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Pilot scale production of a phospholipid-enriched dairy ingredient by means of an optimised integrated process employing enzymatic hydrolysis, ultrafiltration and super-critical fluid extraction

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ABSTRACT

Pilot scale production of a dairy ingredient enriched in phospholipids (PLs) was generated from a buttermilk powder (BMP) substrate utilising a combined process of targeted enzymatic hydrolysis of the innate milk proteins followed by ultrafiltration with a 50 kDa membrane. An 8.5 fold increase in PL was achieved in the 50 kDa retentate (50 R) compared to the BMP, 11.05 ± 0.02% and 1.30 ± 0.00% total PL, respectively. Simultaneously, total lipid content in the retentate increased 8.7 fold with reference to the BMP, 60.07 ± 0.54% and 6.84 ± 0.17% total lipid respectively. Protein reduced to 10.58 ± 0.09% (50 R) from 31.40 ± 0.57% in BMP. Supercritical CO_2 fluid extraction (SFE) was employed to generate a purified lipid fraction. SFE with ethanol as a co-solvent yielded a purified lipid extract with enriched PLs level of 56.24 ± 0.07% on a dry matter basis.

Industrial relevance: PLs have many associated health and nutritional benefits including those related to cognitive development and repair. Generation of an ingredient enriched in dairy PLs would be advantageous from an industrial view to allow fortification of nutritionals, both infant and geriatric, in promoting brain health. The present work demonstrates a novel pilot scale process for the generation of a PL enriched ingredient from a BMP substrate. Utilising a combined process of targeted protein hydrolysis followed by selective removal by ultrafiltration of the smaller molecular weight peptide material, an ingredient with 8.5 fold increase in PL material was achieved. SFE technology was utilised to generate a purified lipid extract with greater PL levels for future applications in biological assays to determine these pathways. The need for investigate and further develop the knowledge relating to the modes of action of these bioactive compounds would be beneficial from a nutritional perspective.

1. Introduction

The milk fat globule membrane (MFGM) is a unique biophysical system (Lopez, 2011; Singh, 2006) surrounding milk fat globules that facilitates their dispersal in milk. This surface active multi-layered membrane is derived from the apical membrane of the secretary cell in the mammary gland (Heid & Keenan, 2005) and is composed of numerous bioactive molecules of which 90% of MFGM dry weight is represented by proteins and polar lipids (Lopez, 2011). Phospholipids (PLs), a complex class of polar lipids, whose structure comprises of a hydrophilic head group and a hydrophobic fatty acid tail (Contarini & Povolo, 2013), are amphiphilic in nature. This distinctive polar property is responsible for the structural architecture of MFGM which, in turn, aids in the emulsification of fat in milk (Barry, Dinan, Murray, & Kelly, 2016; Contarini & Povolo, 2013; Sánchez-Juanes, F., Alonso, J. M., Zancada, L.,, & Hueso, P., 2009).

Buttermilk, an aqueous serum phase released during cream de-emulsification/churning, possesses PL concentrations approx. 15 × that of whole milk (Barry et al., 2016; Rombaut, van Camp, & Dewettinck, 2005) and, hence, is a preferred substrate for the PL enrichment. Dairy PLs are of considerable interest due to their particular prominence of sphingomyelin (SM) and phosphatidylserine (PS) (Burling & Graverholt, 2008). The technological and nutritional functionalities of PLs have been extensively studied (Contarini & Povolo, 2013; Dewettinck et al., 2008). PLs, due to their emulsification and stabilisation properties afforded by their inherent amphiphilic nature are widely utilised by the food and dairy industry for their associated physiochemical roles in dairy products that include emulsification, heat stability, wettability

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and viscosity (Contarini & Povolo, 2013; Corredig & Dalgleish, 1997; Corredig & Dalgleish, 1998; Dewettinck et al., 2008).

Likewise, there have been numerous health and nutritional benefits associated with PLs e.g. reduction in the incidence of cardiovascular disease, tolerance of depression and stress and myelination of the central nervous system (Contarini & Povolo, 2013; Dewettinck et al., 2008; Oshida et al., 2003). Sphingomyelin (SM), mainly through its sphingosine and ceramide metabolites, has been shown to affect anticancer activity notably in the suppression of colon tumours (Berra, Colombo, Scottocornola, & Giacosa, 2002; Hertervig, Nilsson, Cheng, & Duan, 2003; Kuchta- Noctor, A. M., Murray, B. M., Stanton, C., Devery, R., & Kelly, P. M., 2016; Spitsberg, 2005). The biological activity of PLs has also been associated with cognitive development and repair. Phosphatidylserine, pre-eminently, has been implicated in the treatment of Alzheimer's disease (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Dewettinck et al., 2008). In more recent years, studies on infant milk formula fortified with PLs have proved positive in terms of immunological defences (Timby et al., 2015), and also infant neurodevelopment (Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014). Thus, there is considerable interest and demand for the generation of PL concentrates from dairy sources.

Technological approaches utilised to concentrate PLs in buttermilk (BM) have been primarily membrane filtration based, particularly microfiltration (MF) (Astaire, Ward, German, & Jiménez- Flores, R., 2003; Corredig, Roesch, & Dalgleish, 2003; Holzmüller & Kulozik, 2016; Morin, Britten, & Jiménez- Flores, R.,, & Pouliot, Y., 2007; Morin, Pouliot, & Jiménez- Flores, R., 2006) due to its greater porosity. However, similarity in the size of the MFGM components and casein (Morin et al., 2007) limited the effectiveness of MF as a selective separation process. Complementary measures adopted to enhance MF include: cream washing prior to ultrafiltration (UF) (Morin et al., 2007), rennet induced coagulation of the caseins prior to MF (Sachdeva & Buchheim, 1997), and disruption of the casein micelle with citrate prior to MF (Corredig et al., 2003; Roesch, Rincon, & Corredig, 2004; Rombaut, Dejonckheere, & Dewettinck, 2006). A more recent avenue of investigation employed the use of enzymes to pre-digest the milk proteins prior to UF (Barry, Dinan, & Kelly, 2017). Hydrolysis of milk proteins prior to UF enables selective transmission of smaller peptides through the membrane, thus, selectively concentrating PLs in the retentate. Barry et al. (2017) reported that PLs losses through the UF membrane using this novel process were non-detectable.

A more advanced separation technology in the form of supercritical fluid extraction (SFE) has been periodically investigated to selectively extract unique higher value lipids, in particular from primary food substrates including BM sources (Astaire et al., 2003; Costa, & Elias-Argote, X. E.,, & Jiménez- Flores, R., 2010; Spence, & Jiménez- Flores, R., Qian, M.,, & Goddik, L., 2009). Astaire et al. (2003) were able to selectively remove non-polar lipids from the buttermilk by coupling MF with SFC, thus, allowing concentration of the polar lipids in the buttermilk. Spence, and Jiménez- Flores, R., Qian, M.,, and Goddik, L. (2009) concentrated dairy PLs 5-fold when applying SFC to MF pre-processed samples.

The objective of the present study was to apply SFE while upscaling a laboratory-based enzymatic hydrolysis/ultrafiltration process previously outlined by the authors (Barry et al., 2017), in order to produce a purified milk lipid extract with elevated levels of PLs relative to non-polar milk lipids.

2. Materials and methods

2.1. Materials

Spray dried buttermilk powder (BMP) was sourced from Tipperary Co-operative (Tipperary, Ireland) with compositional analysis by standard IDF protocols determining 6.84% fat, 31.40% protein, 48.00% lactose, and 7.40% minerals. The digestive enzyme Alcalase (E.E. 3.4.21.62) was procured from Novozymes (Bagsværd, Denmark). Alcalase is a serine type endoprotease with esterase activity enabling it to catalyse the stereoselective hydrolysis of amino esters and selective esters. Alcalase has a minimum activity of 2.4 Anson units per gram of composition (2.4 AU-A g^{-1}) at pH 7.50, with optimal conditions for alcalase activity within 35–60 °C and pH 7–9. Synder PES spiral wound membranes with a nominal molecular weight cut off (MWCO) of 50 kDa were purchased from David Kellett & Partners Ltd. (Hereford, UK). Acetonitrile was procured from ThermoFisher Scientific (Waltham, MA, USA) and all other chemicals were HPLC grade, > 99.9%, and purchased from Sigma Aldrich (Arklow, Ireland).

2.2. Enzymatic hydrolysis of BMP substrate protein fraction

The degree of hydrolysis (% DH) is interpreted as the number of peptide bonds (*h*) that are cleaved as a percentage of the total peptide bonds (*htot*) all of which is related to the consumption of base as a result of proton release during the hydrolysis reaction according Adler-Nissen's (1986) equation (Eq. (1)).

$$\%DH = 100 \times B \times N_B \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \quad (1)$$

where, B is the volume of base in mL, N_B is the normality of the base, 1/α is the average degree of dissociation of the α-NH groups (= 1.13 at pH 8.00, 50 °C), MP is the molecular weight of the protein and *htot* is the number of peptide bonds in the protein substance (= 8.2 for casein/whey mix).

For all hydrolysis reactions, BMP was reconstituted to 10% total solids in reverse osmosis (RO) water, 360 Kg total weight (37.29 Kg BMP and 322.7005 kg RO water), and allowed to hydrate over night at 4 °C with gentle agitation. The BMP dispersions were heated gently to 50 °C with a water-jacket (to avoid burn on of the BMP to the walls of the tank) and subsequently pH corrected with 4 M NaOH to pH 8.00 (original pH 6.45). The hydrolysis reaction was initiated by addition of alcalase dissolved in RO water at an enzyme to substrate ratio (E:S) of 1: 100, w w^{-1}, (1%) on a protein equivalent basis with the temperature, 50 °C, and pH, pH 8.00, maintained throughout the reaction. The pH was controlled by addition of 4 M NaOH and the reaction was agitated throughout utilising the inherent tank impeller. The hydrolysis reaction was terminated by means of pH inactivation with 2 M citric acid until a pH of 6.5 was achieved. All hydrolysis reactions were performed in duplicate.

Determination of the end-point DH was measured by modification of the trinitrobenzenesulfonic acid (TNBS) reaction outlined by Adler-Nissen (1979) which involves measuring the reaction of liberated α-amino groups (α-NH_2) with 2, 4, 6-trinitrobenzene 1- sulfonic acid. Briefly, samples with a protein concentration of 3 mg mL^{-1} were prepared in 1% SDS, 1% SDS alone serving as a blank, and 0.2125 M phosphate buffer was added. 2 mL of TNBS was added to both the samples and blank with light omitted, and the reaction was incubated for 1 h at 50 °C. 4 mL of 0.1 M HCl was added to terminate the reaction and the samples were cooled for 30 min prior to absorbance at 314 nm being read using a Varian Cary 1 dual beam UV-visible spectrometer (Varian Ltd., Walton-on-Thames, U·K). UV readings were determined against a standard curve generated using L-leucine at concentrations ranging from 20 to 250 mg L^{-1}. DH values were calculated using the following equation (Spadaro, Draghetta, del Lama, Camargo, & Greene, 1979) (Eq. 2)

$$\%DH = \frac{AN_2 - AN_1}{N_{pb}} \times 100 \quad (2)$$

where, AN_1 represents the amino nitrogen content of the protein substrate (unhydrolysed) (mg g^{-1} protein), AN_2 represents the amino nitrogen content of the hydrolysed protein substrate and Npb represents the nitrogen content of the peptide bonds on the protein substrate (mg