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Improvement, by dry-heating, of the emulsion-stabilizing properties of a whey protein concentrate obtained through carboxymethylcellulose complexation

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Abstract

A protein concentrate (WPC) obtained from milk whey by complexation with carboxymethylcellulose (CMC) (protein: 66.2%, CMC: 23.9%) was incubated at 60 °C for time periods of up to 5 weeks and the emulsion-stabilizing ability against creaming and against heat-induced aggregation of the incubated WPC was investigated. Incubation appears to bring about a remarkable improvement in the emulsion-stabilizing properties of the protein concentrate. The results are considered in terms of a conjugate formation during dry-heating between the whey protein and the polysaccharide which, following adsorption to the droplet surfaces, enhances the strength of steric stabilization interaction forces operating between overlapping protein layers adsorbed at the surfaces of neighbouring droplets.

Keywords: Whey protein concentrate; Carboxymethylcellulose; Protein–polysaccharide interactions; Maillard-type conjugate; Heat-induced aggregation

1. Introduction

Protein precipitation at the isoelectric point is an important step in the process of protein isolate preparation from soybean or other sources. Recovery, however, of proteins by isoelectric precipitation from milk whey, a fluid by-product resulting during cheese or casein manufacture, cannot be easily achieved, due to the very low protein content of whey and the high whey protein solubility (Cayot & Lovient, 1997). As a result, a protein precipitation aid has to be employed in order to effect whey protein precipitation by pH manipulation. Anionic polysaccharides, such as carboxymethylcellulose (CMC), are very effective for precipitating these proteins at low pH environments where the formation of an insoluble protein–polysaccharide ionic complex takes place.

As was reported by Hansen, Hidalgo, and Gould (1971), the protein content of the dehydrated whey protein–CMC precipitate was about 65% and may thus be characterized as protein concentrate (WPC). According to Damianou and Kiosseoglou (2006), the polysaccharide content of a WPC recovered by CMC complexation is 23.9%. The relatively high CMC/protein ratio in WPC is bound to influence the functional properties of the whey protein, especially in emulsion systems where, depending on environmental parameters, such as pH and/or NaCl concentration, the presence of polysaccharide molecules may affect droplet interaction and hence emulsion stability against creaming, either directly (through "bridging") or indirectly (through depletion). As reported by Damianou and Kiosseoglou (2006), due to lack of attraction between the protein and the polysaccharide molecules at neutral pH values, destabilization and rapid creaming of emulsion systems, prepared with a WPC obtained through CMC complexation, may take place as a result of the presence of the (non-adsorbing to the droplet surfaces) polysaccharide molecules that enhance depletion phenomena, leading to droplet flocculation. This, in turn, is bound to limit the range of product pH over which a WPC obtained through

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ionic complexation with CMC could be used as an emulsifier and emulsion stabilizer.

When a protein–polysaccharide mixture is dry-heated under controlled temperature and relative humidity conditions, a covalent protein–polysaccharide conjugate results, which exhibits improved functionality compared to the initial biopolymer mixture (Kato, Minaki, & Kobayashi, 1993; Kato, Nakamura, Takasaki, & Maki, 1996; Mishra, Mann, & Joshi, 2001). Diftis and Kiosseoglou (2003, 2006) reported that a conjugate may be obtained when soybean protein isolate is dry-heated in admixture with CMC or dextran. The conjugate may competitively adsorb to emulsion droplet surfaces, along with the non-conjugated soy protein molecules of the dry-heated mixture, or in the presence of low molecular weight emulsifiers (Diftis & Kiosseoglou, 2004), in spite of its relatively large hydrodynamic diameter. The outcome of conjugate adsorption to the droplet surfaces is the enhancement of steric stabilization forces, leading to the preparation of emulsions with small droplet diameter and increased creaming stability. The increased stability of the emulsions may also be connected with the diminishing of depletion phenomena due to polysaccharide adsorption to the droplet surfaces, irrespective of the pH and/or the ionic strength.

The present work aims at investigating the possibility of a whey protein–CMC conjugate formation by dry-heating of the WPC obtained through CMC complexation, in an attempt to control emulsion droplet flocculation and destabilization resulting from the presence in the system of non-adsorbing CMC molecules. As the main target of this study was to control depletion droplet interaction phenomena, resulting from the presence in the emulsion continuous phase of free polysaccharide molecules, the WPI obtained through CMC complexation was heat-treated, without first increasing the polysaccharide/protein ratio to values above 1, where maximum conjugation and, hence, enhanced emulsifying performance results (Diftis & Kiosseoglou, 2003).

2. Materials and methods

2.1. Materials

Pasteurized bovine skim milk and refined corn oil were bought from the local market. Medium viscosity sodium CMC (degree of substitution 0.70–0.80) was obtained from Fluka AG (Buks, Switzerland). The molecular weight of the polysaccharide, determined by viscometry, was around 340,000. All the reagents used in the experiments were of analytical grade.

2.2. Methods

2.2.1. Preparation of the WPC

The skim milk was initially incubated at 80 °C, following pH adjustment to 4.5 with 1 N HCl to precipitate the casein, and the whey was recovered by centrifugation. The protein content of the whey was then determined, according to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), and a suitable quantity of a 2% (w/v) CMC solution (CMC/protein (w/w): 1/3) was added, followed by pH adjustment to 3.2.The precipitated whey protein was recovered by centrifugation and washed twice with distilled water; the pH was adjusted to 7 and the slurry was dehydrated by freeze-drying. The resulting WPC powder was analysed for protein by Kjeldahl (66.2%), for ash (5.9%) and moisture (3.4%) by the oven method (AOAC, 1994) and for CMC (23.9%) by the 2,7-naphthalenediol colorimetric method (Graham, 1971). Application of SDS-PAGE to the concentrate indicated that the β-lactoglobulin and α-lactalbumin contents were 51.8% and 17.8% (w/w), respectively.

Samples of 0.5 g of WPC were then incubated at 60 °C for time periods of 3 and 5 weeks, in a desiccator containing saturated KBr solution at the bottom (Kato et al., 1993).

2.2.2. SDS-PAGE electrophoresis

Both the incubated and the control (non-heated) WPC samples were analysed by SDS-PAGE, according to Laemmli (1970), using 10% and 3% acrylamide separating and stacking gels, respectively. Electrophoresis was conducted in the presence of mercaptoethanol, using an Apelex Model ST 1006T vertical electrophoresis apparatus (Scieplus, France).

2.2.3. Emulsion preparation and stability determination

The incubated samples were carefully dispersed in distilled water by continuous agitation for 1 h to obtain solutions of 0.5% in WPC. The solution pH was adjusted to the value of 6.5 using 0.5 N NaOH. Crude oil-in-water (o/w) emulsions were then prepared by drop- wise addition of corn oil under continuous agitation with a mechanical stirrer, while NaN_3 (0.001%) was added as preservative. The crude emulsions (10% v/v in oil) were homogenized for 3 min at 700 bar with the aid of an APV-2000 pressure homogenizer (APV, Albertslund, Denmark) and the average droplet diameter $d_{3,2}$ was determined using a Malvern Mastersizer 2000, by applying the following optical parameters: corn oil and water refractive indices: 1.4673 and 1.33, respectively; absorbance: ($\lambda_{max} = 0.002$).

The creaming stability of emulsions was assessed visually from the height of the serum phase developing with storage of 10 ml emulsion samples in cylindrical glass containers.

The stability of emulsions against heat-induced aggregation was determined by heating (at 100 °C) of small quantities (1 ml) of samples in test tubes for predetermined time periods of up to 8 min and taking into account that each sample required about 1 min to reach 100 °C. The size of the resulting aggregates or isolated droplets was determined using the Malvern Mastersizer, following dispersion by stirring in distilled water. The volume average diameter, $d_{3,0}$, was calculated from:

$$d_{3,0} = \left(\sum \frac{nidi^3}{ni}\right)^{1/3}$$

where *ni* is the number of droplets with a diameter, *di*. The volume average diameter data were then employed to calculate the ratio of the sum of the numbers of the single emulsion droplets and droplet aggregates at time 0 (N_0) and t (N_t) according to equation (Euston, Finnigan, & Hirst, 2000):

$$N_t/N_0 = [(d3,0)0/(d3,0)t]^3$$

2.2.4. Amount of protein and polysaccharide absorbed in emulsions

Following centrifugation of emulsion samples at 10,000×*g* for 30 min, the cream was discarded while the continuous phase was centrifuged twice more to remove all the oil droplets from the surface. Small samples from the clear serum phase were then obtained with the aid of a syringe and analysed for protein and CMC according to the Lowry (Lowry et al., 1951) and the 2,7-naphthalenediol (Graham, 1971) methods, respectively.

The amount of protein and CMC adsorbed per unit surface of emulsion droplets was determined using the expression:

$$\Gamma = \Gamma_T/S_T$$

where Γ_T is the total amount of protein (or CMC) adsorbed in 100 ml of emulsion and S_T is the total emulsion surface per 100 ml (10 ml oil) calculated from:

$$S_T = 10 \cdot S$$

where S is the average surface area (m^2/ml oil) derived by Walstra (1983):

$$S = 6/d_{3,2}$$

2.2.5. Viscosity measurements

A Brookfield DV II, LV viscometer (Brookfield Engineering Laboratories, Stoughton) was employed to determine the emulsion viscosity at different shear rates. The SC4-18/13R small sample adapter (concentric cylinder geometry) was used for the measurements which were conducted at 25 °C.

2.3. Statistical analysis

All the experiments were conducted at least three times. The data were analyzed using the one-way anova programme and significant differences between means were identified by the LSD procedure.

3. Results and discussion

As shown in Fig. 1, the emulsion, prepared with the non-incubated WPC sample, exhibited a relatively short "delay period", which is the time needed for the first sign of serum separation to become apparent (around 2 h), and a very high creaming rate that led, following storage for only 100 h, to a highly compressed cream layer. On the other hand, the emulsions prepared with the (incubated for 3 or 5 weeks) WPC exhibited much higher stability against creaming during storage. As reported by Damianou and Kiosseoglou (2006), the low stability of the control emulsion should be attributed to the absence of whey protein–CMC interactions at pH 6.5, since the molecules of both biopolymers carry a net negative charge and, therefore, to the enhancement of depletion effects, originating from non-adsorbing at the emulsion droplet surface CMC molecules. According to Blijdenstein, van Winden, van Vliet, van der Linden, and van Aken (2004), such weakly-aggregated oil droplet networks may suffer extensive droplet rearrangements, leading to a more compact network and serum separation.

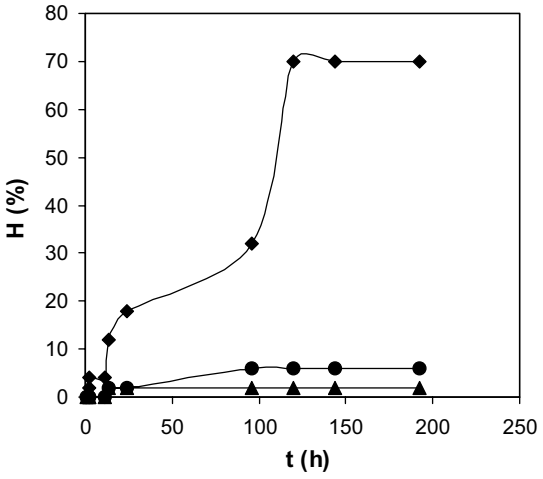

Fig. 1. Effect of WPC dry-heating at 60 °C on creaming stability of emulsions (10% oil, 0.5% WPC). Key: ♦, control; ●, dry-heated for 3 weeks; ▲, dry heated for 5 weeks.

Incubation of WPC at 60 °C for prolonged time periods probably led to Maillard type conjugate formation between the whey protein molecules on the one hand and the polysaccharide molecules on the other. As Fig. 2 shows, dry-heating of the concentrate for 3 or 5 weeks resulted in the appearance of a broad band near the top of the running gel of the SDS-PAGE electrophoregrams, indicating the formation of reaction products exhibiting a wide distribution of molecular weights. Considering that the concentrate may contain a low percentage of lactose (not higher than 0.6%, according to its composition with respect to protein, ash, CMC and moisture), the whey proteins may also become involved, to some extent, in Maillard-type reactions with the lactose molecules. As, however, the lactosylated proteins must constitute only a minor fraction of the total conjugated protein mixture they are not expected to significantly affect its functionality.The involvement of CMC in conjugate formation with proteins was also reported by Diftis and Kiosseoglou (2003) for dry-heated mixtures of CMC with soybean protein isolate,

Fig. 2. SDS-PAGE patterns for non-incubated (A) and dry-heated for 3 weeks WPC emulsions (B).

although the protein/CMC ratio in that study was relatively low (1/1 and 1/3 as opposed to the ratio of 3/1 in this study). Due to the low moisture content and lack of electrostatic interactions between the two biopolymers at the neutral pH environment, separation into protein– and polysaccharide-rich phases probably takes place in the dehydrated mixture. The protein–CMC conjugate formation upon prolonged heating, therefore, is expected to take place only at the interface separating the two phases. Additionally, protein–protein aggregate formation, involving disulfide and/or isopeptide bonds, is also bound to take place to some extent (Diftis & Kiosseoglou, 2003). Protein aggregate formation following WPC incubation may explain the increase in the amount of protein adsorbed per unit surface of emulsion droplets (Fig. 3). Although, however, the increase in the amount of protein was relatively marginal, WPC dry-heating brought about a spectacular rise in the amount of polysaccharide adsorbed per unit surface of emulsion droplets (Fig. 3).This finding presents indirect but very strong evidence for the formation of a whey protein–CMC conjugate upon prolonged dry-heating of the WPC. Following emulsification, the polysaccharide molecules adsorbed to the droplet surface indirectly, as they accompanied the protein molecules to which they were covalently bound. As Fig. 4 shows, an appreciable proportion of the polysaccharide became adsorbed, suggesting that extensive interaction between the protein and the CMC molecules took place during the dry-heating period.

Whey protein–CMC conjugation, however, does not appear to improve the emulsifying but only the stabilizing ability of the WPC since, when intensive stirring was

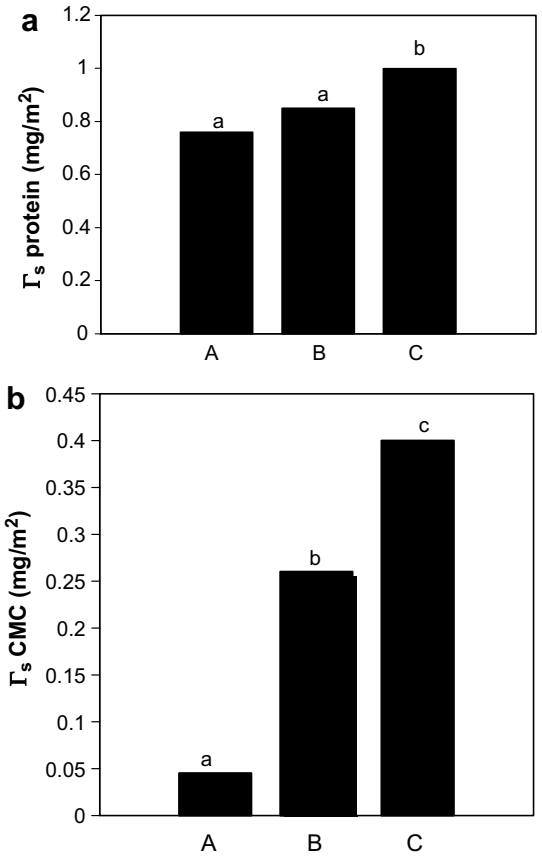

Fig. 3. Effect of WPC dry-heating at 60 °C on the amount of protein (a) and CMC (b) absorbed per unit droplet surface in emulsions (10% oil, 0.5% WPC). a–c: means with different superscripts are significantly different ($P < 0.05$). Key: A, control; B, dry-heated for 3 weeks; C, dry-heated for 5 weeks.

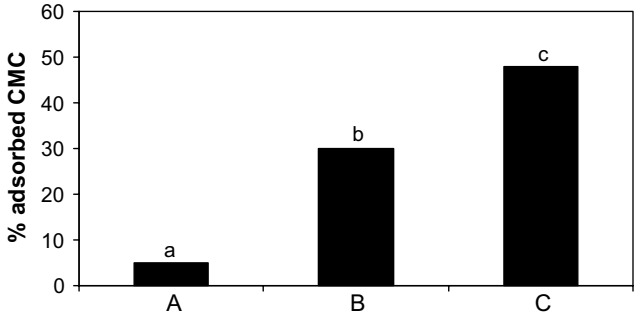

Fig. 4. Effect of WPC dry-heating at 60 °C on the percentage of total CMC adsorbed at the droplet surfaces, in emulsions (10% oil, 0.5% WPC). Key: A, control; B, dry-heated for 3 weeks; C, dry-heated for 5 weeks.

applied to the sample dispersions in water before measurement, the droplet size of the emulsions prepared with the dry-heated WPC was only marginally larger than that of the emulsion prepared with the non-incubated WPC (Fig. 5). The larger droplet size of the emulsions, based on the dry-heat treated WPC, could have been due to the presence in the dispersion of droplet aggregates which were not completely dispersed by agitation since, as Fig. 6 shows, a combination of SDS and mercaptoethanol

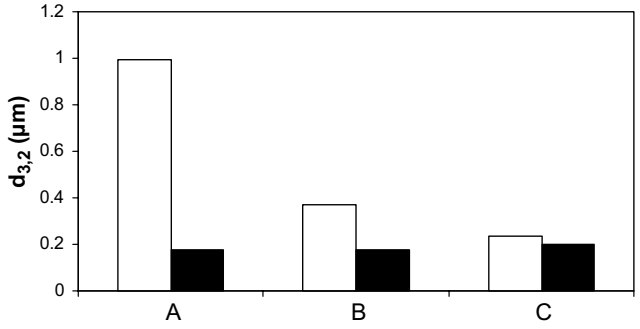

Fig. 5. Effect of WPC dry-heating at 60 °C on mean droplet size, $d_{3,2}$, of emulsions (10% oil, 0.5% WPC) determined after gentle (□) or vigorous agitation (■). Key: A, control; B, dry-heated for 3 weeks; C, dry-heated for 5 weeks.

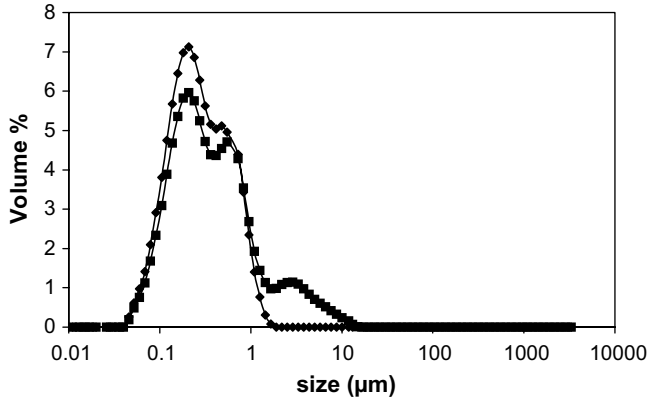

Fig. 6. Droplet size distribution of emulsions (10%) prepared with incubated (for 5 weeks) WPC.Key: ■, treated with 1% SDS, ♦; treated with SDS and 20 mM MeSH at 50 °C.

treatment was needed to disperse the aggregates into singe droplets. The actual mean droplet size of the emulsions was the same, irrespective of whether the WPC was incubated or not (around 0.170 mm). Diftis and Kiosseoglou (2003), on the other hand, reported that soybean protein isolate–CMC conjugation by dry-heating led to an improvement of the emulsifying ability of the initial mixture. This was attributed to steric forces arising from the presence of adsorbed polysaccharide molecules which stabilized the newly-formed droplets immediately after their formation and produced emulsions with a smaller droplet size. This difference in behaviour between the two protein–polysaccharide mixtures should be attributed to the much lower polysaccharide content of the WPC in combination with the much higher molecular weight of the SBPI protein constituents and, therefore, the relatively lower number of protein molecules involved in conjugate formation following incubation. Thus, although the WPC-CMC conjugate is expected to become adsorbed to the droplet surfaces and enhance steric stabilization interactions (Dickinson & Galazka, 1991), its influence during the initial stages of the emulsification process, when continuous droplet formation and coalescence takes place, is not significant. Many of the whey protein molecules of WPC are not in the conjugated form and it is these molecules that determine the emulsifying performance of the incubated WPC.

When a whey protein o/w emulsion with a relatively low oil content is flocculated, as a result of pH and/or NaCl content adjustment, it exhibits a high creaming rate and increased instability (Demetriades, Coupland, & McClements, 1997). In the presence of free CMC molecules, as is the case for the control emulsion in the present investigation, droplet flocculation takes place, even at pH values above 6.0, where the system was expected to be relatively stable in the absence of polysaccharide. Flocculation under these conditions should have been the result of attractive depletion interactions between the oil droplets. Droplet aggregation in turn may lead to overlap of the adsorbed (on neighbouring droplet surfaces) protein layers and to intensification of the aggregation process through protein–protein hydrophobic interactions. As Fig. 4 shows, when the droplet size of the control emulsion was measured after gentle dispersion in water in the measuring vessel of the Malvern, the apparent droplet size corresponded to that of small aggregates. Since these aggregates were dispersed by more intense agitation, one may hypothesize that the droplets in these aggregates were flocculated by depletion forces only. Depletion effects ceased to operate when a homogeneous (with respect to polysaccharide content) diluted continuous phase was obtained. The absence of hydrophobic interactions at a pH above 6 should be attributed, according to Demetriades et al. (1997), to the net negative charge of the droplet surfaces that forces the droplets to flocculate at a separation distance corresponding to a relatively shallow secondary minimum of the interaction potential curve. At this distance, overlapping between the adsorbed protein layers on droplets in close proximity should be negligible. In the case of emulsions prepared with the incubated WPC, flocculation between the droplets appears to take place to a limited extent, probably as a result of the presence, at the droplet surfaces, of protein aggregates formed during the incubation process. Since the strength of covalent disulfide bonds in combination with hydrophobic interactions is relatively high, droplet rearrangements are not expected to take place during ageing. The emulsion system, therefore, appears to be made up of single droplets and small-sized droplet aggregates. As reported by Diftis, Biliaderis, and Kiosseoglou (2005), such systems exhibit a liquid-like behaviour and the creaming process is haulted when a pseudoequilibrium state is reached after ageing with a network structure that does not yield to buoyancy forces, thus not allowing the cream to compress further.

The formation of a relatively homogeneous droplet network in the case of the systems prepared with the dry-heat treated WPC may explain its higher viscosity compared to the control emulsion (Fig. 7). Although the latter was more flocculated and, according to Demetriades et al. (1997), should have been more viscous, it appears that the low volume fraction of the emulsion does not allow extensive

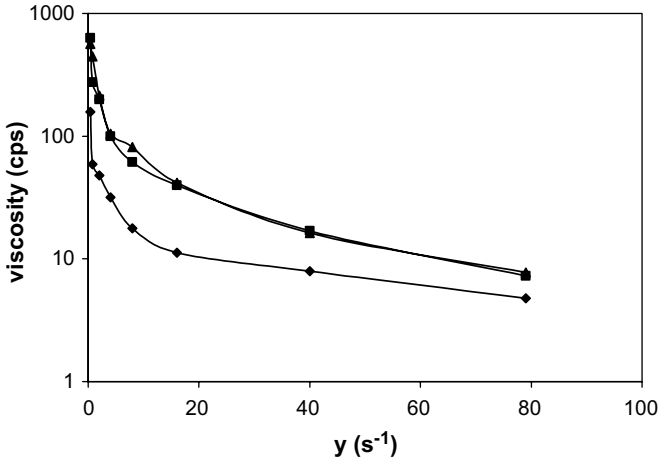

Fig. 7. Effect of WPC dry-heating at 60 °C on the viscosity-rate of shear relationship of emulsions (10%, 0.5% WPC). Key: ♦, control; ■, dry-heated for 3 weeks; ▲, dry-heated for 5 weeks.

inter-aggregate interaction. The viscosity of the emulsion, therefore, is determined only by the clashes between (moving towards the direction of flow) single droplets and aggregates which are bound to be more numerous in the case of the less extensively flocculated system.

Following heating, in boiling water, of the emulsion prepared with the non-incubated WPC, an increase in the mean droplet volume diameter, $d_{3,0}$, with heating time was observed while no appreciable change in the mean droplet diameter of the emulsions containing the incubated (for 3 or 5 weeks) WPC was noted during the same heating time period (Table 1). Whey protein-stabilized emulsions tend to aggregate when subjected to heat treatments at temperatures above the denaturation temperature of the whey proteins (Euston et al., 2000). Euston et al. (2000) suggested that the aggregation process was the result of the presence, in the continuous phase, of non-adsorbed whey protein molecules which, following heating, became denatured and aggregated, and the aggregates formed acted as "glue" to "bridge" neighbouring droplets, leading to flocculation. Additionally, in the presence of depletion-inducing polysaccharides, such as xanthan, the rate of heat-induced aggregation increases markedly, even at polysaccharide contents as low as 0.02–0.04% (Euston, Finnigan, & Hirst, 2002).

Table 1
Influence of heating (100°C) time on the mean droplet size, $d_{3,0}$, of oil-in-water emulsions prepared with non-incubated WPC and incubated at 60°C for 3 or 5 weeks

t (min)	$d_{3,0}$ (μm)		
	WPC	WPC incubated for 3 weeks	WPC incubated for 5 weeks
0	0.133	0.124	0.123
0.5	0.144	0.125	0.125
1.5	0.178	0.126	0.125
3	0.196	0.126	0.126
4	0.203	0.125	0.126
5	0.207	0.142	0.127
7	0.236	0.125	0.126

As discussed above, at pH 6.5, the presence of free CMC molecules in the continuous phase of the emulsion prepared with the non-incubated WPC, is expected to lead to depletion flocculation. When the emulsion is heated in boiling water, therefore, droplet aggregation is expected to become more extensive. To determine the kinetics of aggregation, data for $(N_t/N_0)^{-1}$, for $(N_t/N_0)^{-0.5}$ and for $\ln(N_t/N_0)$ were plotted versus heating time. The best fit was obtained for the $(N_t/N_0)^{-0.5}$ versus $t(R^2 = 0.945)$ and for $(N_t/N_0)^{-1}$ versus time (R^2=0.970) (Fig. 8), suggesting that the aggregation process might have been of the order of 1.5 or 2. According to Euston et al. (2002), the order for heated whey protein-stabilized emulsions was 1.5, irrespective of the presence or absence of depletion-inducing polysaccharides. Diftis and Kiosseoglou (2006) reported that the order of the aggregation process of soybean protein isolate emulsions increased from 1.5 to 2 when dextran was incorporated in the system. This might have also been the case for the emulsions studied in the present investigation. Reaction orders of the droplet aggregation reaction ranging between 1 and 2 are expected, assuming that the aggregation reaction is a two step process, i.e. a first order protein denaturation step, followed by a second order aggregation step. The reaction rate constant determined from Fig. 8, assuming that the order of the process was 1.5, turned out to be around 6×10^{-3}, which is double the value reported by Euston et al. (2000) for emulsions stabilized by 0.5% whey protein and having an oil volume of fraction of 0.20 (as opposed to $\Phi = 0.10$ of the present study). This clearly indicates that the presence of non-adsorbed CMC molecules in the emulsion continuous phase resulted in the intensification, probably through depletion, of heat-induced aggregation phenomena. It should also be stressed that the aggregation rate constant value is WPC source- dependent and could be affected by the variabilities in composition between the various whey protein preparations.

In the case of emulsions prepared with the incubated WPC, the remarkable stability against heat-induced aggregation should be attributed to the enhancement of steric stabilization interaction forces between the droplets, due to adsorption of whey proteins conjugated to polysaccharide molecules. Additionally, since an appreciable proportion of the CMC molecules appears to become adsorbed to the droplet surfaces, the depletion effects that intensify the heat-induced aggregation process, are expected to become diminished. The resistance to denaturation of the whey proteins is expected to increase, due to the protective effect of the conjugated polysaccharide moiety. This may also contribute to the stability of emulsions against aggregation upon heating and should also be taken into account, although most of the interface is expected to be occupied by non-conjugated protein molecules.

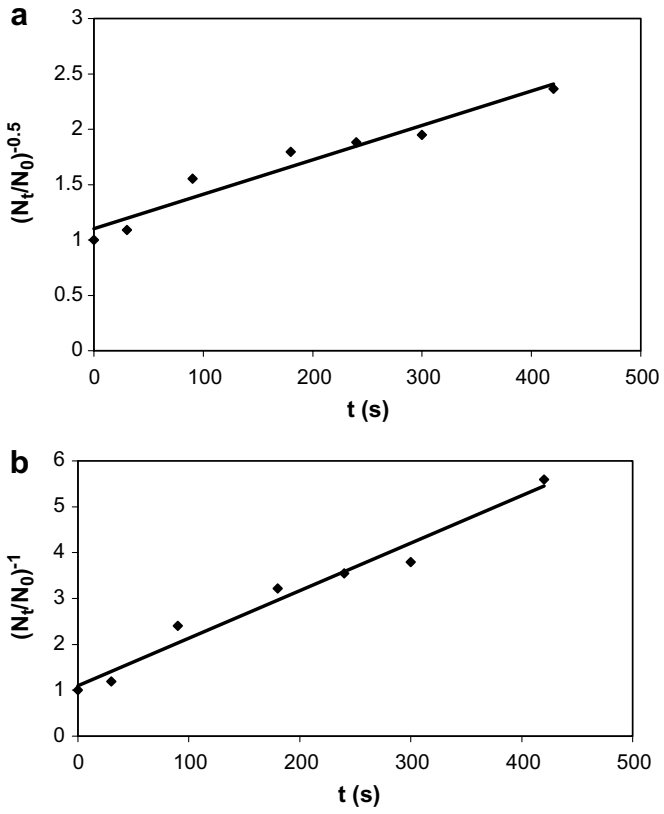

Fig. 8. Plots of $(N_t/N_0)^{-0.5}$ (a) and $(N_t/N_0)^{-1}$ (b) versus heating time for heat-treated (at 100 °C) emulsions stabilized with non-incubated WPC.

4. Conclusion

Prolonged dry-heating at 60 °C of a WPC, precipitated from skim milk with the aid of CMC, resulted in a dramatic enhancement of its emulsion-stabilizing ability against creaming. This was attributed to the formation of a Maillard-type conjugate between the whey proteins and the CMC molecules of the concentrate. Conjugation of whey proteins to CMC probably led to an enhancement of the repulsive steric interaction forces operating between the emulsion droplets, due to the presence of the polysaccharide moiety of the hybrid at the droplet surface. Additionally, incubation of the WPC brought about an increase in its ability to stabilize the emulsions against heat-induced aggregation.

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