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Heat stability and freeze–thaw stability of oil-in-water emulsions stabilised by sodium caseinate–maltodextrin conjugates

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ABSTRACT

A sodium caseinate (NaCN)–maltodextrin (Md100) conjugate was prepared by a Maillard-type reaction by dry heat treatment of a NaCN–Md100 mixture at 60 °C and 79% relative humidity for 4 days. Conjugation resulted in a 35.7% loss of available amino groups in the NaCN and a 25.9% loss of available reducing groups in the Md100. The crude conjugate was purified by batch anion exchange chromatography to remove non-conjugated Md100. Purification reduced the available reducing groups in the conjugate from 74.1% to 23.7% and increased the protein content from 45.6% to 83.9%. The emulsifying properties of the conjugates were assessed in oil-in-water (o/w) emulsions; crude and purified conjugate stabilised emulsions had improved storage stability and freeze–thaw stability when compared to NaCN stabilised emulsions. Purified conjugate stabilised emulsions had better thermal stability than NaCN, NaCN–Md mixture and non-purified conjugate stabilised emulsions. These results indicate a potential for these NaCN–Md conjugates as speciality functional food ingredients.

1. Introduction

The rising cost of and functional demands on milk protein ingredients have created a need for speciality milk protein ingredients which possess requisite functional properties for utilisation in specific food products. Speciality milk protein ingredients may be produced by exploiting the potential to alter the physico-chemical properties of milk proteins by physical, chemical and/or enzymatic modification. One modification recognised as being suitable for producing speciality ingredients for food applications is protein–polysaccharide conjugation *via* a Maillard-type reaction (Kato, 2002).

The dry heating method is the preferred method of protein–polysaccharide conjugation as this method is carried out at the optimum water activity (a_w) for the reaction, thus, less time is required for the reaction to occur in comparison to the wet heating method. This method involves heating a dry protein–polysaccharide mixture at controlled temperature and relative humidity for a given time, inducing the condensation of the carbonyl group of the reducing sugar with the ε-amino groups of the protein, resulting in production of an Amadori product *via* the formation of a Schiff base with the release of water (Ames, 1992). Conjugation of different milk proteins with various polysaccharides has been shown to enhance the functional properties of the proteins, including increasing protein solubility, particularly around the isoelectric pH of the protein, and improving heat stability compared to the unmodified protein (Kato, 2002; Oliver, Melton, & Stanley, 2006; O'Regan & Mulvihill, 2009). The most noteworthy functional characteristic of protein–polysaccharide conjugates is their enhanced emulsifying properties when used as the emulsifying agent in place of the native protein (Kato, 2002; Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004). The enhanced emulsifying properties of protein–polysaccharide conjugates has been attributed to the conjugated protein molecules forming a bulky polymeric layer on the droplet surface, with the polysaccharide portion protruding outwards into the continuous phase, thus conferring enhanced steric stabilisation to the emulsion droplets (Dickinson & Galazka, 1991).

Both proteins and polysaccharides play important structural and stabilisation roles in food formulations (Dickinson, 2009). In food emulsion systems, milk proteins are often used because of their excellent surface active properties; they absorb as an excess layer at the oil–water interface and lower the interfacial tension between the phases. The excess surface layer of protein on the surface of the droplets may generate sufficient steric and electrostatic repulsion interactions which can result in the formation of a stable emulsion. In general terms, polysaccharides have poor surface active properties and therefore do not absorb particularly well at an oil–water interface, but polysaccharides are often added to emulsions because they can provide a stabilisation effect *via* viscosity modification or gelation of the continuous phase of an emul-

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sion (Dickinson, 2003). The type and concentration of the polysaccharide added to or in a food emulsion can be either beneficial or detrimental to the stability of the emulsion (Semenova, Belyakova, Antipova, & Jubanova, 1999). Some polysaccharides can associate with the stabilising protein layer at the emulsion oil–water interface; if association is strong between the protein and the polysaccharide, a secondary layer of steric stabilising polysaccharide may form around the protein-coated oil droplets; when the polysaccharide is present at high concentrations and association with the protein is strong, a gel network may be formed which promotes emulsion stability (Dickinson & Galazka, 1991). If association with the protein layer at the emulsion interface is weak and/or the concentration of absorbed polysaccharide at the droplet interface is low and does not saturate the surface of the oil droplets, emulsion destabilisation may occur as a result of bridging flocculation (Dickinson, 2003).

The presence of non-absorbing polysaccharides in the continuous phase of an emulsion may enhance attraction between the droplets, which can induce the destabilisation of an emulsion by depletion flocculation (McClements, 2004). The rate of depletion flocculation initially increases as the concentration of the non-absorbing polysaccharide is increased; at higher concentrations of non-absorbed polysaccharide, an increase in viscosity of the continuous phase conferred by the polysaccharide can result in the retardation of Brownian motion of the droplets thus reducing droplet collision frequency and improving emulsion stability (Appelqvist, Golding, Vreeker, & Zuidam, 2007; McClements, 2004). Therefore, it is important that the influence of any non-conjugated polysaccharide present must be considered when assessing the potential of protein–polysaccharide conjugates as emulsifying agents.

Conjugation of a polysaccharide to a protein improves the emulsifying properties when compared to the protein itself, indicating a potential for protein–polysaccharide conjugates as speciality functional food ingredients. To improve their quality and shelf life, many food emulsions undergo thermal treatments including heating, chilling and/or freezing during their manufacture and/or storage (Mun, Younghee, Decker, & McClements, 2008). Typical thermal treatments of emulsions include ultra high temperature treatments (135–150 °C for 3–5 s) or in-container retort sterilisation (120 °C for 5–10 min) or a combination of these processes (McSweeney, 2008). Emulsions are often frozen shortly after manufacture and stored in the frozen state for prolonged periods of time to protect them from microbial growth and undesirable chemical reactions. Whilst these thermal processing operations are a necessity than can often affect the stability of a food emulsion; therefore it is important to use an appropriate emulsifier to avoid any product stability problems.

The objectives of this research were to prepare a sodium caseinate–maltodextrin conjugate and to prepare a purified conjugate by removal of the non-conjugated maltodextrin from the crude conjugate. The crude conjugate and purified conjugate were used to stabilise model oil-in-water emulsions and these emulsions were characterised by assessing their storage stability, heat stability and freeze–thaw stability in comparison to sodium caseinate stabilised emulsions.

2. Materials and methods

2.1. Materials

Sodium caseinate (NaCN) containing 90.4% protein was obtained from Kerry Ingredients (Listowel, Co. Kerry, Ireland). Maltodextrin, Maltrin100 (Md100) with a DE of 10.9, was obtained from Grain Processing Corporation (Muscatine, IA, USA). Soya oil was obtained from a local supermarket. DEAE cellulose was purchased from Sigma–Aldrich (Ireland). Dialysis membrane with a molecular weight cut off of 12–14 kDa was purchased from Medicell International Ltd. (UK). All other chemicals were analytical grade and were commercially available (Sigma–Aldrich, Ireland).

2.2. Preparation of the sodium caseinate–maltodextrin conjugate

Maltodextrin (Md100) was dispersed at 10%, (w/v) in Milli-Q water. The Md solution was placed inside the 12–14 kDa dialysis membrane and dialysed against Milli-Q water (20 volumes, with two water changes) containing 0.01%, (w/v) sodium azide at 4 °C for 48 h for the removal of low molecular weight sugars and dextrans. The dialysed solution was then freeze dried. The loss of low molecular weight sugars and dextrins on dialysis was determined by the difference in dry weight of the Md before and after dialysis and expressed as a percentage loss of Md.

A NaCN–Md100 conjugate was prepared according to the method of Shepherd, Robertson, and Ofman (2000). The dialysed, freeze dried Md prepared as described above or NaCN were individually dissolved in Milli-Q water, containing 0.01%, (w/v) sodium azide at 5%, (w/v). Equal volumes of the aqueous solutions were mixed together under moderate magnetic stirring for 1 h at ambient temperature. The NaCN–Md100 mixture was then freeze dried, dry heat treated at 60 °C and 79% relative humidity over a saturated KBr solution for 96 h to induce conjugation, after which the conjugate was freeze dried and stored at 4 °C for further analysis.

2.3. Conjugate purification

Non-conjugated maltodextrin was removed from the crude NaCN–Md100 conjugate by batch DEAE anion exchange chromatography as follows: the crude conjugate was dispersed at 5%, (w/v) in a 0.01 M Imidazole HCl buffer, pH 7.4, containing 3.3 M Urea, 2 mM dithiothreitol (DTT) and 0.01%, (w/v) sodium azide (Wei & Whitney, 1985). DEAE cellulose (50 g, dry weight) was preswollen and equilibrated in the same Imidazole HCl buffer (200 ml) by gentle stirring (300 rpm) for 30 min. Excess buffer was then removed from the DEAE cellulose under vacuum through a Buckner funnel using Whatman No. 41 filter paper and the equilibrated DEAE cellulose filter cake was recovered. This DEAE cellulose filter cake was then added to the crude NaCN–Md conjugate/buffer dispersion (200 ml) and mixed using an overhead stirrer at 300 rpm for 30 min in a waterbath at 4 °C, to allow binding of the conjugated protein to the DEAE anion exchanger. The DEAE cellulose/conjugated protein mixture was then vacuum filtered as previously described above. The recovered DEAE cellulose filter cake containing the bound conjugated protein was again added to the recovered filtrate to facilitate improved conjugated protein adsorption. This cycle was repeated twice and the filtrate recovered at the end of the third cycle contained non-conjugated Md and the filter cake recovered contained the DEAE anion exchanger with bound conjugated protein.

The purified NaCN–Md conjugate was eluted from the DEAE anion exchanger by redispersing the DEAE cellulose filter cake containing the bound conjugated protein in 200 ml of 0.5 M NaCl and mixing for 30 min in a waterbath at 4 °C, followed by filtration through a Buckner funnel as described above and the eluate was collected. The recovered filter cake was again redispersed in 200 ml of 0.5 M NaCl as above, and the dispersion was mixed and filtered as already described. This procedure was repeated twice. The eluates collected were added together (~600 ml) and dialysed using the 12–14 kDa dialysis membrane against Milli-Q water (20 volumes, with two water changes) containing 0.01%, (w/v) sodium azide for 2 days at 4 °C for the removal of NaCl used to elute the purified conjugate. The purified NaCN–Md100 conjugate solution was then freeze dried and the dried powder was stored at 4 °C for further analysis.

2.4. Determination of protein content

The total protein content (Nitrogen content × 6.38) of NaCN, NaCN–Md100 mixture, crude conjugate and purified conjugate was determined by the Kjeldahl method (AOAC, 1995).

2.5. Determination of available amino groups

The concentration of available amino groups in NaCN, NaCN–Md100 mixture, crude conjugate and purified conjugate was determined by the trinitrobenzenesulfonate method (TNBS) as described previously (O'Regan & Mulvihill, 2009).

2.6. Determination of available reducing groups

The concentration of available reducing groups in the Md100, NaCN–Md100 mixture, crude conjugate and purified conjugate was determined using a modified Chloramine-T assay as described previously (O'Regan & Mulvihill, 2009).

2.7. Determination of colour

The influence of conjugation and conjugate purification on the colour of the conjugates was determined by measuring Hunter chromaticity coordinates ($L^* a^* b^*$) with a Minolta chromameter CR-300 (Minolta Limited, Milton Keynes, UK). The instrument was calibrated with a standard white tile ($Y = 93.6$, $x = 0.3130$ and $y = 0.3193$) before measurement. Dry NaCN, NaCN–Md100 mixture, crude conjugate or purified conjugate was placed into a Petri-dish and its $L^* a^* b^*$ colour coordinates were measured as described previously (O'Regan & Mulvihill, 2009).

2.8. SDS–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) using 10% w/v, and 3% w/v, acrylamide separating gel and stacking gel, respectively, both containing 0.1% w/v, SDS as described previously (O'Regan & Mulvihill, 2009). The gel was stained with either Coomassie Brilliant Blue R-250 or Periodic Acid Stain/Fuchsin reagent (Thornton, Carlstedt, & Sheehan, 1994) for protein and carbohydrate detection, respectively.

2.9. Preparation of model oil-in-water emulsions

Model oil-in-water (o/w) emulsions were prepared by reconstituting NaCN, NaCN–Md100 mixture, crude conjugate or purified conjugate (0.8% w/w, protein normally, 0.6% w/w, protein for freeze–thaw studies) in sodium phosphate buffer (0.1 M, pH 7) containing 0.01% w/v, sodium azide at ambient temperature under moderate magnetic stirring conditions for 1 h. The oil phase (20%, w/w) was pure soya oil. The emulsions were prepared by adding the oil phase to the aqueous phase and allowing the blend to temper at 50 °C for 30 min. The blend was then pre-homogenised using an Ultra-Turrax (10,000 rpm for 15 s), and homogenised by passing the pre-emulsion through a two stage laboratory scale homogeniser (APV 1000 Homogeniser, APV AS, Albertslund, Denmark) for five passes at 50 °C at a first stage pressure of 20 MPa and a second stage pressure of 5 MPa.

2.10. Characterisation of the model o/w emulsions

2.10.1. Determination of emulsion storage stability

The fat globule size distribution and mean fat globule size of each emulsion was determined on emulsion formation and following storage of the emulsion in glass containers under accelerated shelf life testing conditions at 45 °C for up to 20 days (Lynch & Mulvihill, 1997) by dynamic light scattering using a Malvern Particle Size Analyser (Mastersizer 2000S, Malvern Instruments Ltd., UK) equipped with a He–Ne laser ($\lambda = 623$ nm). The optical parameters selected were, a dispersed phase refractive index of 1.449, a droplet absorbance of 0.001 and a continuous phase refractive index of 1.333. The results are reported as a fat globule size distribution (size range measured from 0.05 to 878.67 μm) and the volume weighted mean fat globule size, d_{43}:

$$d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

where n_i is the number of droplets of a diameter d_i.

2.10.2. Determination of emulsion heat stability

The heat stability of each emulsion was evaluated by heating at 140 °C for selected time intervals up to 20 min as described by McSweeney, Mulvihill and O'Callaghan (2004). Aliquots (2 ml) of emulsion were transferred to glass tubes, sealed with silicone bungs and placed in an oil bath thermostatically controlled at 140 °C. A glass tube containing emulsion was removed following heating for 2.5, 5, 10, 15 or 20 min, and immediately placed in an ice-bath to cool. The treated emulsion was allowed to equilibrate to room temperature and held for 1 h. The heat stability of each emulsion was assessed by monitoring changes in the fat globule size distribution and mean fat globule size (d_{43}) as a function of heating time.

2.10.3. Determination of emulsion freeze–thaw stability

Emulsions (20 ml) were prepared essentially as described above, except that the aqueous phase contained a protein content of 0.6% (w/w), were placed in cryogenic tubes (internal diameter 23 mm and height 100 mm), and frozen by placing the tube into a −20 °C freezer for 46 h (Mun et al., 2008). The emulsions were then thawed by leaving them at room temperature for 30 min and then placing them into a waterbath at 40 °C for 1.5 h. These freeze–thaw cycles were repeated four times. The freeze–thaw stability of each emulsion was assessed by monitoring changes in the fat globule size distribution and mean fat globule size (d_{43}).

All experiments were carried out in triplicate and the results presented are representative of the trends observed.

3. Results and discussion

3.1. Conjugate characterisation

3.1.1. The effect of conjugation on the protein content and on the available amino groups in NaCN and the available reducing groups in Md

On mixing NaCN (protein content of ~90.4%) with Md100, the protein content was reduced to ~45%, as expected (Table 1). The NaCN–Md100 mixture had effectively the same level of available amino groups as in the NaCN and the same level of available reducing groups as in the Md100. Conjugation of the NaCN–Md100 mixture resulted in no significant change in protein content (~45%); however, conjugation resulted in a loss of ~35.7% of the available amino groups in the NaCN, and a loss of ~25.9% of the available reducing groups in the Md100 (Table 1, NaCN–Md100 crude conjugate). These results show that conjugation occurred while also indicating that a high proportion (~74%) of the available reducing groups of Md100 were still reactive in the crude conjugate.

3.1.2. Purification of the NaCN–Md100 crude conjugate

The batch anion exchange chromatography process used to purify the NaCN–Md100 crude conjugate did not influence the level of available amino groups; in comparison to the crude conjugate, the

Table 1
The loss of available amino groups in the NaCN, the available reducing groups in the Md on conjugation, the protein content and the colour (L^* a^* b^*) of NaCN, NaCN–Md mixture, NaCN–Md crude conjugate and NaCN–Md purified conjugate.

Sample	Protein content (%)	Loss of available amino groups (%)	Loss of available reducing groups (%)	L^*	a^*	b^*
(a) NaCN	90.4 ± 0.2	0[a]	–	97.74 ± 0.07	−1.27 ± 0.03	8.16 ± 0.08
(b) Md100	–	–	0[b]	100.31 ± 0.46	−0.04 ± 0.03	0.39 ± 0.04
(c) NaCN–Md100 mixture	45.3 ± 0.2	0.6 ± 0.4	0.3 ± 0.2	95.50 ± 0.21	−0.40 ± 0.03	3.61 ± 0.12
(d) NaCN–Md100 crude conjugate	45.6 ± 0.2	35.7 ± 0.8	25.9 ± 1.3	91.41 ± 0.27	1.32 ± 0.06	17.67 ± 0.18
(e) NaCN–Md100 purified conjugate	83.9 ± 0.3	35.3 ± 0.2	76.3 ± 1.7	91.21 ± 0.36	1.10 ± 0.07	13.81 ± 0.30

[a] Unreacted NaCN assumed to represent 100% available amino groups.
[b] Untreated Md assumed to represent 100% available reducing groups.

purified conjugate had a similar level of available amino groups (Table 1). However, conjugate purification did result in a large change in the level of available reducing groups; in comparison to the crude conjugate which had ~74% of the reducing groups of the Md100 available (~26% loss on conjugation), the purified conjugate had only ~24% of the reducing groups of the Md100 available (~76% loss), confirming the removal of a large proportion of the non-conjugated Md100 on purification. The removal of the non-conjugated Md100 was also confirmed by the change in protein content (Table 1); the NaCN–Md100 mixture and crude conjugate each had a protein content of ~45, while the purified conjugate had a protein content of ~84%. The reduction in the available reducing groups combined with the increase in protein content show that batch anion exchange chromatography successfully reduced the concentration of non-conjugated Md100, thus, purifying the NaCN–Md100 crude conjugate.

3.1.3. Conjugate colour

As conjugates are formed *via* the Maillard reaction, the colour of conjugates is very important for potential applications of these ingredients. Previous research (O'Regan & Mulvihill, 2009) has shown that the removal of low molecular weight sugars and dextrans from the Mds by membrane dialysis prior to mixing with NaCN and conjugation significantly reduced the colour that developed on conjugation. As in the previous study (O'Regan & Mulvihill, 2009) conjugation of Md100 with NaCN resulted in an increase in the a^* and b^* values and a decrease in the L^* value in comparison to the NaCN and Md100 (Table 1). Purification of the conjugate resulted in a decrease in both the a^* and b^* values and a slight decrease in the L^* value in comparison to the NaCN–Md100 crude conjugate; these results indicates that purification improved the colour of the conjugate product which may be attributed to the removal of some Maillard products formed during the conjugation process because of their non-adsorption to the ion exchange resin.

3.1.4. SDS–PAGE profile of the conjugates

SDS–PAGE was performed to confirm conjugation and to estimate the molecular size of polymers in the NaCN–Md100 crude conjugate and purified conjugate. The SDS–PAGE gel stained for protein with Coomassie Brilliant Blue stain R-250 is shown in Fig. 1a. The typical monomeric caseins (α_s-, β- and κ-casein) were resolved in the NaCN and NaCN–Md100 mixture (lanes 2, 4). The characteristic native casein bands disappeared on conjugation, with a distinct shift to a broad range of high molecular weight conjugated proteins (lane 5). Some protein staining material present in the NaCN–Md100 crude conjugate also remained at the interface of the stacking and separating gel, which suggests the presence of high molecular weight products that were too large to penetrate the separating gel. Conjugate purification had little effect on the protein stained electrophoretic pattern (lane 6); however, the material that was too large to penetrate the separating gel was essentially absent in the purified conjugate, suggesting that this material which was removed by purification, was primarily composed of high molecular weight Maillard reaction products; removal of this material may coincide with the improvement in the colour of the conjugate on purification as discussed above.

Fig. 1b shows the SDS gel stained for protein and for polysaccharide with Periodic Acid/Fuchsin reagent. The Md present in the native Md100 (lane 3) and in the NaCN–Md100 mixture (lane 4) remained relatively immobile and migrated close to the loading well, while the Md100 present in the NaCN–Md100 crude conjugate (lane 5) and NaCN–Md100 purified conjugate (lane 6) migrated through the gel with the protein as indicated by the migrated purple stain, indicating that conjugation had occurred and that the Md was covalently attached to the NaCN. In comparison to the crude conjugate, the purified conjugate showed no obvious difference in its polysaccharide stained electrophoretic pattern.

3.2. Emulsifying properties of the conjugates

3.2.1. Emulsion storage stability

The fat globule size distribution (Fig. 2a) and mean fat globule size (0.47–0.49 μm) of model oil-in-water emulsions stabilised with NaCN, NaCN–Md100 mixture, crude conjugate and purified conjugate were essentially similar immediately after emulsion preparation. After 20 days of storage at 45 °C, a shift in the fat globule size distribution and an increase in the mean fat globule size was observed for all of the emulsions (Fig. 2b). The NaCN stabilised emulsion was the least stable; there was a large shift in the fat

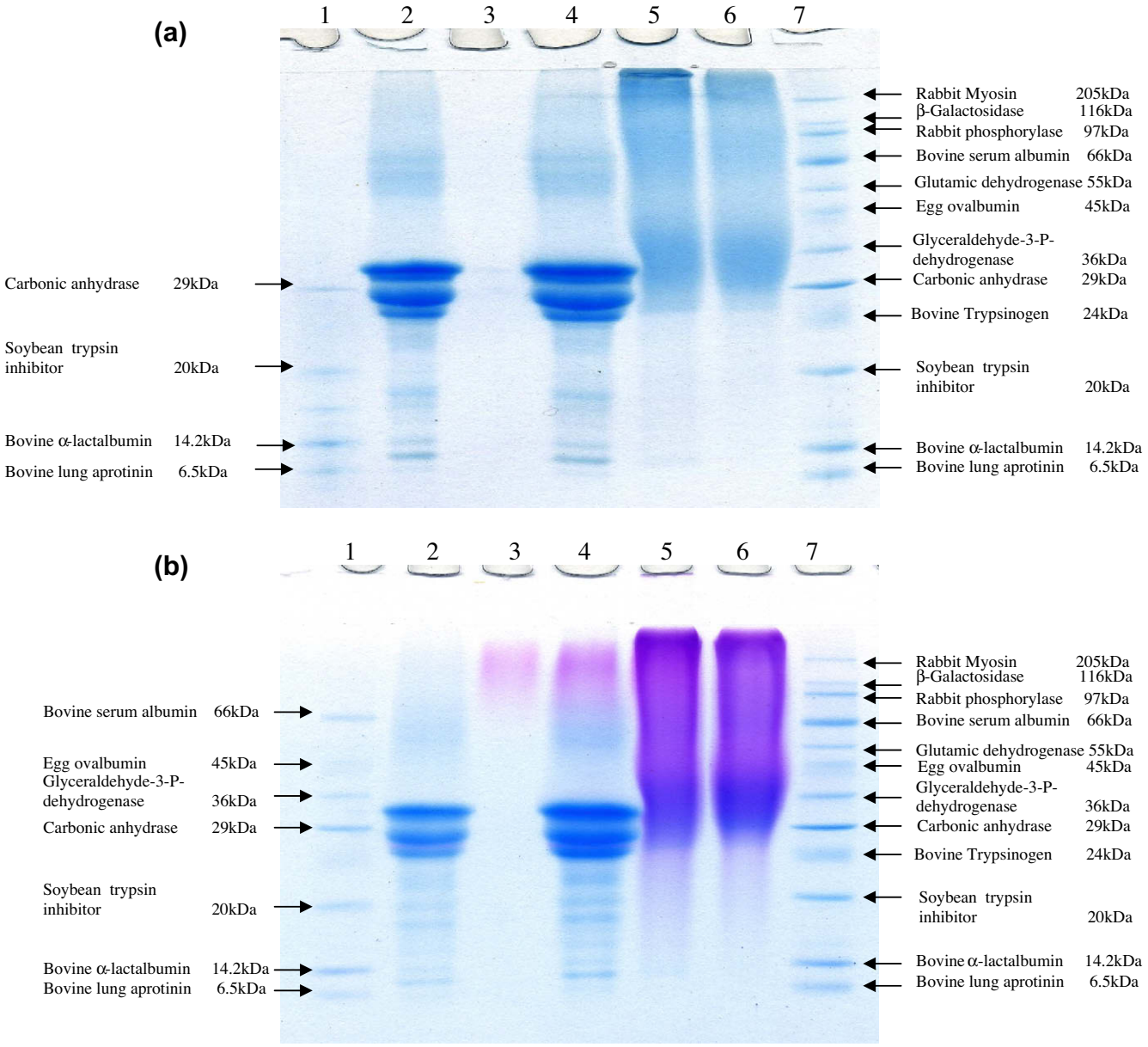

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoretogram stained for protein with Coomasie Brilliant Blue R-250 (a) and stained for protein (as above) and for carbohydrate with Periodic Acid/Fuschin reagent. (b) The labelled lanes are (1) Molecular weight marker (low range); (2) NaCN; (3) Md100; (4) NaCN–Md100 mixture; (5) NaCN–Md100 crude conjugate; (6) NaCN–Md100 purified conjugate; (9) Molecular weight marker (wide range).

globule size distribution and a large increase in the mean fat globule size from 0.49 to 70.7 μm on storage. The NaCN–Md100 mixture stabilised emulsion had a slightly better emulsion stability with an essentially similar shift in fat globule size distribution and a slightly lower increase in mean fat globule size from 0.49 to 61.2 μm; this may be attributed to the presence of non-absorbed Md100 in the continuous phase of the NaCN–Md100 mixture stabilised emulsion which would increase the continuous phase viscosity slightly resulting in reduced Brownian motion of the droplets (O'Regan & Mulvihill, 2009), thus reducing droplet collision frequency and slightly improving storage stability (McClements, 2004). The NaCN–Md100 crude conjugate stabilised emulsion had the smallest shift in fat globule size distribution and mean fat globule size from 0.47 to 0.77 μm and thus had better storage stability than the NaCN and NaCN–Md100 mixture stabilised emulsions. The improved stability of NaCN–Md100 crude conjugate stabilised emulsions is attributed to the conjugated protein molecules forming a more bulky polymeric layer than the non-conjugated protein on the droplet surface, with the Md100 portion protruding outwards into the continuous phase providing better steric stabilisation, thus preventing droplet aggregation and coalescence (Akhtar & Dickinson, 2003; O'Regan & Mulvihill, 2009). This emulsion also had an appreciable level of non-conjugated Md in the continuous phase contributing to enhanced continuous phase viscosity as discussed above. The purified NaCN–Md100 conjugate stabilised emulsion also had a moderate shift in fat globule size distribution and mean fat globule size from 0.47 to 4.13 μm and thus had a better storage stability than the NaCN and NaCN–Md100 mixture stabilised emulsions which is attributed to the conjugated protein molecules at the droplet surface providing better steric stabilisation; however, the purified conjugate stabilised emulsion was less stable than the crude conjugate stabilised emulsion which may be explained by the lower levels of non-conjugated Md in the continuous phase of this emulsion than in the

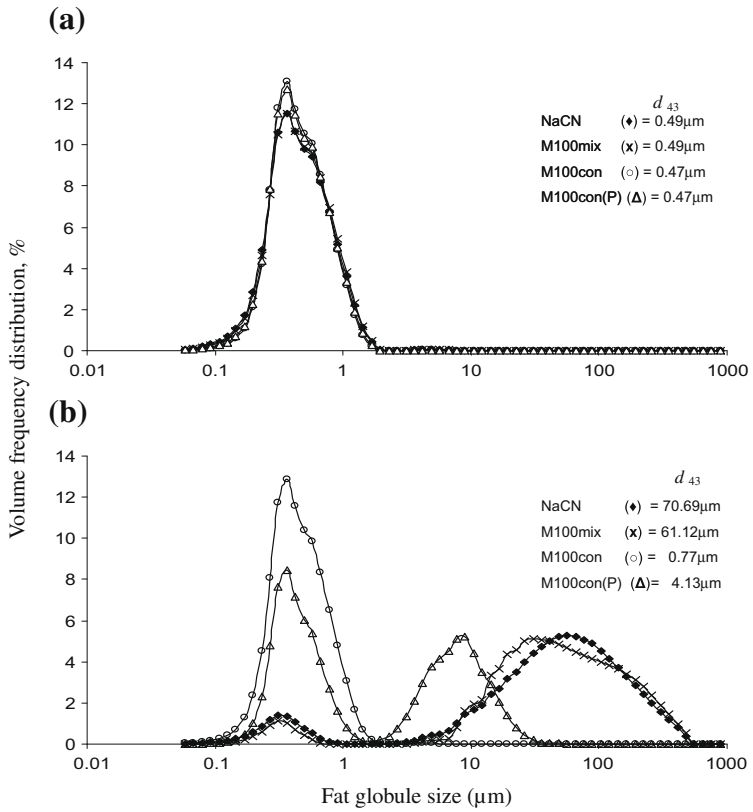

Fig. 2. Fat globule size distribution and mean fat globule size (d_{43}) immediately after emulsification (a) and following storage at 45 °C for 20 days (b) of model o/w emulsions stabilised by NaCN (♦); NaCN–Md100 mixture (×); NaCN–Md100 crude conjugate (○) and NaCN–Md100 purified (P) conjugate (△).

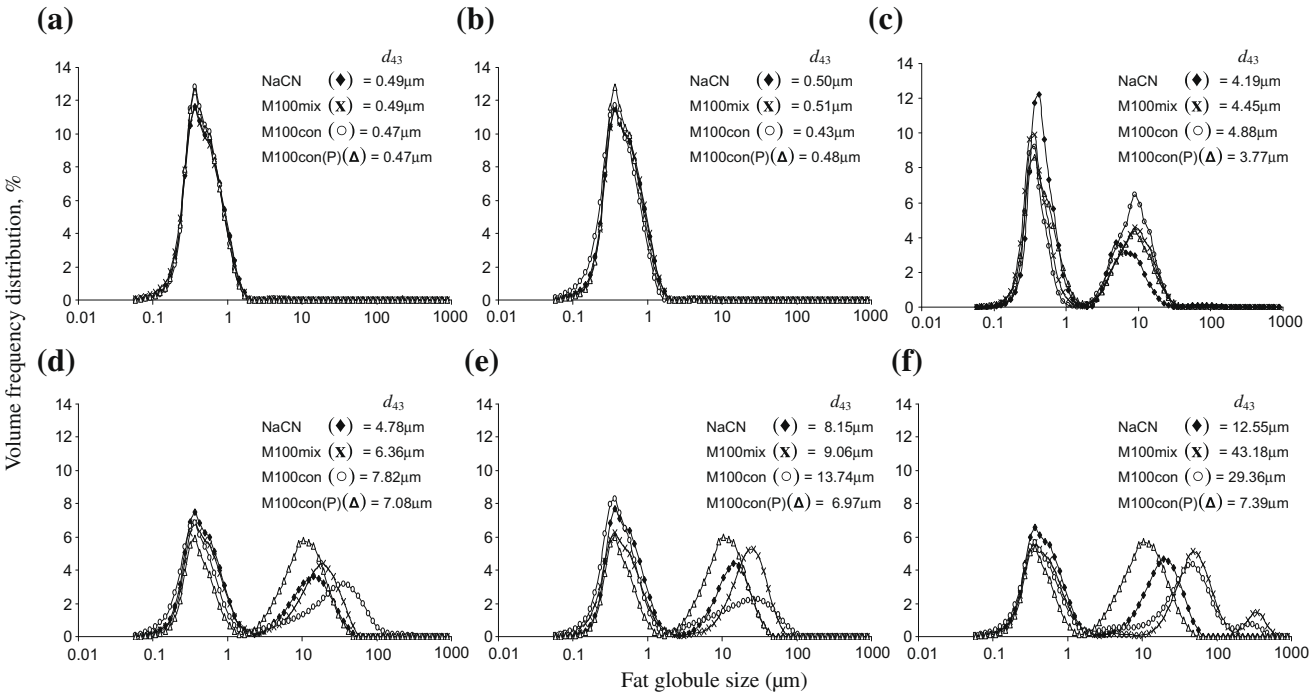

Fig. 3. Fat globule size distribution and mean fat globule size (d_{43}) of model o/w emulsions stabilised with (0.8%, w/w protein) NaCN (♦); NaCN–Md100 mixture (×); NaCN–Md100 crude conjugate (○); NaCN–Md100 purified (P) conjugate (△), prior to heating (a) and following heating at 140 °C for 2.5 min (b), 5 min (c), 10 min (d), 15 min (e) and 20 min (f).

crude conjugate stabilised emulsion, thus contributing less to the enhanced viscosity of the continuous phase and allowing enhanced Brownian motion of the droplets leading to increased droplet–droplet interactions.

3.2.2. Heat stability of o/w emulsions

Conjugation with polysaccharides has been shown to improve the heat stability of whey proteins (Jiménez-Castaño, López-Fandiño, Olano, & Villamiel, 2005; Jiménez-Castaño, Villamiel, & López-Fandiño, 2007), soy protein (Diftis & Kiosseoglou, 2006), ovalbumin (Kato, Aoki, Kato, Nakamura, & Matsuda, 1995) and lysozyme (Aoki et al., 1999; Shu, Sahara, Nakamura, & Kato, 1996). NaCN–Md mixture, crude conjugate and purified conjugate stabilised o/w emulsions were as thermally stable as NaCN stabilised emulsions on heating for up to 2.5 min at 140 °C, as minimal changes in the fat globule size distributions and mean fat globule size were observed (Fig. 3a and b) on heating for up to this time. Extending the heating time to between 5 and 20 min progressively shifted the fat globule size distribution and mean fat globule size to larger distribution/sizes for all of the emulsions. The NaCN stabilised emulsion was more heat stable than both the NaCN–Md100 mixture and crude conjugate stabilised emulsions as the shift in distribution and size was smaller for it than for the other emulsions. This may be attributed to the non-conjugated Md in the continuous phase of the NaCN–Md100 mixture and NaCN–Md100 crude conjugate stabilised emulsions remaining unabsorbed at the droplet interface, and enhancing droplet–droplet interactions by depletion flocculation (Diftis & Kiosseoglou, 2006; Euston, Finnigan, & Hirst, 2002). Also prolonged heating of the non-conjugated Md containing emulsions may have resulted in further progression of the Maillard reaction leading to droplets aggregation/interactions resulting in increases in mean fat globule size. The purified NaCN–Md conjugate stabilised emulsion had better heat stability than the NaCN–Md mixture and crude conjugate stabilised emulsions, which may be attributed to the lower levels of non-conjugated Md in the continuous phase of this emulsion leading to less formation of Maillard reaction products on heating of this emulsion resulting in less droplet aggregation/interactions and thus lower mean fat globule sizes on prolonged heating. The purified NaCN–Md conjugate stabilised emulsion also had better heat stability than the NaCN stabilised emulsion which is attributed to the conjugate protein molecules at the droplet surface providing better steric repulsion and thus improving the emulsion heat stability.

3.2.3. Freeze–thaw stability of o/w emulsions

The influence of freeze–thaw cycling on the fat globule size distribution and mean fat globule size of NaCN, NaCN–Md100 mixture, crude conjugate and purified conjugate stabilised emulsions are shown in Fig. 4. The initial emulsions had similar fat globule size distributions and mean fat globule sizes (Fig. 4a). On progressive freeze–thaw cycling there was a shift in the fat globule size distribution to larger fat globules and an increase in mean fat globule size (Fig. 4b–e). The results show that the NaCN stabilised emulsions were the least stable to freeze–thaw cycling; the NaCN–Md100 mixture stabilised emulsion had the highest stability to the freeze–thaw cycling, while both the NaCN–Md100 crude conjugate and purified conjugate stabilised emulsions had better freeze–thaw stability than the NaCN stabilised emulsion. The results show that the stability of emulsions to freeze–thaw cycling increased with increasing concentrations of non-conjugated Md in the continuous phase of the emulsion.

On freezing of an o/w emulsion the following physiochemical processes that promote emulsion destabilisation, may occur: (i) ice crystals form which results in the oil droplets being forced closer together; (ii) as a result of the ice formation there may be insufficient free water to maintain the emulsifier molecules at the surface of the oil droplets fully hydrated; (iii) ice crystallisation leads to an increase in the concentration of soluble constituents in the non-frozen aqueous phase surrounding oil droplets (freeze concentration), any increase in ion concentration would screen electrostatic charge repulsion forces between the oil droplets; (iv) ice crystals formed on freezing may penetrate and disrupt the interfacial membrane surrounding the oil droplets leading to coalescence of the droplets on thawing; (v) the fat phase within the droplets may solidify and protruding fat crystals may penetrate and disrupt the interfacial membrane surrounding the oil droplets

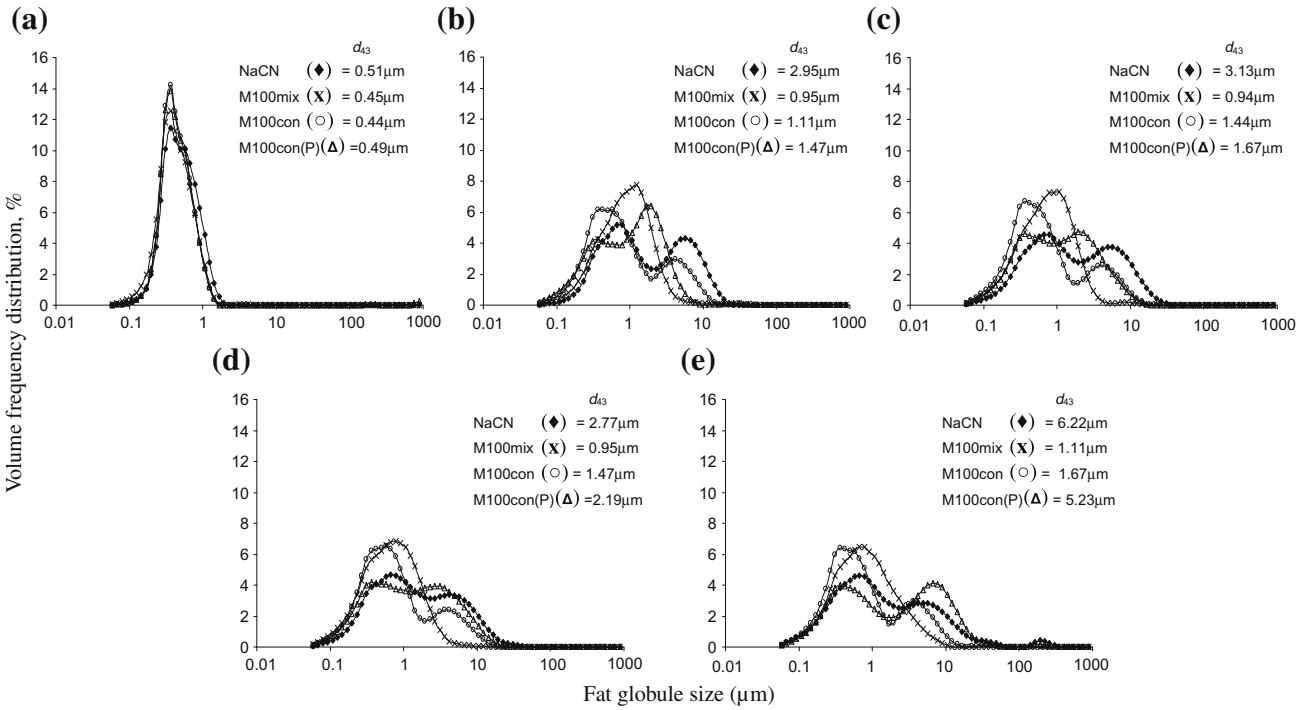

Fig. 4. Fat globule size distribution and mean fat globule size (d_{43}) of model o/w emulsions stabilised with (0.6%, w/w, protein) NaCN (♦); NaCN–Md100 mixture (×); NaCN–Md100 crude conjugate (○); NaCN–Md100 purified (P) conjugate (△), on manufacture (a) and following 1 (b), 2 (c), 3 (d), and 4 (e) freeze–thaw cycles.

leading to partial coalescence of the oil droplets at low temperatures and full coalescence on thawing (McClements, 2004).

The aqueous phase used to prepare all of the emulsions used in the freeze–thaw studies contained 0.6%, w/w, NaCN; the aqueous phase used to prepare the NaCN stabilised emulsion contained no Md; the aqueous phase used to prepare the NaCN–Md100 mixture stabilised emulsion contained a total level of 0.6% Md all of which was free in the aqueous phase of the emulsion; the aqueous phase used to prepare the NaCN–Md crude conjugate stabilised emulsion also had a total level of 0.6% Md with ~74% (~0.44%, w/w) of this free in the aqueous phase of the emulsion and ~26% (~0.16%, w/w) conjugated to NaCN. If an assumption is made that the aqueous phase used to prepare the NaCN–Md purified conjugate stabilised emulsion contained the same level of Md conjugated to NaCN as in the crude conjugate stabilised emulsion while it contained ~24% as much Md free in the aqueous phase as in the NaCN Md100 mixture stabilised emulsion, then it has a total Md level of ~0.3%, w/w, with ~0.16%, w/w, conjugated to casein and ~0.14%, w/w, free in the aqueous phase of the emulsion. Therefore, the emulsions stabilised by NaCN, NaCN–Md purified conjugate, NaCN–Md crude conjugate and NaCN–Md100 mixture had ~0.0%, 0.14%, 0.44% and 0.60%, w/w, Md, respectively, free in the aqueous phase of their, respectively stabilised emulsions.

Because of the low overall concentrations of Md free in the aqueous phases of the emulsions and its high molecular weight, the differences in the level of Md free in the aqueous phases of the different emulsions would have had little effect of the initial freezing temperature of the aqueous phases of the emulsions. On freezing of the emulsions, Md free in the aqueous phases would have become more concentrated in the unfrozen aqueous phase of all the emulsions and this may have eventually led to sufficient differences in levels of Md that would have led to differences in freezing temperature depression and thus slight differences in levels of unfrozen water in the aqueous phases of the different emulsions (Chronakis, 1998; Radosta & Schierbaum, 1990). Also, because of Md hydration, the level of water remaining unfrozen in the aqueous phases of the emulsions would increase with increasing concentrations of Md free in the aqueous phase of the emulsions (Chronakis, 1998). As even a small difference in the volume of water remaining unfrozen has been shown to contribute to differences in freeze–thaw stability of emulsions (Ghosh, Cramp, & Coupland, 2006), the differences observed in freeze–thaw stability in this study may be partly attributed to the above effects of different levels of free Md on the level of unfrozen water. Any increase in the volume of water remaining unfrozen around emulsion droplets would enhance emulsion freeze–thaw stability by (i) maintaining a larger hydration space around the oil droplets and thus reducing the compressive force on the droplets on freezing (Ghosh et al., 2006); (ii) maintaining ice crystals formed on freezing further away from the oil droplet interfacial membrane and thus preventing rupture of the membrane (Mun et al., 2008); (iii) maintaining the protein emulsifier at the oil droplet interface more fully hydrated (Gu, Decker, & McClements, 2007); (iv) reducing the freeze concentration effect and thus reducing any screening of electrostatic charge repulsion forces between the droplets (Thanasukarn, Pongsawatmanit, & McClements, 2004).

The NaCN–Md conjugate absorbed at the interface of the oil droplet also partly contributed to the improved freeze–thaw stability of the crude conjugate and purified conjugate stabilised emulsions in comparison to the NaCN stabilised emulsion. The NaCN–Md conjugate molecules form a more bulky polymeric layer than the non-conjugated protein on the oil droplet surface, with the Md portion extruding outwards into the continuous phase providing better hydrodynamic and steric stabilisation (Akhtar & Dickinson, 2003), thus preventing the oil droplets from coming close enough to coalesce when droplets are forced together on freezing. The bulky polymeric layer of conjugated protein at the oil droplet surface would also make it more difficult for any fat crystals formed on freezing to penetrate and rupturing the interfacial membrane surrounding the oil droplets, thus preventing partial coalescence on freezing and full coalescence on thawing (Hagiwara, Hartel, & Matsukawa, 2006). However, the formation of fat crystals on freezing of the emulsions used in this study would have been minimal as the bulk freezing temperature of oil phase used in the emulsions was −20 °C and emulsified oil has been shown to have a significantly lower freezing temperature than oil in the bulk form (Dickinson & McClements, 1996). Thus, the conjugated Md at the droplet surfaces partially contributed to the improved freeze–thaw stability of the conjugated stabilised emulsions by hydrodynamic and steric stabilisation effects rather that by inhibiting partial coalescence because of fat crystallisation.

4. Conclusion

This study shows that a NaCN–Md100 conjugate, produced *via* a non-toxic, naturally occurring Maillard-type reaction, can be purified to reduce the level of non-conjugated Md by a batch anion exchange chromatography process. Purification of the conjugate by this method also reduced the level of Maillard products and produced a conjugate with a neutral colour, which may be an advantage for use in many food applications.

Oil-in-water emulsions stabilised by the crude or purified conjugate had improved storage stability in comparison to NaCN and NaCN–Md mixture stabilised emulsions after storage for 20 days at 45 °C. Purified conjugate stabilised emulsions had better thermal stability in comparison to the NaCN, crude conjugate and NaCN–Md100 mixture stabilised emulsions. Both the crude conjugate and purified conjugate stabilised emulsions also had better stability to freeze–thaw cycling than NaCN stabilised emulsions. Overall, both the crude and purified NaCN–Md100 conjugates had better emulsifying properties than NaCN; this indicates a potential for these conjugates as effective emulsifiers in formulating food emulsion products.

References

AOAC (1995). *Official methods of analysis* (16th ed.). Arlington, VA, USA: Association of Official Analytical Chemists (chapter 33, pp. 1–75).

Akhtar, M., & Dickinson, E. (2003). Emulsifying properties of whey protein–dextrin conjugates at low pH and different salt concentrations. *Colloids and Surfaces B: Biointerfaces, 31*, 125–132.

Ames, J. M. (1992). The Maillard reaction. In B. J. F. Hudson (Ed.), *Biochemistry of food proteins* (pp. 99–153). London: Elsevier Science Publishers.

Aoki, T., Hiidome, Y., Kitahata, K., Sugimoto, Y., Ibrahim, H. R., & Kato, Y. (1999). Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. *Food Research International, 32*, 129–133.

Appelqvist, I. A. M., Golding, M., Vreeker, R., & Zuidam, J. (2007). Emulsions as delivery systems in foods. *Encapsulation and Controlled Release Technologies in Food Systems, 3*, 41–81.

Chronakis, I. S. (1998). On the molecular characteristics, composition properties, and structural–functional mechanisms of maltodextrins: A review. *Critical Reviews in Food Science, 38*, 599–637.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17*, 25–39.

Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids, 23*, 1473–1482.

Dickinson, E., & Galazka, V. B. (1991). Emulsion stabilisation by ionic and covalent complexes of β-lactoglobulin with polysaccharides. *Food Hydrocolloids, 5*, 281–296.

Dickinson, E., & McClements, D. J. (1996). Fat crystallization in oil-in-water emulsions. In *Advances in food colloids* (pp. 211–246). UK, Glasgow: Blackie Academic & Professional, Chapman & Hall.

Diftis, N., & Kiosseoglou, V. (2006). Physicochemical properties of dry-heated soy protein isolate-dextrin mixtures. *Food Chemistry, 96*, 228–233.

Euston, S. R., Finnigan, S. R., & Hirst, R. L. (2002). Kinetics of droplet aggregation in heated whey protein-stabilised emulsions: Effect of polysaccharides. *Food Hydrocolloids, 16*, 499–505.

Ghosh, S., Cramp, G. L., & Coupland, J. N. (2006). Effects of aqueous composition on the freeze thaw stability of emulsions. *Colloids and Surfaces A: Physicochemical Engineering Aspects, 272*, 82–88.

Gu, Y. S., Decker, E. A., & McClements, D. J. (2007). Application of multi-component biopolymer layers to improve the freeze–thaw stability of oil-in-water emulsions: β-Lactoglobulin-ι-carrageenan-gelatin. *Journal of Food Engineering, 80*, 1246–1254.

Hagiwara, T., Hartel, R. W., & Matsukawa, D. J. (2006). Relationship between recrystallization rate of ice crystals in sugar solutions and water mobility in freeze-concentrated matrix. *Food Biophysics, 1*, 74–82.

Jiménez-Castaño, L., López-Fandiño, R., Olano, A., & Villamiel, M. (2005). Study on β-lactoglobulin glycosylation with dextran: Effect on solubility and heat stability. *Food Chemistry, 93*, 689–695.

Jiménez-Castaño, L., Villamiel, M., & López-Fandiño, R. (2007). Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. *Food Hydrocolloids, 21*, 433–443.

Kato, A. (2002). Industrial applications of Maillard-type protein–polysaccharide conjugates. *Food Science and Technology Research, 8*, 193–199.

Kato, A., Aoki, T., Kato, N., Nakamura, R., & Matsuda, T. (1995). Modification of ovalbumin with glucose-6-phosphate by amino-carbonyl reaction. Improvement of protein heat stability and emulsifying activity. *Journal of Agricultural and Food Chemistry, 43*, 301–305.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head to bacteriophage T4. *Nature, 227*, 680–685.

Lynch, A. G., & Mulvihill, D. M. (1997). Effect of sodium caseinate on the stability of cream liqueurs. *International Journal of Dairy Technology, 50*, 1–7.

McClements, D. J. (2004). *Food emulsions: Principles practice and techniques* (2nd ed.). Boca Raton: CRC Press.

McSweeney, S. L. (2008). Stability of model ready-to-feed infant formula emulsions. Ph.D. thesis, National University of Ireland, University College Cork, Cork, Ireland.

McSweeney, S. L., Mulvihill, D. M., & O'Callaghan, D. M. (2004). The influence of pH on the heat induced aggregation of model like protein ingredient systems and model infant formula emulsions stabilised by milk protein ingredients. *Food Hydrocolloids, 18*, 109–125.

Mun, S., Younghee, C., Decker, E. A., & McClements, D. J. (2008). Utilization of polysaccharide coatings to improve freeze–thaw and freeze-dry stability of protein-coated lipid droplets. *Journal of Food Engineering, 86*, 508–518.

Neirynck, N., Van der Meeren, P., Bayarri Gorbe, S., Dierckx, S., & Dewettinck, K. (2004). Improved emulsion stabilizing properties of whey protein isolate by conjugation with pectins. *Food Hydrocolloids, 18*, 949–957.

Oliver, C. M., Melton, L. D., & Stanley, R. A. (2006). Creating proteins with novel functionality via the Maillard reaction. *Critical Reviews in Food Science and Nutrition, 46*, 1–14.

O'Regan, J., & Mulvihill, D. M. (2009). Preparation, characterisation and selected functional properties of sodium caseinate–maltodextrin conjugates. *Food Chemistry, 115*, 1257–1267.

Radosta, S., & Schierbaum, F. (1990). Polymer–water interactions of maltodextrins. Part III. Non-freezable water in maltodextrin solutions and gels. *Starch/Stärke, 42*, 142–147.

Semenova, M. G., Belyakova, L. E., Antipova, A. S., & Jubanova, M. A. (1999). Influence of maltodextrins with different dextrose equivalents on the thermodynamic properties of legumin in a bulk and at the air–water interface. *Colloids and Surfaces B: Biointerfaces, 12*, 287–297.

Shepherd, R., Robertson, A., & Ofman, D. (2000). Dairy glycoconjugate emulsifiers: Casein-Maltodextrins. *Food Hydrocolloids, 14*, 281–286.

Shu, Y. W., Sahara, S., Nakamura, S., & Kato, A. (1996). Effects of the length of polysaccharide chains on the functional properties of the Maillard-type lysozyme–polysaccharide conjugate. *Journal of Agricultural and Food Chemistry, 46*, 2433–2438.

Thanasukarn, P., Pongsawatmanit, R., & McClements, D. J. (2004). Impact of fat and water crystallization on the stability of hydrogenated palm oil-in-water emulsions stabilised by whey protein isolate. *Colloids and Surfaces A: Physicochemical Engineering Aspects, 246*, 49–59.

Thornton, D. J., Carlstedt, I., & Sheehan, J. K. (1994). Identification of glycoproteins on nitrocellulose membranes and gels. In J. M. Walker (Ed.), *Methods in molecular biology. Basic protein and peptide protocols* (Vol. 32, pp. 119–128). Totowa, NJ: Springer.

Wei, T. M., & Whitney, R. McL. (1985). Batch fractionation of bovine caseins with diethylaminoethyl cellulose. *Journal of Dairy Science, 68*, 1630–1636.