Emulsifying and emulsion stabilising properties of soy protein hydrolysates, covalently bonded to polysaccharides: The impact of enzyme choice and the degree of hydrolysis

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Abstract

Emulsifying and emulsion stabilising properties of fragments, derived from vegetable proteins (soy protein isolate) and covalently linked to maltodextrin have been studied. The dual role of the degree of hydrolysis (DH) in improving the solubility of soy protein isolate (SPI) on one hand and difficulties of linking hydrolysates to polysaccharide on the other, have been highlighted. The findings have been compared to results obtained for whey protein isolate (WPI) fragments, undergoing the same enzymatic fragmentation and subsequent Maillard reaction. All experiments were conducted with two very different enzymes. Trypsin is rather selective of peptide bonds it cleaves, while alcalase is less specific. At the same DH, the actions of these two enzymes on the behaviour of the resulting conjugates were found to be broadly similar for the whey protein hydrolysates. In contrast, the trypsin generated soy protein fragments showed distinctly superior emulsifying properties than those produced by alcalase. These differences were related to the physical form of SPI existing as small colloidal aggregates in solution at the time of hydrolysis, whereas WPI was present as a molecularly well-dissolved protein. It is shown that the improved solubility as a result of a higher level of hydrolysis is offset by the deterioration of the functionality of polypeptides due to further fragmentation. This leads to an optimum value for the DH of vegetable proteins for synthesising the most suitable Maillard based emulsifiers. For commercial SPI used in this study this was found to be around 8%.

1. Introduction

Animal based proteins, particularly milk proteins, are effective emulsifiers by virtue of their amphiphilic characteristics which enable them to adsorb strongly to the oil-water interfaces. Some milk proteins (particularly caseins) have disordered coil-like structures that further aid their rapid adsorption (Dickinson, 1992b; Dickinson, Horne, Phipps, & Richardson, 1993). Though they can mediate some level of steric interactions, the stability of emulsion droplets fabricated with these biopolymers is mainly due to the electrostatic repulsion, provided by virtue of the charge on the adsorbed proteins (Dickinson, 2009, 2015). Therefore, in cases involving high ionic strength or when the pH approaches pI of the protein, the colloidal stability is often lost. In order to improve the stability of protein based emulsions under such unfavourable conditions, polysaccharides are commonly incorporated to form a second protective layer deposited on top of the primary protein layer, enhancing the steric component of the repulsive forces between the droplets (Dickinson, 2008, 2015).

This idea can be realised either by forming electrostatically-driven protein-polysaccharide complexes (McClements, 2010; Xu, Luo, Liu, & McClements, 2017), or by the use of covalently bonded protein-polysaccharide conjugates (Dickinson & Semenova, 1992; Evans, Ratcliffe, & Williams, 2013; Wooster & Augustin, 2007). The first approach can give rise to several issues of its own, including the bridging and depletion flocculation during the deposition of polysaccharide layers (Dickinson, 2008), the breakdown of the structural integrity of the complexes induced by large pH shifts (Ettelaie, Zengin, & Lishchuk, 2017; Guzey & McClements, 2006), and also the gradual mutual diffusion of biopolymers layers to form a single mixed film rather than the desired layer-by-layer preparation (Ettelaie, Akinshina, & Maurer, 2012; Ettelaie, et al., 2017). In contrast, protein-polysaccharide conjugates behave somewhat like a copolymer, where the non-adsorbing polysaccharides protrude outwards away from the surface, thus effectively forming a second outer layer surrounding the oil droplets. Nonetheless, the polysaccharides remain on the surface of oil droplets, kept there by the strongly adsorbed proteins to which they have been covalently attached. This design aims to keep the integrity of the composite macromolecules, while avoiding any bridging flocculation arising from separate loading of polysaccharides as happens in the layer-by-layer approach, irrespective of changes in the environmental conditions (Akhtar & Ding, 2017; Dickinson, 2008, 2015, 2019).

The covalently-linked conjugates of protein and polysaccharide can be achieved by means of physical (Guan, Qiu, Liu, Hua, & Ma, 2006; Mu, et al., 2010), chemical (Hattori, 2002; Marshall & Rabinowitz, 1976) and enzymatic treatments (Chen, H., et al., 2018; Liu, Selig, Yadav, Yin, & Abbaspourrad, 2018), among which the dry-heating Maillard reaction is regarded as the most effective and straightforward. Maillard reaction is a naturally occurring process in cooking, where covalent bonds form between an amino group of protein, usually Lysine, and a reducing carboxylic group of a polysaccharide. The reaction is usually conducted under controlled temperature and humidity, with no requirement for additional chemicals (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Kato, 2002; Oliver, Melton, & Stanley, 2006). Reported glycoproteins made in this way involve a diverse combination of ionic and non-ionic polysaccharides, combined with animal-based proteins (Al-Hakkak & Al-Hakkak, 2010; Kim, Choi, Shin, & Moon, 2003; O'Regan & Mulvihill, 2010; Wooster, et al., 2007). Numerous studies in the literature have shown enhanced functionalities of such conjugates, as for instance the superior emulsifying and stabilizing ability under harsh environmental conditions (i.e. low pH, high salt concentration, temperature cycling) when compared to their non-conjugated counterparts (Akhtar & Dickinson, 2007; Hou, Wu, Xia, Phillips, & Cui, 2017; O'Regan, et al., 2010; Wooster, et al., 2007). This demonstrates the much stronger role of steric repulsion in keeping the droplets apart.

In recent years, the ''green'' trends in the pharmaceutical, cosmetic and food industries have motivated a significant level of research interest in achieving completely plant based protein-polysaccharide conjugates (Burgos-Díaz, Wandersleben, Marqués, & Rubilar, 2016; Chen, Chen, Ren, & Zhao, 2011b; Chen, Chen, Wu, & Yu, 2016; Nesterenko, Alric, Silvestre, & Durrieu, 2013). Plant proteins considered for this purpose have included rice protein (Li, et al., 2013), potato protein (Delahaije, Gruppen, van Nieuwenhuijzen, Giuseppin, & Wierenga, 2013), wheat protein (Wong, Day, & Augustin, 2011) and pea protein (Zha, Dong, Rao, & Chen, 2019), with the most popular being isolated soy protein due to its high nutritional value, abundancy and biodegradability.

Soy protein isolate (SPI) is the by-product of the soybean oil industry. They are amphiphilic in nature. However, they exhibit poor emulsifying capability compared to milk derived proteins such as casein or whey protein, due to their large molecular weight, compact globular structure and limited solubility (Dickinson, 2019; Tang, 2017). Recent progress on achieving emulsifiers from SPI and SPI derived materials has mainly focused on Pickering-type aggregated protein emulsifiers or microgel particles (Chen, Chen, Ren, & Zhao, 2011a; Dickinson, 2019; Guo, et al., 2016; Hao, Peng, & Tang, 2020; Liu & Tang, 2013, 2014; Luo, Liu, & Tang, 2013; Matsumiya & Murray, 2016; Peng, Xu, Liu, &

Tang, 2018; Tang, 2017). These reported emulsions are not stabilised by molecularly adsorbed protein layers (i.e. not in the same manner as for example with sodium caseinate), but rather by protein in highly aggregated particulate form (Nishinari, Fang, Guo, & Phillips, 2014) covering the surface of the droplets.

Some researchers have used the covalent bonding of soy protein isolate with already surface active polysaccharides as a means to enhance the functional properties of the polysaccharides. These polysaccharides include gum Arabic (Xue, Li, Zhu, Wang, & Pan, 2013), pectin (Ma, et al., 2020) or soy soluble polysaccharide (Nakamura, Yoshida, Maeda, & Corredig, 2006; Yang, et al., 2015), all of which to greater or lesser extent already exhibit some degree of emulsifying ability (McNamee, O'Riorda, & O'Sullivan, 1998; Nakamura, Takahashi, Yoshida, Maeda, & Corredig, 2004; Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015).

Solubility of the protein is not only critical in producing fine emulsions, but is also a key issue in synthesizing suitable covalent complexes with polysaccharides. It is crucial that a well-mixed blend of the two biopolymers is achieved in the solution in the first instance, and remains so once the solution is freeze-dried prior to the heat induced Maillard reaction. Thus, the lack of sufficient solubility of many plant proteins becomes a major stumbling block in obtaining such an intimate mix, leading to a reduction in the efficiency of bond formation between protein and the polysaccharide molecules.

To overcome this problem, one possible solution is to only use the relatively soluble components of soy proteins. For instance, Xu and Yao (2009) conjugated acid soluble soy protein (ASSP) with dextran, and produced emulsions with submicron-sized droplets under both low pH conditions and high NaCl concentrations. In the studies of Kasran, Cui, and Goff (2013a) and Kasran, Cui, and Goff (2013b), soy whey protein which has an excellent solubility was conjugated with fenugreek gum. Emulsions with an average droplet size around 2 μm were produced at pH 4.0 and stayed stable for 28 days. Another example is the work by Zhang, Wu, Yang, He, and Wang (2012), where β-conglycinin (a glycoprotein that has a much better emulsifying ability than the other components of SPI) is modified with dextran via Maillard reaction. This was then hydrolysed by an enzyme, leading to a product with good emulsifying and emulsion stabilizing performance at acidic pH conditions. These studies are satisfactory examples in terms of utilization of soy proteins on a lab scale. However, the soluble components of soy proteins are either very tedious and expensive to isolate, or are not as

commercially abundant as SPI (Kasran, et al., 2013a; Nagano, Hirotsuka, Mori, Kohyama, & Nishinari, 1992; Thanh, Okubo, & Shibasaki, 1975; Vu Huu & Shibasaki, 1979) due to only making up a small fraction of the whole soy proteins. As such their use remains infeasible in any large scale industrial application of soy proteins. Nevertheless, the important insight gained from the above studies is that in order for any modified soy protein to be efficient emulsifying agent, enhanced protein solubility is a prerequisite.

Our approach to overcome the poor solubility of commercial SPI is to hydrolyse it prior to its conjugation with polysaccharides. The hypothesis is to unfold the protein structure and to also produce smaller polypeptides which are expected to be more soluble and surface active (Chen, et al., 2011a; Ettelaie, Zengin, & Lee, 2014; Ettelaie, et al., 2017). The covalent bonding of these polypeptides with polysaccharides may therefore be a promising way to produce molecular level plant-based emulsifying agents. The idea is not entirely new and has been explored in a few studies in relation to the interfacial adsorption behaviour (Li, et al., 2016), emulsion stability during freeze-thaw cycles (Yu, et al., 2018) and protection against oxidation offered by these composite macromolecules (Zhang, et al., 2014). However, the focus of these studies, unlike the present work, had not been the long-term emulsion stability, particularly at acidic conditions. The synthesis of current type of conjugates based on the use of plant protein fragments, also requires an understanding of the role of DH and the type of enzyme used in producing the polypeptides. To the best of our knowledge, very few studies have systematically investigated these factors.

In the present work, we consider modification of soy protein as suitable emulsifying and stabilizing agent for fabricating fine conventional-type submicron O/W emulsion systems. Soy protein fragments were obtained at various degrees of hydrolysis by two very different enzymes, trypsin and alcalase. The first acts on a rather selective set of peptide bonds, while the latter is much more indiscriminate. We examined emulsions stabilised by the polypeptides resulting from the action of these enzymes, both prior to and post formation of covalent bonds with an electrically neutral, otherwise surface inactive polysaccharide, namely maltodextrin. We also carefully compared the observed behaviour to those seen for the whey protein hydrolysates, undergoing exactly the same enzyme treatment, degree of hydrolysis and the subsequent Maillard reaction process.

2. Materials and Methods

2.1 Materials

Commercial isolated soy protein (SPI) powder was purchased from Shandong Yuwang Industrial Co. (China). The sample contains approximately 90% (w/w) protein, ash (4.8%) and moisture (4.6%), according to the supplier. The commercial isolated whey protein (WPI) was obtained from Davisco Foods International (USA). The WPI has a protein content of at least 95% (w/w) with a composition of 66% β-lactoglobulin (β-LG), 22% α-lactalbumin (α-LA) and 6% bovine serum albumin (BSA), as provided by the supplier. Porcine trypsin (T7409) in the form of lyophilized powder, and alcalase 2.4L (from Bacillus licheniformis) in the form of aqueous solution, as well as all the other chemicals were purchased from Sigma-Aldrich. The materials required for electrophoresis analysis were all purchased from ThermoFisher Scientific Co. (USA), which included the pre-casted gel sheets, sample buffer, running buffer and molecular weight ladder. The deionised water from a Milli-Q water system (Millipore Co., USA) was used in all the experiments, including preparations of samples, buffers and reagents.

2.2 Hydrolysis of SPI and WPI by Trypsin and Alcalase

2.5% (w/v) SPI dispersion was prepared by dissolving SPI powder in deionised water for 2 h with gentle stirring. The dispersion was then allowed to hydrate overnight at 4°C. Before enzymatic hydrolysis was conducted, the dispersion (100 mL/batch, contained in a cylinder beaker of 150 mL) was treated with ultrasonication (200W, 25kHz) for 10 min. The probe of the sonicator was sunk 4 cm into the protein dispersion. Ice bath was used to control the temperature during the treatment. For hydrolysis by trypsin, the dispersion was preheated to 37°C and the pH was adjusted to 8.5. Accordingly, for preliminary tests, trypsin was added at enzyme/substrate (E/S) ratio (w/w) of 1/200, 1/100, 1/50 to achieve three different degrees of hydrolysis (DH), obtained within approximately 2 h. In the case of alcalase, the SPI dispersion was preheated to 50°C and pH adjusted to 8.5. Different amounts of alcalase solution (i.e. 3, 7 and 15 μL/100 mL protein dispersion) were added respectively, again to provide different levels of hydrolysis.

For each case, protein was hydrolysed under constant temperature and pH, controlled by a water bath and Metrohm 902 Titrando system (Metrohm Co., USA). The DH was determined according to the pH-stat method proposed in the study of Adler-Nissen, Eriksen, and Olsen (1983).

When the desired DH (i.e. 2.5%, 5.5%, 8.0%) was reached, the enzyme activity was immediately stopped by diluting the dispersion to 1.0% (w/v) with 4°C deionized water and incubating in the ice bath with gentle stirring for 0.5 h. The protein hydrolysates were then freeze dried over 48 h. A mild heat treatment (80˚C, 10 min) was performed to all the freeze-dried samples, in order to ensure a complete inactivation of the residual enzyme activity.

WPI hydrolysates were prepared generally in the same way as SPI hydrolysates, except that no ultrasonication treatment was applied since WPI is able to dissolve well in water without the need for such a process. Also, the amount of required trypsin and alcalase was reduced, as they were found to be more effective in hydrolysing WPI. Based on preliminary tests, the E/S ratio was lowered to 1/300, 1/150 and 1/80 for trypsin, and the amount of alcalase to 2.5, 4.5 and 7.5 μL /100 mL protein dispersion, for desired DH values to be reached in approximately 2 h.

Ultrasonicated soy protein hydrolysates (SSPHs) by trypsin and alcalase at different values of DH (2.5%, 5.5%, 8.0%) were labelled as SST1, SST2, SST3 and SSA1, SSA2, SSA3, respectively. Whey protein hydrolysates (WPHs) were denoted in the same way, i.e. WT1, WT2, WT3 and WA1, WA2, WA3.

2.3 Preparation of protein-polysaccharide conjugates

The Maillard reaction products (MRPs) were prepared by dry heating according to Akhtar, et al. (2007); Xu, et al. (2009). First, maltodextrin DE16.5-19.5 (MD, M_w = 8.7 kDa) in powder form was added to 1.0% (w/v) dispersions of sonicated SPI (SSPI) and SSPI hydrolysates (SSPHs) with different DH, as fragmented by either trypsin (SST1, SST2, SST3) or alcalase (SSA1, SSA2, SSA3). The ratio of added maltodextrin (MD) to protein was 2:1 by weight. The protein-maltodextrin mixture was stirred for 1 h at room temperature, and the pH was adjusted to 7.5, before being subjected to freeze drying process for 48 h. Freeze-dried samples were placed in a desiccator with a saturated NaCl solution to control the relative humidity. Then the desiccator was either incubated at 90°C for 3 h, or at 60°C for 24 h, allowing to investigate whether these two commonly used heating practices would result in any differences regarding the emulsifying and stabilizing properties of the produced conjugates.

The MRPs are denoted in here starting with the type of the protein/peptides, followed by polysaccharides. For example, the MRPs made from SST1 with maltodextrin DE16.5-19.5 is marked and referred to as SST1-MD throughout the paper. The conjugated whey protein materials were produced and labelled in the same way as conjugates made from soy protein materials.

2.4 Particle sizing of protein/peptide dispersions

Freeze-dried (and also heated) SSPI and SSPHs samples with different degrees of hydrolysis (DH = 2.5%, 5.5% and 8.0%) caused by trypsin (SST1, SST2, SST3) and alcalase (SSA1, SSA2, SSA3) were reconstituted in and diluted with deionised water, and the pH was adjusted to 7.5 with 1 M NaOH. Protein particle size was measured by Nano ZS Zetasizer (Malvern, UK) and was given as Z-average diameter (nm). The measurements were conducted at 25°C. The refractive indices used for protein and aqueous phase were 1.45 and 1.33, respectively.

2.5 Electrophoresis analysis

SDS-PAGE was performed under reduced conditions on pre-casted Bolt™ Bis-Tris Plus Mini Gel 4-12%. The 65 μL of tested samples (0.15% of protein) were thoroughly mixed with 25 μL Bolt™ LDS sample buffer and 10 μL 0.5 M dithiothreitol (DTT). The resulting solutions were then heated in a water bath at 70°C for 10 min. A running buffer (1× Bolt™ MES SDS) was added into the chamber. Then 20 μL of each heated sample solution was loaded per lane. An unstained broad-range protein ladder (2.5∼200 kDa) was used to estimate the molecular weight of the protein materials in the samples. The electrophoresis was carried out at a constant voltage of 200 V for 22 min. The gel sheet was stained for protein by Coomassie brilliant blue for 2 h.

2.6 Protein solubility

The soluble protein content was determined according to Biuret method (Gornall, Bardawill, & David, 1949; Kim, Park, & Rhee, 1990). Samples were prepared at a protein concentration of 1.0% (w/v) and adjusted to five different pH conditions (pH 7.5, 6.0, 4.5, 3.0 and 2.0). Then the samples were centrifuged at 12,000 g for 15 min. A volume of 200 μL supernatant was incubated with 1 mL Biuret reagent for 1 h. The absorbance was read at 540 nm using UV-VIS spectrophotometer UV-2600 (Shimadzu, Japan). The protein content in the supernatant (g/L) was taken as the solubility of the protein. In order to convert the absorbance into protein content, a standard curve was produced using bovine serum albumin (BSA) as a reference protein.

2.7 Dissociation of insoluble MRPs made from SSPI

Since the MRPs made from SSPI (SSPI-MD) were found to be quite insoluble, despite their hydrophilic polysaccharide attachment, the interactions involved in the formation of SSPI-MD were evaluated using a method according to the study of Liu, et al. (2014). An amount of 0.05 g SSPI-MD sample was

incubated in 10 mL of several different solvents: buffer (pH 9.0, containing 0.086 M Tris, 0.090 M Glycine), SDS (5% SDS in buffer), DTT (0.5 M DTT in buffer) and SDS + DTT (5% SDS plus 0.5 M DTT in buffer). The incubation was allowed for 3 h at 25 °C with gentle stirring. Then the improvement in the solubility of tested samples in different solvents was visually assessed.

2.8 Preparation of emulsions

1.0% (w/v, based on protein content) unconjugated and conjugated protein samples were prepared in deionised water and mixed for 2 h and then left for hydration overnight at 4°C. Sodium azide (0.02%) was added to prevent the microbial activity. Then the pH of the dispersion was adjusted to 7.5. An oil-in-water emulsion (10 vol.% sunflower oil) was prepared in two steps, by a first pre-homogenization (12,000 rpm, 5 min) followed by two passes through Leeds Jet homogenizer at 300 bar (Akhtar & Dickinson, 2003; Dickinson & Stainsby, 1988). The pH of the freshly made emulsions was then adjusted to various desired values. The emulsion samples were stored at 4°C for further investigations.

2.9 Storage stability of emulsions at acidic pH conditions

The stability of emulsion was assessed according to different measures. These included the mean droplet size $D_{4,3}$ and the size distribution of emulsions, both obtained by Mastersizer 3000 (Malvern, UK), the rheological flow properties of emulsions, determined using Kinexus Ultra rheometer (Malvern, UK), the ζ-potential measurements of the emulsion droplets (at ionic strength of 20 mM), using Nano ZS Zetasizer (Malvern, UK) and microstructure of emulsions by optical microscopy. The assessments were performed at various stages during the storage period.

More specifically, to measure droplet size and its distribution, the emulsion under test was diluted by adding 2∼3 drops (approximately 0.5 mL) of sample into the measuring tank of the instrument containing ∼400 mL deionised water. The rheological behaviour was conducted using a double gap cylinder geometry (DG25). The emulsion sample was gently mixed before loading into a temperature-controlled cell. The temperature was allowed to equilibrate at 25°C for 20 min prior to any measurements. The viscosity of emulsion was measured at shear rates ranging from 1 to 100 s^{-1}, using the continuous shear mode of the operation for the rheometer.

2.10 Statistical analysis

All the measurements were done in triplicate. The obtained data were averaged and reported as a mean value in each case. The error bars were added as standard deviations. All the calculations were analysed by Microsoft Excel 2016.

3. Results and Discussions

In this section, we shall mainly focus on the data obtained for proteins that were conjugated with maltodextrin at 90°C for 3 h. The results obtained for these conjugated biopolymers are compared with those for the unconjugated equivalents. No significant differences in respect of the functional properties were found between conjugates made at 90°C for 3 h and those produced at 60°C for 24 h, except for a slightly lower level of solubility for the latter (results not shown). Therefore, unless specifically stated, the discussion concerning the conjugates formed at 90°C for 3 h is understood to also largely apply to those made at 60°C for 24 h.

3.1 Protein particle size

Fig.1A shows the visual appearances of the intact, as well as partially hydrolysed, SPI dispersions in water. As can be seen from the sample SPI in **Fig. 1A**, the intact SPI dispersion rapidly settled down because of its very poor solubility. Due to various processing conditions applied to the commercial SPI, the extracted proteins become totally or partially denatured (Adler-Nissen, 1976). Resultant exposure of the hydrophobic amino acid residues leads to significant clustering of the protein molecules. Thus, commercial SPI is normally present in a highly aggregated form, leading to its poor solubility and inferior dispersibility (Dickinson, 2019; Tang, 2017; Wagner, Sorgentini, & Añón, 2000).

After ultrasonication treatment (see SSPI in **Fig.1A**), a stable and homogenous dispersion formed. The size of protein aggregates was found to reduce to 226 nm in diameter (see SSPI in **Fig.1B**). Ultrasonication is a low-cost treatment which is known to be able to break up the noncovalent inter- and intra-molecular interactions (e.g. hydrogen bonding, hydrophobic interactions), resulting in protein denaturation as well as dissociation of protein aggregates. Therefore, sonicated proteins have been reported to be more accessible for enzymatic hydrolysis than their untreated counterparts (Chen, et al., 2011a; Jia, et al., 2010).

SSPI was then hydrolysed by two distinctly different enzymes, trypsin and alcalase. Our choice of these two enzymes was based on their differing levels of selectivity to cleave various peptide bonds. Trypsin is one of the most specific enzymes, which tends to only break the peptide bonds at the *C*-terminal of lysine (Lys) and arginine (Arg) (Tavano, 2013). On the other hand, alcalase has a much broader range of amino acid substrates as compared to trypsin (Doucet, Otter, Gauthier, & Foegeding, 2003). The dispersions of SSPHs samples obtained from different levels of hydrolysis by trypsin (i.e. SST1 at DH 2.5%, SST2 at DH 5.5% and SST3 at DH 8.0%), exhibited a marked reduction in their degree of turbidity. This was particularly noticeable at DH 5.5%, and even more at DH 8.0%. In comparison, those samples hydrolysed by alcalase (i.e. SSA1 at DH 2.5%, SSA2 at DH 5.5% and SSA3 at DH 8.0%) continued to remain rather opaque (**Fig 1A**).

The observed changes in turbidity are the result of a reduction in aggregated protein particle size (**Fig. 1B** and **1C**). The mean protein particle size was reduced from 226 nm (SSPI) down to 84 nm (SST3) by trypsin. In contrast, the protein particle size was only slightly reduced to around 200 nm at the early stage of alcalase hydrolysis (SSA1), and then maintained more or less unchanged as hydrolysis proceeded further (SSA2 and SSA3).

At a low degree of hydrolysis, one would presume that most of the cleavable bonds will reside close to the surface of aggregated protein particles. As DH increases, this continues to be the case for alcalase, given its less selective nature and higher ability to break peptide bonds of various types. On the other hand, trypsin will begin to run out of specific bonds it can hydrolyse near the surface. If it is to achieve the same degree of hydrolysis, trypsin is required to diffuse deeper into the core of the protein particles to find further bonds to break. Though it may take longer to achieve the same value of DH (i.e. the same number of broken bonds), the breakage would be more uniformly distributed for trypsin case, which aids the progressive breakup of the aggregated protein particles and the reduction in their size. This is indeed what we see in **Fig.1A** and **Fig.1B** when hydrolysis took place using trypsin. In contrast, for the protein aggregates exposed to alcalase, most of the cleaved bonds occur close to the surface of aggregates and the core of the protein particles remains much less affected. Therefore, a smaller reduction in particle size was found, at least at the levels of hydrolysis considered here (**Fig. 1C**). Alternatively, possible coagulation of hydrolysates generated by alcalase may also contribute to the turbidity of the dispersions (Inouye, Nagai, & Takita, 2002; Nagai & Inouye, 2004).

Fig.S1 in supplementary material, shows a schematic picture summarising the proposed differences in the size reduction of the aggregates and the resulting generated polypeptides, arising from the actions of our two contrasting enzymes. The molecular weight distribution of produced fragments is discussed in the next section.

3.2 Molecular weight profiles

The composition of soy protein isolate has been extensively reviewed by the excellent review of Nishinari, et al. (2014) on the subject. The profile of hydrolysed soy proteins was analysed by reducing SDS-PAGE. The two major components of intact SPI (Kuipers, 2007; Nishinari, et al., 2014; Samoto, et al., 2007), i.e. 7S (β-conglycinin) and 11S (glycinin) including their constituent subunits (i.e. α, α' and β of 7S, acidic and basic subunits of 11S), were marked in Lane 0 of **Fig.2** for comparison. It is clearly seen that trypsin and alcalase generated polypeptides with distinct profiles. Similar to the observation by Kim, et al. (1990), we found that trypsin gradually broke soy protein down. This was seen as a shift of bands towards lower molecular weight range with increasing DH (lane 1-3 in **Fig.2**). As for alcalase, the profiles of hydrolysates (lane 4-6 in **Fig.2**) did not show a distinct difference with increasing DH beyond 2.5%. Moreover, under reducing conditions, all the associations between peptides are broken (including disulphide bonds and hydrophobic interactions). A large number of small peptides less than 2.5 kDa, would have also been expected to be released. However, these would be too small to show up on the gel sheet used here and therefore were not detected. The profiles of fragments of whey protein (see **Fig.S2** in supplementary), obtained from treatment by trypsin and alcalase, exhibited similar patterns to the corresponding ones produced for soy protein hydrolysates.

These results for SSPHs from SDS-PAGE were consistent with the discussion in section 3.1, indicating that the mixture of protein fragments obtained by trypsin treatment contained intermediate-sized polypeptides. Due to the highly selective nature of peptide bonds broken by trypsin, this enzyme has to get deep into the core of protein aggregate structures in order to achieve the required degree of hydrolysis. This means that the chains are broken down throughout the whole body of protein aggregate particles. On the other hand, alcalase hydrolysis produced large contents of very small fragments, which we suspect are predominantly produced from a subset of protein chains residing close to the surface of the protein aggregates, due to the low selectivity of this enzyme (Tamm, Herbst, Brodkorb, & Drusch, 2016).

Last but not least, the successful formation of conjugates was confirmed using SDS-PAGE analysis. The presented result here is limited to conjugates formed using SST3, though similar data (not shown) were also obtained for other hydrolysed samples too. In comparison to the equivalent unmodified protein fragments (lane 7 in **Fig.2**), a noticeable shift in molecular weight, towards higher values, was observed for conjugated SST3 (lane 8 in **Fig.2**). This increase in molecular weight is the result of covalent bonding of maltodextrin with the protein fragments.

3.3 Solubility

A reasonable level of solubility is known to be a key requirement for satisfactory functioning of any good molecular (i.e. non-Pickering type) emulsifier (Dickinson, 1992a). One may correctly suppose that the covalent bonding of a protein with a highly soluble polysaccharide, such as maltodextrin or dextran, would render the conjugated biopolymer a sufficient level of solubility in an aqueous medium. This continues to be the case even when the solubility of the original protein may not be particularly high, as might be the case for milk based proteins at their pI. While this assertion is true, unfortunately it does not mitigate the requirement for protein to have a reasonable degree of solubility to begin with, when it comes to synthesising the conjugated emulsifier/colloidal stabiliser. It is important that the protein, or hydrolysates as the case may be, and polysaccharide molecules are in intimate contact, distributed homogenously in the mixture. It is only then that the Maillard reaction between the two can proceed to an extent that a sufficient number of conjugated emulsifiers are produced. This important point is sometimes overlooked by the research work in the literature.

Additional requirements, such as preventing possible segregative phase separation (Banta, et al., 2018; Fang, Li, Inoue, Lundin, & Appelqvist, 2006) may also need some consideration, but normally can be avoided if uncharged polysaccharides are used.

Given the importance of the initial solubility of the protein materials, in this section we shall present and discuss the results of the solubility measurements, both for hydrolysates prior to and post conjugation with maltodextrin.

3.3.1 Solubility of unconjugated proteins/peptides samples

As shown in **Fig.3**, ultrasonication treatment of intact soy protein broke up the non-covalent intermolecular interactions within large protein aggregates. This produced an apparent improvement

in the solubility of the protein ($p<0.05$) at all tested pH conditions, with the exception of pH 4.5 (i.e. the isoelectric point of SPI). We use the word apparent here, as it is suspected that a large portion of ultrasonicated proteins still remain in the form of aggregates, but with a much-reduced particle size ~226 nm (see **Fig.1**). Protein material in these aggregates is not truly dissolved. At pH conditions away from pI, the fine protein particles are sufficiently charged to stay colloidally stable. In fact, the aggregates are small enough not to be completely separated by the centrifugation process. Their continued presence in the supernatant leads to a higher perceived level of solubility, then otherwise the case if they could have been removed. Of course, this issue does not arise at pH 4.5 where the total net charge on both SPI and SSPI is largely lost. This leads to complete precipitation, with neither any small aggregates nor individual protein molecules remaining in the solution.

The enzymatic hydrolysis, particularly by trypsin, noticeably enhanced the protein solubility at all pH values. This was especially so at pI, irrespective of which enzyme was used (see **Fig.3A**). At pH 4.5, the solubility improved to around 5~6 g/L from a value well below 1 g/L for SSPI. In contrast, whey protein fragments, produced by either enzyme, displayed reduced level of solubility at the entire tested pH range, compared to intact WPI (see **Fig.S3** in supplementary).

The contrasting impact of protein fragmentation on the solubility of soy and whey protein can be rationalised as follows. The process of hydrolysis produces various polypeptides with a variety of different pI values. Instead of a sharp well-defined pH value associated with the isoelectric point of the intact protein, now one has a more smeared distribution of pI values for various fragmented chains, following hydrolysis. Thus, at any pH, some fragments are away from their respective pI so as to be reasonably soluble, while others are not. For the whey protein sample, which is already highly soluble, this effect tends to reduce the solubility of protein hydrolysates compared to the intact protein. However, by the same token, the averaging effect induced by fragmentation tends to improve the solubility, if the original protein is not especially water soluble to begin with. This is indeed the behaviour we observed here for soy protein. The improved solubility of SSPHs throughout the entire tested pH range, as in comparison to SSPI, is also partially attributed to the breakdown of soy protein aggregate particles. The measured apparent solubility is expected to increase when these aggregates are broken down more effectively. This is why soy fragments hydrolysed by trypsin exhibited a higher solubility (5~8 g/L) in comparison to those produced by alcalase (4~6 g/L). Kim, et al. (1990) also found a similar result that soy peptides obtained by trypsin hydrolysis were more soluble than those obtained by alcalase.

382 The visual appearance of 1% (w/v) SST3 sample as a function of pH was shown in **Fig.3B**. At pH values
383 below 6.0, we observed a substantial formation of precipitates due to reduced electrostatic repulsion
384 between the fragmented chains. But a stable and homogenous particulate protein dispersion was
385 formed at higher pH. The situation was the same for all the SSPHs samples. Therefore, the mixture
386 of soy protein hydrolysates and maltodextrin in water was produced at pH 7.5, in order to ensure a
387 homogenous and well-mixed system of the two biopolymers, prior to its drying.

388 **3.3.2 Solubility of conjugated proteins/peptides samples**

389 The synthesis of conjugates was carried out according to the procedure outlined in section 2.3. The
390 solubility of various conjugated samples was displayed in **Fig.4**.

391 The conjugates (SSPI-MD), formed between the ultrasonicated soy protein and maltodextrin, may
392 have been expected to have a better solubility than SSPI on its own. Instead, we found a dramatic
393 decrease in the dispersibility from ~5 g/L for SSPI (**Fig.3A**) to ~2 g/L for SSPI-MD (**Fig.4A**) at pH 7.5,
394 with highly insoluble products formed from the dry-heating Maillard reaction (**Fig.5**). This result was
395 replicated for conjugates formed at 90°C, as well as at 60°C.

396 The formation of such kind of insoluble products has already been reported in the literature (Akhtar,
397 et al., 2007; Xu, et al., 2009). In order to investigate this issue further, SSPI-MD was dissolved in
398 various denaturing solvents, including those with added SDS and DTT (**Fig.5**). This helps the
399 dissociation and breakup of different types of inter- and intra-molecular bonds, such as hydrophobic
400 interactions and disulphide bonds. The SSPI-MD conjugates remained insoluble in Tris-Glycine buffer
401 at pH 9.0. This indicates that the poor solubility of SSPI-MD is not merely due to the lack of
402 electrostatic repulsions between the conjugated biopolymers, since they would have acquired
403 sufficient charges at such alkaline condition. In a buffer solution with the presence of 5% SDS,
404 insoluble flakes of SSPI-MD started breaking into smaller pieces, due to the disturbance of the
405 hydrophobic associations by SDS (Ren, Tang, Zhang, & Guo, 2009). The inclusion of 0.5 M DTT, which
406 aids to break the disulphide bonds under alkaline conditions (pH > 8.0) (Liu, et al., 2014; Singh,
407 Lamoureux, Lees, & Whitesides, 1995), also proved helpful in dispersing SSPI-MD aggregates.
408 However, the effect was not quite as strong as that seen with SDS. When both SDS and DTT were
409 present, SSPI-MD aggregates were broken down into much smaller particles as can be seen in **Fig.5**.

410 These results taken together, suggest that hydrophobic interactions are likely the main driving force
411 in the extensive aggregation of SSPI-MD, occurring during the dry-heating Maillard reaction phase.

412 Exchange of disulphide bonds provides further contribution to this process. Nevertheless, no matter
413 how much denaturing agents were added, SSPI-MD could never be made to completely dissolve. This
414 indicates the rather tight and dense structure of the formed SSPI-MD aggregates, which does not
415 allow for easy penetration of small-molecular-weight reagents (i.e. SDS and DTT) deep into the
416 aggregates, at least not within the time scale of current experiments here (~3 h).

417 Unlike insoluble SSPI-MD, the conjugated soy hydrolysates with maltodextrin stayed easily
418 dispersible, without any noticeable formation of insoluble products as that seen in SSPI-MD post
419 Maillard reaction. However, they also did not show any improvement in their solubility relative to
420 the unreacted fragments either (compare **Fig.3A** and **Fig.4A**). Visible aggregates were observed both
421 at pI and other acidic pH conditions (**Fig.4B**).

422 Separately, the solubility of conjugates made from WPI/WPHs and maltodextrin was seen to improve
423 (**Fig.S4** in supplementary), with conjugates forming a clear golden-brown solution at pI of whey
424 protein materials (i.e. pH 4.5). In contrast, the unconjugated equivalents lacking the sufficient charge
425 under this pH condition, settled down out of the solution (**Fig.S3** in supplementary). The absence of
426 precipitation at pI confirmed the formation of covalent bonds between WPI/WPHs and maltodextrin.

427 As for conjugated soy fragments, the formation of large visible aggregates at pI and other low pH
428 values generally, could be an indication of the fact that a sizable portion of protein fragments did not
429 form the required covalent bonds with maltodextrin, at least not under the same heating regime as
430 that used for the WPI/WPHs + maltodextrin. Prolonged heating time and addition of a higher amount
431 of maltodextrin (i.e. the weight ratio of protein/maltodextrin increasing to 1:3, 1:4 and 1:5), were
432 both tried, in the hope of facilitating conjugation between soy protein fragments and maltodextrin.
433 However, these did not improve the situation dramatically, neither with respect to the solubility nor
434 the emulsifying and stabilizing abilities of conjugates.

435 The difficulty for soy protein to form covalent bonds with maltodextrin, in contrast to whey protein,
436 is presumably related to its distinct and more complex structure. When protein conjugates are made
437 via heating of the dry mixture of protein and polysaccharide, two main competing processes occur
438 simultaneously in the system. Firstly, the required Maillard reaction between protein and
439 polysaccharide, involving free α-NH_2 groups of the protein. The second is the undesirable heat-
440 induced protein aggregation (Akhtar, et al., 2007; Dickinson, et al., 1992). It is suggested that due to
441 the structural characteristics and the aggregated state of soy protein materials, the heat-induced

associations between protein molecules via hydrophobic interactions, as well as exchange of disulphide bonds, tend to take place at a much more rapid and intense rate than the Maillard reaction.

Substantial associations between soy protein molecules tends to shield the chemically reactive sites on proteins (i.e. free α-NH_2), making the bonding between protein and maltodextrin much harder (Mulcahy, Fargier-Lagrange, Mulvihill, & O'Mahony, 2017). Under such circumstances, insoluble products are formed, which are simply aggregates of protein molecules, rather than the desired MRPs. It is obvious that improving this situation needs a homogeneous dry mixture, with intimate contacts between the two biopolymers on length scales of individual chains. Existence of large protein particles during the preparation of protein + polysaccharide solution, present even before the drying phase, is clearly not conducive in achieving a complete and efficient synthesis of conjugates.

This situation seems to improve for hydrolysed soy protein (SSPHs). As the compact structure of soy protein is broken down, protein particulate aggregates fall apart. Then the preparation of a well-blended homogenous mixture of protein fragments + maltodextrin becomes more feasible. Additionally, protein fragmentation also causes unfolding and allows for more reactive sites on protein chains to become exposed. This again increases the chance for bonding between the two biopolymers.

However, it must also be mentioned that extensive hydrolysis above a certain level can have a detrimental effect on the emulsifying and stabilizing properties of protein fragments, as well as their conjugated derivatives. The exact reasons for this will become clearer in the discussion of the next section. For now, we merely note that this implies a possible optimum value for DH, where the above-mentioned benefits of hydrolysis are achieved, but yet the resulting polypeptide fragments are still not made too small to lose their functionalities.

3.4 Morphology and stability of emulsions at acidic pH conditions

The size, the peptide distribution profile and the solubility, as investigated in the previous sections, focused on some of the key features that could influence the functional properties of a protein material as a suitable emulsifier/colloidal stabiliser. In this section, we discuss the emulsifying/stabilizing abilities of the different protein materials, in the light of the observed attributes studied in the last sections, as well as other possible relevant parameters. The focus of attention will be SSPHs and SSPHs bonded to maltodextrin. However, since we also wish to compare

the behaviours of soy protein materials to their whey protein counterparts, we briefly summarise the key results obtained for whey protein based materials. Consequently, we have refrained from providing all the data in detail for the whey protein based materials for the sake of brevity. These can be found in section 5.1 and 5.2 of the supplementary material, though some have also been included in **Fig.6** to **Fig.9** for the purpose of a clearer comparison with soy protein counterparts.

3.4.1 Emulsions based on unconjugated soy protein/peptides

The relatively large aggregated protein particles in SPI and SSPI samples manifested themselves in the rather poor emulsifying abilities. The droplet sizes obtained for intact soy protein, used for fabricating emulsions in the absence of or with prior ultrasonication treatment, were $D_{4,3}$ = 28.4 μm and 9.8 μm at pH 7.5, respectively (**Fig.6A**).

The droplet size decreased dramatically as soy protein was progressively broken down by trypsin, before its use as emulsifiers (**Fig.6A**). From the micrographs (see S-7.5-1 in **Fig.7A**), it was observed that soy fragments with the highest DH 8.0% (SST3) were able to produce a finely dispersed submicron-sized emulsion. The average size $D_{4,3}$ was found to be 0.608 μm, at pH 7.5. This is comparable to the emulsions stabilized by WPHs (e.g. $D_{4,3}$ = 0.628 μm for WT1 in **Fig.7B**). On the other hand, alcalase digestion progressively worsened the emulsifying capacities of SSPI, leading to the formation of larger droplets ($D_{4,3}$ = 9.8 μm, 14.6 μm, 18.4 μm and 21.4 μm, for fresh emulsions made at pH 7.5 by SSPI, SSA1, SSA2 and SSA3, respectively). It was clear that the more the soy protein was hydrolysed with alcalase, the poorer its emulsifying performance became.

On the contrary, for whey protein materials (**Fig.6B**), hydrolysis by trypsin up to DH of 2.5% moderately enhanced the emulsifying/stabilizing capacities of WPI. This trend did not continue with further fragmentation, where the required surface properties were instead seen to suffer. The worsening of the functional properties of whey proteins was observed from the very onset for alcalase treatment, even at DH 2.5%. Despite this small difference at DH = 2.5%, the overall trend between the performances of hydrolysates produced by the two enzymes upon increasing DH was otherwise similar.

The striking contrast in the observed performances of SSPHs and WPHs, resulting from the action of two different types of enzymes, trypsin and alcalase, have to be sought in the distinct structures of SSPI and WPI when present in the solution. The former exists in the form of dispersed protein

aggregates of particle size ∽226 nm (after ultrasonication), which remain hard to break down further. WPI on the other hand is relatively well dissolved. As discussed in previous sections, at the DH values studied here, alcalase generated rather small peptides from the exterior of soy protein aggregates. While the remaining unhydrolyzed parts of soy protein aggregates, post alcalase digestion, are still of fairly large size (see section 3.1). While it has been shown that these particles are able to stabilize oil droplets through Pickering type action (Liu, et al., 2013, 2014), it is not clear that they will be equally useful in fabrication of submicron-sized fine oil droplets. It seems that the simultaneous presence of small peptides and protein aggregates contributes to a deterioration of functional properties of SSPI so far as their non-Pickering type emulsifying behaviours are concerned. In contrast, at the same comparable DH, trypsin digestion, where the protein chains are broken in a more uniform manner, resulting in a mixture of intermediate-sized polypeptides, proves to be a much better approach for modification of soy proteins. The differences between the actions of the two enzymes did not arise for WPI, because whey protein is well dissolved. Thus, for WPHs with the same DH, the emulsifying abilities remained broadly comparable for fragments generated by trypsin and alcalase.

From the above results, one also notes the unfavourable effect of excessive hydrolysis on the emulsifying and stabilizing properties of proteins. Small fragments released by enzymatic digestion at high DH levels, may well be flexible and surface active, but are not able to provide colloidal stability effectively. They are capable of gradually disturbing and displacing the larger adsorbed fragments from the interface during storage of emulsions. Those larger chains can potentially provide better steric stabilizing ability and form stronger interfacial films (Chen, et al., 2019; Ipsen, et al., 2001; Schröder, Berton-Carabin, Venema, & Cornacchia, 2017). Hence, their displacement from the surface of droplets is not desired and may lead to colloidal instability (e.g. coalescence). The weakening in the stabilizing abilities at higher levels of fragmentation is not only confined to whey proteins (Schröder, et al., 2017), but also has been reported for other proteins such as casein (Luo, Pan, & Zhong, 2014), soy proteins (Chen, et al., 2011a, 2011b; Qi, Hettiarachchy, & Kalapathy, 1997) and peanut protein (Chen, Chen, Yu, Wu, & Zhao, 2018). Note that this conclusion does not apply to situations where the aim is to make Pickering type emulsifiers, based either on protein aggregates or using microgels route. For these, more extensive hydrolysis may well prove beneficial in forming more appropriate particles, with higher adsorption energies or more suitable contact angles, which can improve the ability to stabilize hydrophobic-hydrophilic interfaces.

Let us now turn attention to the long-term storage stability of emulsions stabilised by soy protein fragments generated by the action of trypsin. At pH 7.5, there was a gradual increase of mean droplet size for all SSPHs stabilised emulsions during the storage, with the growth most clearly seen in the emulsion stabilized by SST3 (**Fig.6A**). The droplet size $D_{4,3}$ of SST3 based emulsion sample started to dramatically rise from around day 3, but began to slow down from the 30th day onwards (see **Fig.S5** in supplementary). In the micrograph S-7.5-60 in **Fig.7A**, quite a few large droplets were visible, with the average size $D_{4,3}$ measured to be 4.22 μm at the end of 60 days of storage. The size distribution was also observed to become bimodal after this period. Nonetheless, it was also noticed that this emulsion had a relatively Newtonian flow behaviour (see **Fig.S6** in supplementary) and the droplets remained highly charged, with ζ-potential around -46∽-50 mV (**Table 1**). Thus, it was unlikely for such growth of droplets to be the result of emulsion flocculation, and indeed no significant evidence for any droplet aggregation was seen in the micrograph for this sample.

Furthermore, in protein-stabilized emulsions when the droplets are in a non-flocculated state, there is normally a high level of stability against coalescence, too. This is because aggregation of droplets is often a first required step towards their coalescence (though flocculation does not always imply that the droplets will definitely coalesce). Yet, with a high level of surface charge, no evidence for flocculation, and adsorbed proteins forming viscoelastic protective interfacial layers around the droplets, coalescence is less likely in our sample here (Bos & Van Vliet, 2001; Dickinson, Murray, & Stainsby, 1988; Murray, 2011).

It is tempting to associate the coarsening of emulsion droplets to the presence of a small but unavoidable amount of residual soy phospholipids (∼3%, w/w) remaining in the commercial SPI as impurity. These soy phospholipids could disturb the interfacial network of protein layers, and more importantly, may also facilitate the process of indirect Ostwald ripening which enables transportation of oil molecules between dispersed droplets via solubilisation in surfactant micelles. More details and some experimental proofs for these arguments are provided in section 8 of the supplementary material.

So far, our discussion of emulsions stabilised by trypsin fragmented SSPHs was limited to pH values away from pI. Next, we consider the impact of pH variation on the colloidal stability of our SSPHs stabilised systems. Fresh emulsions adjusted to acidic pH conditions, exhibited a marked rapid increase in droplet size (**Fig.6A**). For instance, the droplets became flocculated and the value of $D_{4,3}$

jumped to 12.9 μm at pH 4.5 for SST3-stabilized emulsion sample (see S-4.5-1 in **Fig.7A**). This is expected due to an insufficient level of surface charge, where we found ζ-potential = -6.7 ± 0.9 mV (**Table 1**). Thus, so far, this behaviour is similar to what we also found for WPHs stabilised emulsions (see **Fig.6B**). However, differences arose when the pH was further lowered to 3.0, with the system retained at the intermediate pH of 4.5 for only a short period (< 5 mins). Unlike the WPHs based systems, where the droplets became well dispersed at pH 3.0 once regaining sufficient charge (ζ-potential = +45.9 ± 1.5 mV, see **Table 1**), on this occasion the flocs did not break down into individual oil droplets for any of the emulsion samples stabilised by SSPHs (**Fig.6A**). For example, the droplet size of fresh SST3 stabilized emulsion at pH 3.0 was 14.8 μm, which was not all that different from 12.9 μm at pH 4.5. These values are to be compared to $D_{4,3}$ of 0.608 μm at pH 7.5, prior to any pH adjustment. The same phenomenon also occurred for conjugated SSPHs stabilized emulsions considered in the next section. A discussion of these observations will be provided once the data for the stability of emulsions made by our SSPHs + maltodextrin covalent complexes have also been presented below.

3.4.2 Emulsions based on conjugated soy protein/peptides

When soy protein hydrolysates were conjugated with maltodextrin, all modified SSPHs samples delivered significantly improved emulsifying and stabilizing capabilities, in comparison to their unconjugated counterparts. This was true at all tested pH conditions (**Fig.8A**).

We present as an example the results for soy protein hydrolysates, generated by trypsin digestion at DH = 8.0%. It is seen that initially the conjugated and non-conjugated fragments (i.e. SST3-MD and SST3) produced fine emulsions with similar average droplet sizes of 0.638 μm and 0.608 μm at pH = 7.5 (see micrographs CS-7.5-1 and S-7.5-1 in **Fig.9A** and **Fig.7A**, respectively). The droplet size distributions of the two emulsions closely resembled each other too. However, after 60 days of storage, a significantly higher number of larger droplets was visible in emulsion sample stabilized by non-conjugated SST3. The size distributions of the two emulsions are also seen to diverge. While both emulsions exhibited some degree of coarsening, the average droplet size $D_{4,3}$ was only 2.29 μm for the conjugated polypeptides, whereas it increased to 4.22 μm for the non-bonded fragments. The most likely reason for this superior behaviour of the conjugated system is the provision of enhanced steric repulsion, due to the presence of the maltodextrin part of these composite biopolymers (Dickinson, Murray, et al., 1988; McClements, 2015; Tcholakova, Denkov, Ivanov, & Campbell, 2006).

591 If this assertion is true, it may be possible to further improve the stabilising ability of our emulsifiers
592 by attachment of larger molecular weight (M_w) polysaccharides (Dunlap & Cote, 2005; Wooster &
593 Augustin, 2006; Wooster, et al., 2007). Some experimental support for this view is provided in **Fig.S8**
594 of the supplementary material.

595 The primary reason for conjugating a protein with a polysaccharide is to improve the stabilising
596 properties of the former, particularly at pH values close to its isoelectric point. Recall that our results
597 indicated a poor stability against aggregation at pH = 4.5, when droplets were stabilised by the non-
598 bonded SSPHs, or WPHs. However, for all the whey protein fragments, covalent bonding with
599 maltodextrin was seen to vastly improve the stabilizing capacity of the emulsifiers against flocculation
600 at acidic conditions. Particularly, the emulsion stabilized by conjugated WPHs obtained at low level
601 of DH by trypsin digestion (i.e. WT1-MD) maintain the same level of stability at pH 4.5 as that found
602 at pH 7.5 (see **Fig.8B** and **Fig.9B**). Nonetheless, very small polypeptides generated by excessive
603 degree of hydrolysis, even after possible conjugation, still remained relatively unsuitable as
604 emulsifiers.

605 Let us now turn attention to the stability of emulsions made of SSPHs + maltodextrin conjugates at
606 pH values close to the isoelectric point of protein/peptides. In general, the flocculation stability of
607 fresh emulsions fabricated with conjugated SSPHs remained somewhat poorer at pH = 4.5 relative to
608 that seen at pH = 7.5 (**Fig.8A**). This can also be observed by comparing the results in **Fig.9A** for
609 samples CS-4.5-1 and CS-7.5-1, both produced using SST3-MD emulsifier. Clear evidence for some
610 level of droplet clustering was seen in the micrograph of CS-4.5-1, with the average particle size
611 changing from 0.638 μm to 1.87 μm upon pH adjustment. Further support for the flocculation at pH
612 = 4.5 system came from a study of its rheological behaviour. The low shear viscosity of this emulsion
613 was markedly higher at pH = 4.5 compared to at pH 7.5. Also, the emulsion sample exhibited shear-
614 thinning behaviour (flow behaviour index = 0.492) under acidic conditions, while it was more
615 Newtonian at pH = 7.5 (see **Fig.S6** in supplementary).

616 Despite this result, it has to be said that in comparison with their non-bonded counterparts (i.e. SST3),
617 the conjugated hydrolysates SST3-MD still did offer a significant enhancement in the emulsion
618 stabilising properties against droplet flocculation. The micrograph and the size distribution at pH =
619 4.5 indicate that the majority of droplet clusters were small and less than 3 μm for the SST3-MD
620 based system, with average size $D_{4,3}$ = 1.87 μm (see CS-4.5-1 in **Fig.9A**). On the other hand,

non-conjugated SST3 based system showed a much more extensive level of aggregation, with the measured average particle size at a far larger value at pH 4.5 ($D_{4,3}$ = 12.9 μm, see S-4.5-1 in **Fig.7A**).

Even with its aggregated morphology, the SST3-MD based emulsion at pH = 4.5 showed far less evidence for the formation of large droplets than was the case at pH = 7.5, following 60 days of storage (compare CS-4.5-60 and CS-7.5-60 in **Fig.9A**). As we mentioned before, we believe that the formation of larger droplets at such long storage time is mainly the result of a limited Ostwald ripening process. As such, the more compact and aggregated adsorbed protein films formed at pH = 4.5, may resist Ostwald ripening and the shrinkage of droplets more effectively than the more extended but sparsely configured layers at pH = 7.5 (Graham & Phillips, 1980; Meinders, Kloek, & van Vliet, 2001; Murray, 2002; Pezennec, et al., 2000; Rivas & Sherman, 1984).

The above set of results indicates that the strength of the steric forces provided by adsorbed layers made of SST3-MD, fall somewhat short of those achieved by WT1 (DH = 2.5%) based conjugates. This is likely due to the limited number of covalent bonds formed between soy fragments and maltodextrin, at the value of DH = 8.0%. The conjugated and the unreacted polypeptides will tend to compete with each other for adsorption onto surfaces. This may result in mixed layers, leaving the surface of droplets not sufficiently covered with the desired additional protection from polysaccharide. Thus, as the droplet surface charge is lost at pH 4.5 (ζ-potential = -3.7 $\pm$ 0.5 mV, see **Table 2**), the lack of electrostatic repulsion between the particles, coupled with an insufficient steric force, can no longer prevent aggregation of the droplets.

It is tempting to follow the same recipe as WT1 to produce MRPs by using SSPHs with lower DH. However, one has to remember that fragmentation of soy protein is a necessary step to the breakup of aggregated protein particles to ensure a final homogenous mixture between protein/peptides and maltodextrin prior to Maillard reaction. If the degree of hydrolysis is too low, then this latter requirement would not be met.

In order to improve the degree of conjugation between maltodextrin and soy fragments with high DH of 8.0%, we prepared conjugated SST3 at increased weight ratio of maltodextrin (i.e. the ratio of SST3/MD = 1:3, 1:4 and 1:5). Unfortunately, there was no significant enhancement in the stabilizing abilities against flocculation at pH 4.5 (results not shown). This again indicates the restricted level of Maillard reaction between soy protein/peptides and maltodextrin. We believe that this relatively inefficient reaction between the two biopolymers arises mainly from the aggregated state of soy

protein or its hydrolysates (i.e. the average particle size of various SSPHs samples is around 80∽200 nm). In addition, the presence of non-protein substances in the form of impurities in commercial SPI, may also play a role in further reducing the degree of reaction between polypeptides and maltodextrin. These minor components bind on protein molecules through strong electrostatic and hydrophobic interactions, masking or affecting the availability and reactivity of α-NH_2 on soy protein materials (Genovese, Barbosa, Pinto, & Lajolo, 2007; Nash, Eldridge, & Wolf, 1967; Skorepova & Moresoli, 2007).

For freshly made emulsion samples adjusted to even lower pH conditions (i.e. pH 3.0 and 2.0), clustered droplets already formed at pH 4.5 were not broken down (**Fig.10A** and **Fig.10B**). Even if the sample was brought back to pH 7.5 (ζ-potential = -37.2 $\pm$ 2.6 mV) where the fresh emulsion without acid treatment was well dispersed, the flocs of droplets still remained visible (**Fig.10C**). Recall from the previous section that this same situation did not occur in emulsions based on whey protein materials, so long as the sample was kept just a short time (< a few minutes) at pH = 4.5 before lowering pH to 3.0 or back up to pH 7.5 again. The irreversible association of emulsion droplets stabilized by soy protein materials probably arises from the conformational rearrangements of adsorbed proteins/peptides on the droplet surface, via exposure of their hydrophobic residues during the storage of emulsions (Freer, Yim, Fuller, & Radke, 2004; Kim, Decker, & McClements, 2002a, 2002b; McClements, 2004). These rearrangements and mutual diffusion of the polypeptides between adjacent surface layers could result in interfacial films shared between neighbouring droplets. This can also facilitate the formation of possible disulphide bonds between adjacent layers. Once such bonds are formed, switching back the electrostatic repulsion between the droplets, by adjustment of pH, will no longer be sufficient to redisperse the emulsion system.

4. General Conclusions

The current study aims to understand the possibilities and the complications involved in turning vegetable proteins into suitable biopolymer-based emulsifying agents for producing fine submicron sized O/W emulsion systems, rivalling their animal derived counterparts used for this purpose. Most plant based proteins, when used as non-Pickering type emulsifiers, have inferior emulsifying functionalities arising from their poor solubility and their presence in the form of colloidally sized protein aggregates in the solution. This is to be contrasted with milk proteins which can easily be dissolved down to almost individual protein molecules.

Taking commercial isolated soy protein (SPI) as an example, we investigated the impact of enzymatic hydrolysis, followed by conjugation with maltodextrin, on improving the emulsion stabilising behaviour of this protein under various pH conditions. At each stage, careful comparisons with whey protein materials undergoing exactly the same modification process were made in order to provide a clearer understanding of the impact of the changes made to SPI.

Hydrolysis was attempted with two enzymes having very different levels of selectivity towards cleavage of peptide bonds. Broadly speaking, so long as the degree of hydrolysis was not too extensive (< 10%), trypsin and alcalase produced similar hydrolysates for whey protein at the same DH in terms of their emulsifying and emulsion stabilising abilities. In both cases, increasing DH much beyond 2.5% tended to reduce the desired surface functionality. In contrast, the impact of the two enzymes on SPI was very different. None of the DH values achieved by alcalase digestion in the current study were observed to be particularly useful for fabricating soy hydrolysates which are capable of producing fine submicron-sized, well dispersed liquid-like emulsions. On the other hand, trypsin reduced the size of soy protein aggregates more efficiently, and was found to allow for fabricating fragmented soy protein (at DH = 8.0%) that were able to form fine emulsion droplets comparable to those achieved by whey protein material. Nevertheless, the long-term stability was still not very good, with a significant development of larger emulsion droplets following 60 days of storage.

As with many proteins, the flocculation stability of emulsion droplets fabricated using SPI derived fragments was generally poor at acidic condition (e.g. pH 4.5). Covalent bonding of soy hydrolysates with maltodextrin provided significant improvement. Nonetheless, the stability of emulsion based on conjugated soy hydrolysates (obtained by trypsin digestion at DH 8%) still continued to be inferior to what can be achieved with the Maillard products of whey protein (or its hydrolysates at low DH = 2.5%), covalently bonded with same maltodextrin. However, it is useful to note that despite the aggregated morphology of the emulsion samples fabricated with conjugated SST3, droplets were seen to roughly maintain their original sub-micron sizes, even after a considerable storage period.

Our study highlights the benefits of using highly selective enzymes such as trypsin in producing plant protein fragments with good emulsifying abilities to act as non-Pickering emulsifiers. We also demonstrated the delicate balance needed in the choice of the required degree of hydrolysis. If too little, then the solubility of the vegetable protein would remain poor. Thus, it would not be possible

711 to achieve a molecular-scaled uniform mixture of protein fragments with polysaccharide, which is an
712 important prerequisite for good reaction efficiency to obtain suitable conjugates via a heating
713 process. Yet, a high degree of hydrolysis is equally undesirable as it leads to many small fragments
714 which would lack the desired surface functional properties. For commercial SPI used in this work, a
715 DH value of around 8% was found to be close to optimum. It is important to note that these
716 requirements are quite different to those when the aim is to produce soy derived aggregated protein
717 particles for use as Pickering type emulsifiers. For these latter, significant progress has already been
718 reported in the literature by a number of researchers (Guo, et al., 2016; Liu, et al., 2013, 2014;
719 Matsumiya, et al., 2016).

720

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