). TN and TS contents (expressed as percentages) in each phase were calculated as a function of respective weight phase. Because of the presence of lactose in WPI, whey proteins contributed to 0.016% of TS in the WPI−LMP complexes, whereas pectin contributed to 0.470% of TS. Consequently, to take into account the trace lactose in WPI and the possible interference by protein, a solution containing 0.5% pectin and 2% WPI was used as a standard in TS content determination. During storage, pH was measured by the official standard methods (*23*). The evolution of mesophilic aerobic flora was enumerated on plate count agar and incubated at 30 °C for 48 h under aerobic conditions.

Statistical Methods. The impact of heat treatment on the stability of WPI−LMP complexes was analyzed according to a complete factorial design. For the effect of storage on the stability of WPI−LMP complexes, a split-plot factorial design was applied. Time factor was the subplot. Significant differences were tested at $P < 0.05$. Statistical analysis was carried out with the general linear models procedure of SAS (version 9.1.3, Cary, NC). All measurements were made in triplicate.

RESULTS

Weight Proportions of Phases Obtained after Centrifugation. The weight proportion of each phase obtained after centrifugation is given in **Table 1**. A significant interaction ($P < 0.05$) between type of mixture (COM and WPI or LMP solutions), heat treatment, and pH was observed. For COM25 at pH 4.5, four phases were observed. The SSP and ISP represented almost 68% of total weight. These phases were colorless (the ISP was more turbid than the SSP). The INSP was cloudy and white in color, whereas the IP was white and in a semisolid state at the bottom of the tube. The proportions of the INSP and the IP were 20% and 12%, respectively. Adjusting the pH of COM25 to 7 produced a limpid homogeneous phase (SSP) that represented 99% of the weight. A small insoluble part (1%) was found at the bottom. For COM73 at pH 4.5, two phases were observed: a predominant SSP (84%) and an IP in a proportion similar to that of COM25 at pH 4.5. In addition, the SSP was similar in appearance to the same phase of COM25 at pH 4.5. Three phases were observed in COM73 at pH 7: an SSP, an ISP, and an IP representing 27.7%, 70.8%, and 1.5% of the weight, respectively. For COM85 and COM90 at pH 4.5, four phases were observed after centrifugation, with similar proportions. Dissimilarities between unheated and heated complexes were observed with respect to weight percentages. For instance, the SSP proportion for pH 4.5 was smaller in COM25 than in COM85 and COM90, whereas for the ISP, INSP, and IP, weights were higher in COM25. For COM85 adjusted to pH 7, four phases were still observed, but in comparison with COM85 at pH 4.5, the SSP decreased, the ISP remained stable, and the INSP and IP increased. For COM90 adjusted to pH 7, only three phases were observed. The SSP and IP weight proportions were smaller and the INSP weight proportion was higher than the corresponding phases observed for COM90 at pH 4.5. No ISP was observed.

Only two phases (SSP and IP) were observed for WPI and LMP adjusted to pH 4.5 or 7, irrespective of the heat treatment used. The SSP was the main phase, accounting for more than 92%. For WPI at pH 4.5, the proportion of the SSP decreased significantly with the severity of heat treatment, dropping from 99% for WPI25 to 93% for WPI90. For WPI at pH 7, the proportion of the SSP decreased slightly with the severity of heat treatment. An opposite trend was observed for the IP. In the case of LMP, pH and heat treatments did not affect the proportion of the SSP or the IP.

Nitrogen and Sugar Contents of Phases Obtained after Centrifugation. Nitrogen and sugar distributions in each phase after centrifugation of WPI solutions and WPI−LMP complexes are presented in **Figures 2** and **3**, respectively. Nitrogen and sugar proportions (%) were expressed as the quantity of total nitrogen (TN) or total sugar (TS) as a function of respective weight phase. Nitrogen and sugar quantified in the different phases were significantly affected ($P < 0.05$) by the interactions between the type of mixture, the pH, and the heat treatment. At pH 4.5, the majority of nitrogen was in the SSP in untreated WPI25 (**Figure 2a**). As the heat treatment increased (WPI73, WPI85, and WPI90), nitrogen passed gradually into the IP (**Figure 2b−d**). The adjustment of pH to 7 did not modify the distribution of nitrogen. Similar nitrogen profiles were observed for pH values of 4.5 and 7 (**Figure 2a−d**). Because the concentration of lactose in WPI powder was very low (<4%), the distribution of sugar in WPI solutions was not presented.

When pectin was added to WPI to form WPI−LMP complexes, the distribution of nitrogen was found in four phases after centrifugation (**Figure 3a−d**) and differed from the distribution obtained for WPI (**Figure 2a−d**). In untreated COM25 at pH 4.5, the majority of nitrogen was found in the INSP and especially in the IP. After the pH value was adjusted to 7, all of the nitrogen content came back in the SSP (**Figure 3a**). Similar observations were made for the distribution of sugar in COM25 at pH 4.5 and 7 (**Figure 3e**).

In COM73 at pH 4.5, the majority of nitrogen was found in the IP (**Figure 3b**). In contrast to COM25 (**Figure 3a**), after adjustment of the pH to 7, the nitrogen content was found in the two superior phases (SSP and ISP) (**Figure 3b**). Similar observations were also made for the distribution of sugar in COM73 at pH 4.5 and 7 (**Figure 3f**).

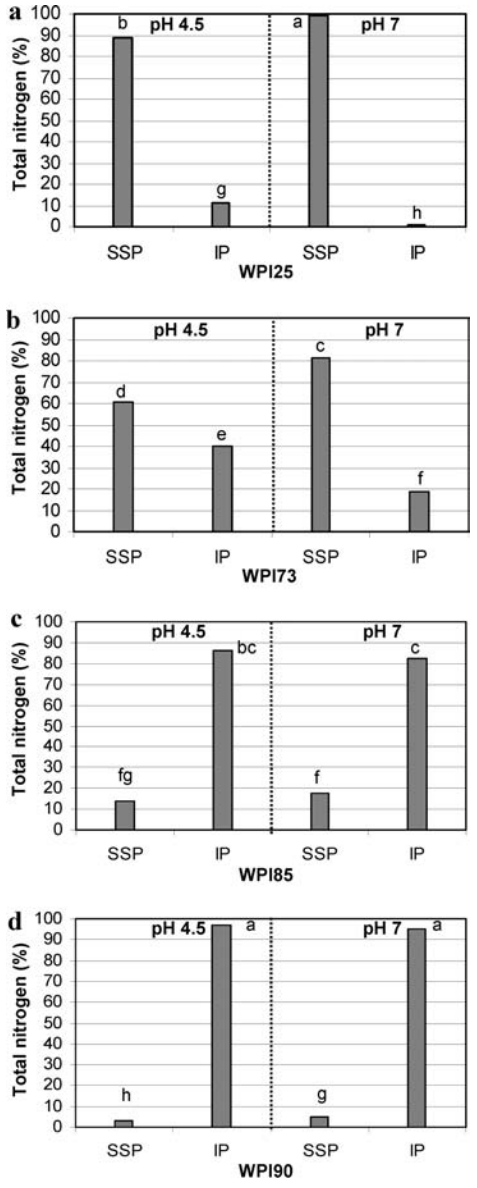

Figure 2. Distribution of nitrogen (%) in superior soluble phase (SSP) and insoluble phase (IP) after centrifugation in WPI solutions at pH 4.5 or pH adjusted to 7 after heat treatment: (a) unheated (WPI25), (b) 73 °C/5 min (WPI73), (c) 85 °C/15 min (WPI85) and (d) 90 °C/2 min (WPI90). Bar charts with the same superscript did not differ at $P < 0.05$.

In COM85 (**Figure 3c**), the distribution of nitrogen differed from that observed for COM25 (**Figure 3a**) and COM73 (**Figure 3b**). At pH 4.5, the majority of nitrogen was also found in the IP, but after adjustment of the pH to 7, the nitrogen still remained in the IP (**Figure 3c**). Sugar distribution in COM85 (**Figure 3g**) also differed from the distribution of nitrogen (**Figure 3c**). At pH 4.5, sugar was mainly found in the SSP and especially in the IP. However, after pH adjustment to 7, the sugar content was mainly in the SSP.

In COM90 (**Figure 3d**), the distribution of nitrogen at pH 4.5 was similar to that observed in COM85 (**Figure 3c**), and the majority of the nitrogen was found in the IP. However, unlike the other complexes, after pH adjustment to 7, the distribution of nitrogen moved to the two inferior phases (INSP and IP). Sugar distribution (**Figure 3h**) differed from nitrogen distribution (**Figure 3d**). However, sugar distribution in COM90 at pH 4.5 was similar to that observed in COM85 (**Figure 3g**), with most being found in the SSP and the IP. After adjustment of the pH to 7, the sugar was mainly found in the SSP and the INSP. This differed from COM25, COM73, and COM85.

The ratios of TS to TN in the various phases obtained after centrifugation are presented in **Table 2**. A significant interaction ($P < 0.05$) between pH and heat treatment was observed. As stated before, WPI−LMP complexes should be found in the INSP and the IP after centrifugation. In addition, the theoretical TS to TN ratio in these phases should be 1.6 (0.486 g of TS and 0.303 g of TN). In COM25 at pH 4.5, the ratio values for the SSP and the ISP were 3.5 and 3.1, respectively. In the INSP and the IP, the TS to TN ratios were close to the targeted value (1.6). For this condition, complexes seemed to be in both bottom phases. In the INSP in COM85 and COM90 at pH 4.5, the TS to TN ratios were slightly lower than the targeted value: 1.3 as compared to 1.6. Application of heat treatment for complexes at pH 4.5 tended to slightly decrease the TS to TN ratio values in the IP relative to the value for COM25 at pH 4.5, except in the case of COM73 at pH 4.5. When the pH of COM25 was adjusted to 7, the TS to TN ratio, in the SSP, was 1.6. However, in the IP, the TS to TN ratio increased to 4.1. A similar profile was found for COM73 adjusted to pH 7: the TS to TN ratios, in the SSP and the ISP, were close to the targeted value. The TS to TN ratios, in the SSP and the ISP, for COM85 and COM90 at pH 4.5 increased relative to the value for COM25 at the same pH. For COM90 at pH 7, the ratio value in the INSP was similar to that at pH 4.5. In the IP, TS to TN ratios for COM90 were not significantly different either at pH 4.5 or after adjustment to pH 7.

Effect of Storage. Evolution of mesophilic aerobic flora and TS to TN ratios in the different WPI−LMP complexes (COM25 at pH 4.5, COM25 at pH 4.5 + sodium azide, COM90 at pH 4.5, and COM90 at pH 7) stored during 28 days at 4 °C is presented in **Table 3**. A significant interaction ($P < 0.05$) with respect to bacterial count was observed between the type of WPI−LMP complexes and time. The bacterial population of COM25 at pH 4.5 gradually increased from 2.39 to 6.35 ($\log \mathrm{CFU \cdot mL^{-1}}$) during storage, whereas in COM25 at pH 4.5 + sodium azide and in COM90 at pH 4.5, no growth was observed. For COM90 at pH 7, a bacterial growth was observed after 21 days of storage. The addition of chemical preservative or a heat treatment at 90 °C for 2 min (COM90) was sufficient to control the growth of undesirable microorganisms. Although no significant difference was observed for pH, in COM25 at pH 4.5, the pH tended to increase slowly during the storage (data not shown). For the other WPI−LMP complexes, the pH did not change over time. The effect of storage on the stability of unheated and heated WPI−LMP complexes is also presented in **Table 3**. The TS to TN ratios of the INSP and the IP only are presented. Prior results showed that theses phases allowed the determination of the stability of complexes. During storage, the stability of WPI−LMP complexes was not affected.

DISCUSSION

This study has shown that the combination of WPI and LMP solutions at pH 4.5 allowed the formation of soluble complexes (no visible sedimentation at the bottom of the beaker after 16 h of storage at 4 °C). On the other hand, for further utilization as a potential new stabilizing agent in yogurt, the WPI−LMP complexes must be stable at pH 7. The method used to estimate the stability of WPI−LMP complexes was the quantification of TS to TN ratio in the different phases obtained after centrifugation. In uncentrifuged WPI−LMP complexes, the TS to TN ratio is 1.6. The interactions between protein and polysaccharide should lead to the formation of larger macromolecular entities (complexes), which should be found at the bottom of the centrifuge tube (INSP

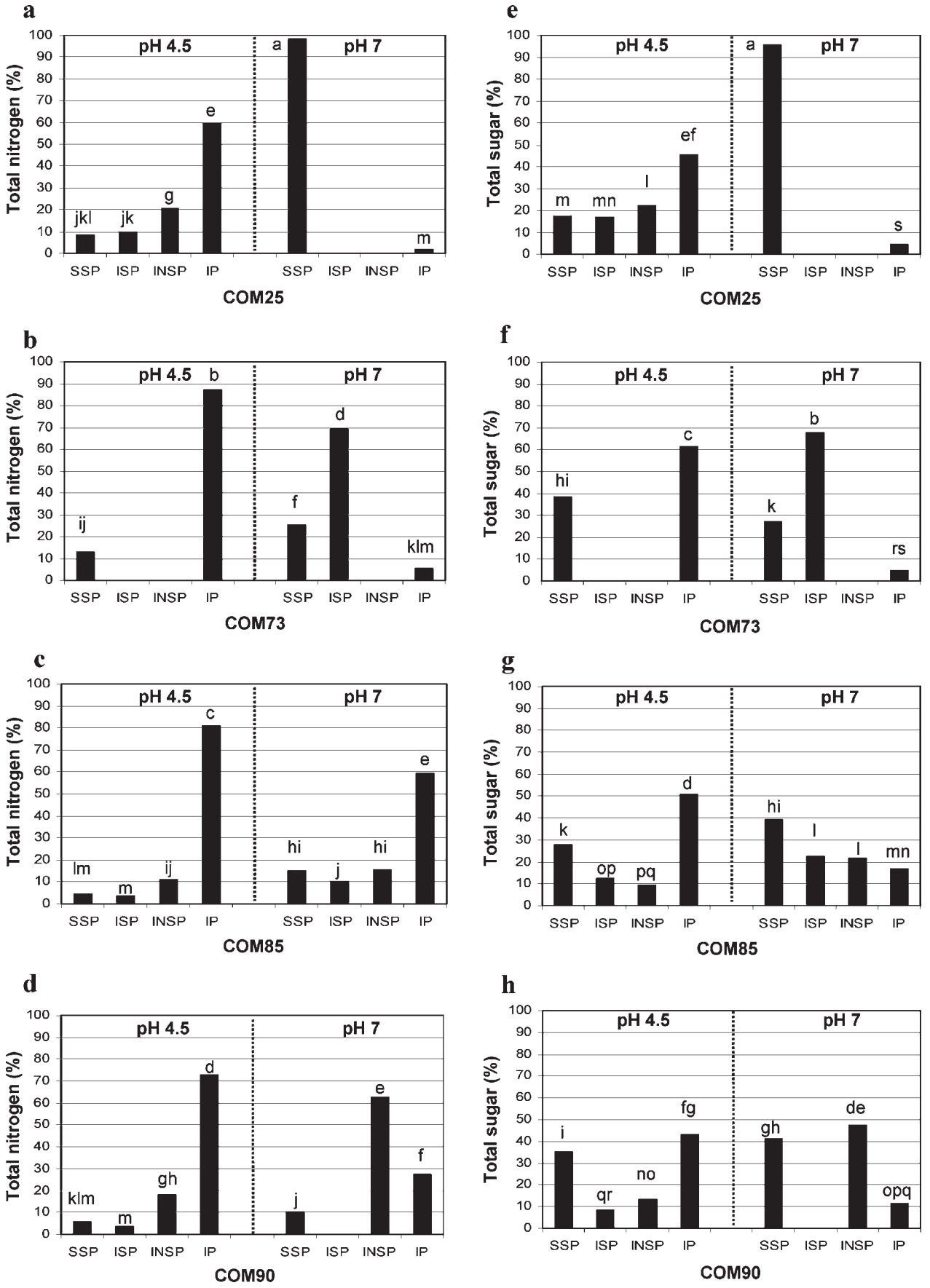

Figure 3. Distribution of nitrogen (%) and sugar (%) in each phase after centrifugation of WPI-LMP complexes at pH 4.5 or pH adjusted to 7 after heat treatment: (a, e) unheated (COM25), (b, f) 73 °C/5 min (COM73), (c, f) 85 °C/15 min (COM85) (d, h) 90 °C/2 min (COM90). Missing columns indicate that no phase was observed. Bar charts with the same superscript did not differ at $P < 0.05$.

Table 2. Total Sugar to Total Nitrogen Ratio for Each Phase after Centrifugation of WPI−LMP Complexes (COM) at pH 4.5 or pH Adjusted to 7 after Heat Treatment

		total sugar to total nitrogen ratio[b]			
mixture[a]	pH	SSP	ISP	INSP	IP
COM25	4.5	3.5 d	3.1 cd	2.1 b	1.3 c
	7	1.6 d	NP[c]	NP	4.1 a
COM73	4.5	5.4 c	NP	NP	1.2 cd
	7	1.9 e	1.7 d	NP	1.7 b
COM85	4.5	9.5 a	5.3 a	1.3 c	1.0 de
	7	4.4 cd	3.7 bc	2.4 a	0.5 f
COM90	4.5	10.7 a	4.0 b	1.3 c	1.0 de
	7	7.1 b	NP	1.3 c	0.7 ef

[a] COM = the number refers to the heat treatment: 25 (no heat treatment), 73 (73 °C/5 min), 85 (85 °C/15 min), and 90 (90 °C/2 min). [b] SSP = superior soluble phase; ISP = intermediate soluble phase; INSP = inferior soluble phase; IP = insoluble phase. [c] NP = no phase was observed. a−f = means with the same superscript in the same column did not differ at $P < 0.05$.

Table 3. Evolution of Mesophilic Aerobic Flora (log CFU·mL^{-1}) and Total Sugar to Total Nitrogen Ratio in the Inferior Soluble Phase (INSP) and the Insoluble Phase (IP) of WPI−LMP Complexes during 28 Days of Storage at 4 °C[a]

			total sugar to total nitrogen ratio	
WPI−LMP complexes	time (days)	aerobic flora	INSP	IP
COM25 at pH 4.5	1	2.39 bc	1.6 a	1.1 a
	14	3.62 b	1.5 a	1.3 a
	28	6.35 a	1.5 a	1.1 a
COM25 at pH 4.5 + sodium azide	1	1.16 cd	1.4 a	1.1 a
	14	0.00 d	1.1 a	1.1 a
	28	0.60 d	1.3 a	1.2 a
COM90 at pH 4.5	1	0.71 d	1.4 a	0.8 a
	14	0.23 d	1.4 a	1.0 a
	28	0.00 d	1.4 a	0.9 a
COM90 at pH 7	1	0.57 d	1.5 a	0.5 a
	14	0.00 d	1.4 a	0.6 a
	28	2.53 bc	1.3 a	0.5 a

[a] a−d = means with the same superscript in the same column did not differ at $P < 0.05$.

and IP) after centrifugation. Moreover, this hypothesis is supported by the fact that below the IEP of proteins, protein−polysaccharide complexes are less soluble (*1*, *3*, *5*). Some impurities and denatured proteins can also be found in phases at the bottom of the tube. Free LMP or WPI molecules that were not involved in complex formation or that came from dissociated complexes should be found in the upper soluble phases at the top of the centrifuge tube (SSP and ISP). In unheated WPI−LMP complexes (COM25) at pH 4.5, complexes were at the bottom of the tube (INSP and IP) since the ratios in these phases are close to the targeted value (1.6). However, the unheated WPI−LMP complexes were not stable after pH adjustment to 7, and more than 95% of the nitrogen and the sugar were solubilized and found in the top of the tube (SSP). Although the TS to TN ratio was 1.6, the presence of one soluble phase indicated that WPI−LMP complexes were dissociated. This result is supported by the literature, which reports the existence of electrostatic interactions between pectin and β-lactoglobulin, the primary driving force of complex formation, between pH 4.5 and pH 6 (*19*, *25*). In this pH range, proteins carry positive charges that allow electrostatic interactions with the anionic pectin. As the pH changes to 6, the whey protein net global charge becomes progressively negative, resulting in repulsive interactions between protein and polysaccharide.

In COM73 at pH 4.5, some interactions between WPI and LMP resisted to heat treatment as the major part of sugar and nitrogen were observed in the IP. The TS to TN ratio was similar to the targeted one. However, when the pH was adjusted to 7, sugar and nitrogen shifted into the SSP and the ISP as observed for COM25. These results could be likely explained by the reversible thermal denaturation of whey proteins at this temperature (*22*). As postulated before, heat treatment could be a way to stabilize WPI−LMP complexes. This treatment will expose new charged groups on the lateral chain of proteins and, then, could help to maximize the interactions between WPI and LMP. A reversible thermal denaturation of proteins means that proteins keep their native state after cooling. Similar results were obtained by Kazmierski et al. (*6*). They have observed that a larger amount of pectin was found in the supernatant after adjusting the pH to 6 of their modified high methoxyl pectin and β-lactoglobulin complexes formed at pH 3.8 and previously heated at 65 °C/20 min. They have also shown by size exclusion that the peak of elution of unheated complexes at 3.8 and after heating at 65 °C/20 min disappeared after adjusting the pH to 6. Therefore, the results showed that a heat treatment at 73 °C (COM73) did not permit stabilization of WPI−LMP complexes at pH 7.

When WPI−LMP complexes at pH 4.5 were heated to temperatures above 73 °C (COM85 and COM90) and a further adjustment was made to bring the pH to 7, most of sugar and nitrogen was found in the INSP and the IP. In addition, the TS to TN ratios in these phases were close to the targeted ratio of 1.6, indicating that WPI−LMP complexes remained stable. Jones et al. (*16*) have demonstrated that heating beet pectin and β-lactoglobulin complexes formed at pH 5 at 83 °C for 15 min was sufficient to stabilize these complexes over pH 3−7. As mentioned before, preliminary results have shown that heating WPI−LMP complexes formed at pH 5 at 90 °C for 2 min led to the dissociation of the complexes (*20*). This difference can be attributed to the pectin used for complex formation. Beet pectin has a higher degree of esterification and contains more protein segments in its backbone than citrus pectin (*16*). These results underlined the importance not only of the pH in complex formation prior to heat treatment but also of the type of biopolymers used as observed by Jones et al. (*15*). Kazmierski et al. (*6*) have also shown that heat treatment at 90 °C for 20 min is sufficient to stabilize modified high methoxyl pectin and β-lactoglobulin complexes after pH adjustment to 6. Jones et al. (*17*) have proposed a mechanism for heat stabilization of protein−polysaccharide complexes. The protein unfolding process in response to heat could lead to the formation of a network consisting of aggregated proteins coated with pectin. Kazmierski et al. (*6*) have also demonstrated that thermal treatment (90 °C) increases protein−protein interactions. Our results tended to endorse this mechanism given that thermal and pH conditions had an effect on the distribution of nitrogen in WPI solutions and WPI−LMP complexes. According to the literature, nitrogen content is largely affected by pH and heat treatment in WPI solutions (*26*−*28*). In general, whey proteins are soluble over a large range of pH (*26*). Above the IEP of proteins (typically pH 5.2−5.4 for whey proteins), macromolecules are soluble (*26*, *27*), but below or close to the IEP of proteins, solubility is reduced. At room temperature, WPI at both pH 4.5 and pH 7 was soluble. The insoluble part was probably attributable to the presence of insoluble materials like denatured proteins or insoluble impurities in the WPI powder (*29*). Heat treatment reversibly (below 75 °C) or irreversibly (up to 75 °C) unfolds whey proteins and causes their aggregation (*22*, *26*). Reversible unfolding was observed when the pH was adjusted to 7 for WPI73. Irreversible thermal denaturation was noted for WPI85 and WPI90 at pH 4.5: the adjustment to pH 7

did not allow solubilization of nitrogen. In contrast to whey proteins in WPI solutions, proteins involved in complex formation did not precipitate after heat treatment or at a pH below the IEP of whey proteins. Some authors have reported that protein−polysaccharide complex formation increases protein solubility over a larger pH range and against heat treatment (*1*, *3*, *5*, *6*, *16*). LMP solutions were stable independently of the heat treatment or the pH condition used. Effectively, weight proportion, distribution of sugar and nitrogen content, and TS to TN ratios did not vary or varied only slightly. These results were in concordance with literature revealing that pectin is relatively stable over a large range of pH and upon heating (*30*). Consequently, stabilization of electrostatic complexes through pH adjustment to 7 by heating will be related to the formation of covalent bonds between proteins during the unfolding process. Polysaccharide could be trapped inside the protein network or participate in the network through protein−polysaccharide interactions.

For industrial purposes, it will be advantageous to use WPI−LMP complexes for long storage periods. This study showed that heat treatment was as efficient as the addition of a chemical preservative (sodium azide) in maintaining the shelf life of WPI−LMP complexes over 28 days of storage. In addition, the stability of WPI−LMP complexes was maintained during storage. Therefore, using the 90 °C/2 min combination was sufficient to destroy mesophilic aerobic flora, as reported in the literature (*22*). Without heat treatment, the COM25 at pH 4.5 could not be used for a long period of time (maximum of 14 days of storage) because microorganisms were able to grow during its storage at 4 °C. Hence, its shelf life was compromised because of the absence of heat treatment.

This study has shown that WPI−LMP complexes formed at pH 4.5 can be stabilized against a pH adjustment to neutrality by heating in conditions allowing irreversible thermal denaturation of proteins. The mechanism responsible for the stabilization and the type of interactions involved upon heating are currently unknown and require further investigation. This study has also demonstrated that heated WPI−LMP complexes can be used for 28 days following production, with no alteration in stability or microbiological quality. This information is essential to the widespread potential application of electrostatic complexes as new stabilizing agents in yogurt manufacture.

ABBREVIATIONS USED

LMP, low methoxyl pectin; WPI, whey protein isolate; COM, complexes; TN, total nitrogen; TS, total sugar; SSP, superior soluble phase; ISP, intermediate soluble phase; INSP, inferior soluble phase; IP, insoluble phase.

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