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# Formation of whey protein isolate hydrolysate stabilised nanoemulsion

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ABSTRACT

Whey proteins and their hydrolysates are valued as important emulsifiers in foods. The purpose of this study was to produce oil-in-water nanoemulsions using whey protein isolate (WPI) hydrolysate as the emulsifier. Factors including the oil phase concentration (4 and 10% [w/w]), enzyme type (chymotrypsin and pepsin), hydrolysate emulsifier concentration (1–4% [w/w]), storage temperature (4 and 25 °C), and storage time (0–7 days) were investigated to determine their effects on the nanoemulsifying ability of WPI hydrolysate. Chymotrypsin–WPI hydrolysate formed nanoemulsion ($d$ = 287.9–192.5 nm) whose diameter decreased as the hydrolysate concentration in the aqueous phase was increased from 1 to 4%, whereas pepsin WPI hydrolysate did not form nanoemulsions ($d$ = > 3900 nm) due to lack of electrostatic repulsive forces between the peptide that makeup the pepsin WPI hydrolysate. However, the droplet sizes of the chymotrypsin nanoemulsions were larger than those formed by unhydrolysed WPI ($d$ = 160.7 nm) and Tween 20 ($d$ = 176.7 nm) but were still within the nanometre range. Additionally, the WPI hydrolysate was a better emulsifier in nanoemulsions at low oil concentration (4%) than at high oil concentration (10%), and better storage properties (minimal changes in droplet size and creaming stability) were exhibited at 4 °C than at 25 °C. This study demonstrated that WPI hydrolysates can be employed as emulsifiers in nanoemulsions that are tailored for food applications.



## 1. Introduction

Nanoemulsions are a nanotechnology with potential food applications. They consist of small droplets covering the size range of about 10–200 nm. This small droplet size allows for efficient and controlled delivery, encapsulation, enhanced solubility, bioavailability and bioaccessibility of various lipophilic food components, such as β-carotene, vitamin E, polyunsaturated fatty acids, antimicrobial and various phyto-phenolic compounds (Donsì, Annunziata, Vincensi, & Ferrari, 2012; Qian & McClements, 2011; Relkin, Jung, & Ollivon, 2009). Nanoemulsion systems tend to be optically transparent or slightly translucent because the dimensions of the droplets are usually smaller than the wavelength of light, so that they scatter light weakly (Lee & McClements, 2010). Consequently, nanoemulsions may be used to incorporate lipophilic bioactive compounds into food beverages and gels without a loss of clarity (Kentish et al., 2008; Lee & McClements, 2010).

The formation and functionality of nanoemulsion systems is dependent on the ingredients used to make the emulsion. Various lipid phases ranging from short to long chain triacylglycerol oils, essential oils and flavour oils have been used to produce nanoemulsions for food and beverage applications (Ahmed, Li, McClements, & Xiao, 2012; McClements & Rao, 2011). More importantly, the type of emulsifier used to stabilise the droplets may also determine the properties of the nanoemulsion (e.g. droplet size, optical and rheological properties), their food applications and other functionalities (e.g. digestibility, transport and control release of active compounds, encapsulation efficiency). To date, most studies on nanoemulsions have focused on the synthetic-low molecular weight emulsifiers/co-emulsifier, such as Tweens and the sugar esters of fatty acids (Kentish et al., 2008; Qian, Decker, Xiao, & McClements, 2012; Rao & McClements, 2011) although nanoemulsions stabilised by proteins and polysaccharides have also been prepared (Lee & McClements, 2010; Qian & McClements, 2011; Relkin, Perla, Shukat, Bourgaux, &

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Meneau, 2011). The synthetic-low molecular weight emulsifiers tend to possess superior interfacial properties when compared to the biopolymers. However, proteins and polysaccharides have greater appeal as emulsifiers in food systems as they may be considered more "natural".

Proteins are important emulsifiers in the food industry because they are amphiphilic (possess both hydrophobic and hydrophilic residues) and surface active, and are capable of forming strong and cohesive membranes around droplets that are sufficient to stabilise emulsion droplets (Lam & Nickerson, 2013). Proteins mainly stabilise emulsion droplets through electrostatic repulsion due to the presence of charged groups on the surface of proteins, as well as by steric effects (McClements, 2005). Proteins such as casein (Relkin et al., 2009; Relkin, Yung, Kalnin, & Ollivon, 2008), whey protein isolate–WPI (Lee & McClements, 2010; Relkin, et al., 2011), whey protein concentrate–WPC (Jafari, He, & Bhandari, 2006), and soy and pea proteins (Chu, Ichikawa, Kanafusa, & Nakajima, 2007; Donsì et al., 2012) have been used to make nanoemulsions tailored for food applications.

Whey proteins (α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoferrins, and immunoglobulins) constitute about 20% of the total protein in milk (~80% caseins) and are valued as important emulsifiers in food due to their amphiphilic properties (Foegeding, Davis, Doucet, & McGuffey, 2002). Limited hydrolysis of whey proteins has been used to produce peptides that are smaller in size, have fewer secondary and tertiary structures, have partially exposed hydrophobic core, and exhibit modified emulsifying properties (Gauthier & Pouliot, 2003; Tirok, Scherze, & Muschiolik, 2001). In general, the hydrolysates have a higher rate of diffusion to the oil/water interface and cover a larger area of the interface than the native protein (Davis, Doucet, & Foegeding, 2005; O'Regan & Mulvihill, 2010). Furthermore, peptides have a high nutritional value, are easy to digest and absorb, and are less allergenic. These characteristics are crucial for infant formulae, sports nutrition diets and also in parental nutrition (Tirok et al., 2001). Nanoemulsion studies using whey proteins and proteins in general have focussed on the native protein but not their hydrolysates.

The purpose of this study was to investigate the emulsifying ability of WPI hydrolysates in nanoemulsions for possible application in food manufacture. Factors including enzyme type (chymotrypsin and pepsin), hydrolysate emulsifier concentration, oil phase concentration, and storage temperature and time were investigated in order to ascertain the potential of WPI hydrolysate as an emulsifier in food nanoemulsions. The knowledge gained from this study may increase the utilisation of WPI hydrolysates and hydrolysates from other food protein sources as emulsifiers in nanoemulsions tailored for food applications.

## 2. Materials and methods

### 2.1. Materials

Food grade whey protein isolate (WPI; Batch: 1412.11, MyoPure Pty Ltd., Petersham, Australia) and canola oil were used in these trials. α-Chymotrypsin (C4129), pepsin (P6887) and Tween 20 (P1379) were obtained from Sigma–Aldrich (Sydney, Australia). All other chemicals were of reagent grade.

### 2.2. Preparation of WPI hydrolysates

WPI was suspended in phosphate buffer (10 mM, pH 7) at a concentration of 5% (w/v), and allowed to hydrate for 30 min at 37 °C with constant stirring. The suspensions were then adjusted to the working pH of the enzymes (pH 2.6 for pepsin and 7.8 for chymotrypsin) with either 2 M HCl or NaOH. Enzymes were added at an enzyme: substrate ratio of 1:40 and the suspensions were incubated for 3 h with constant stirring and without pH adjustment. After the hydrolysis, the hydrolysates were heated at 90 °C for 15 min to inactivate the enzymes. The suspensions were allowed to cool down to room temperature, freeze dried and stored at −20 °C. The degree of hydrolysis (DH) was determined by the OPA method as described by Nielsen, Petersen, and Dambmann (2001).

The molecular weight (MW) distribution of hydrolysates was analysed by size exclusion chromatography under isocratic conditions using a Shodex Protein KW-802.5 column (8.0 mm × 300 mm) fitted with a Protein-Pak 125 Sentry Guard Column (Waters Pty, Sydney, Australia) on an HPLC system as previously described (Adjonu, Doran, Torley, & Agboola, 2013).

The zeta potential of WPI hydrolysates was determined by dispersing hydrolysates in phosphate buffer (10 mM, pH 7) without pH adjustment at concentrations of 1, 2 and 4%. After mixing, the solutions were centrifuge at 4000 *g* to remove insoluble matter. Measurement was performed at 25 °C on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) and in duplicate.

### 2.3. Nanoemulsion formation

Oil-in-water nanoemulsions were prepared by homogenising 4% oil phase with 96% aqueous phase (w/w). The aqueous phase was prepared by dispersing WPI hydrolysates (1–4% [w/w]) in 10 mM phosphate buffer (pH 7) containing 0.02% (w/w) sodium azide as an antimicrobial agent. To ensure complete hydration, the suspension was stirred for an hour at room temperature using a magnetic stirrer. The initial step in preparing nanoemulsions involved production of a coarse premix emulsion by homogenising the oil and aqueous phases using an Ultra Turrax (T25 basic, Janke & Kunkel IKA Labortechnik, Staufen, Germany) for 4 min at room temperature. Nanoemulsions were formed from the crude emulsions by passing the coarse premix through a pneumatically driven high pressure valve homogeniser (EmulsiFlex-C5, Avestin, Inc., Ontario, Canada) for four passes at 100–150 MPa. Nanoemulsion samples were prepared in duplicate.

Nanoemulsions were also produced using unhydrolysed WPI and Tween 20 for comparison purposes. These nanoemulsions were prepared at 1% emulsifier and 4% oil phase concentrations. Another set of nanoemulsion was produced using chymotrypsin–WPI hydrolysate at 10% oil phase concentration in order to determine the effect of the oil concentration on the ability of the WPI hydrolysate to form nanoemulsions. Hydrolysate emulsifier concentration used in study was 1%.

### 2.4. Droplet size (z-average [d]) measurement

The mean droplet size (diameter), size distribution and polydispersity index (PDI) of nanoemulsions were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Measurements were performed at 25 °C with duplicate measurement on each sample. Prior to measurement, the nanoemulsions (50 μL) were diluted with phosphate buffer (4950 μL; pH 7) to avoid multiple scattering during measurements.

### 2.5. Zeta potential measurement

The net electrical charge of the nanoemulsion droplets was determined by measuring the zeta potential of nanoemulsions using a universal 'Dip cell' (ZEN1002, Malvern Instruments, Malvern, UK) on a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Nanoemulsions were diluted (100 μL) in the working buffer (4900 μL) and then equilibrated at 25 °C in the instrument