Rheological Behavior and Droplet Size Distribution of Emulsions Stabilized by Whey Proteins and Chitosan during *ex vivo* Digestion

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ABSTRACT

The effect of type of emulsifier, 2 w/w% whey protein concentrate (WPC) or 2 w/w% WPC and 0.1 w/w% chitosan (CH) on the rheological changes and droplet size distribution during the *ex vivo* digestion of oil in water emulsions (pH 2) containing 1 w/w% rapeseed oil (RSO) were investigated. The procedure was used to mimic the human gastro duodenal digestion in two steps, gastric phase at pH 2 for 30 min followed by the duodenal phase at pH 7 for 60 min. With regard to viscosity, the emulsion, either WPC or WPC-CH showed an increase from gastric phase to duodenal phase ($p<0.001$) and then it was stable until the end of digestion. The viscosity of WPC-CH emulsions during the whole digestion (with/without added HGJ/HDJ) process was higher than the respective WPC emulsions. At pH 7 (duodenal juice addition), the droplet diameters stabilized by WPC emulsion showed a bimodal distribution with droplet sizes in the range of 0.05-1.7 μm and 200-500 μm. A significant change ($p<0.01$) in the overall droplet size distribution was observed by corresponding WPC-CH emulsion (10-200 μm) at that pH. In comparison, at pH 2 the droplet sizes of initial WPC and WPC-CH emulsions were in the lowest range 0.05-1.0 μm except for a very small peak for WPC emulsion (150-700 μm). The increase in droplet sizes during successive digestion from gastric to duodenal phase might be due to flocculation and coalescence. Differences in viscosity and droplet sizes between WPC emulsion and WPC-CH emulsion during digestion may be of importance for the degradation, lipolysis and transit time of the molecules and thereby for their bio accessibility. Our results might indicate a lower digestibility of the emulsion due to the presence of CH in WPC emulsion.

INTRODUCTION

The design of research on oil in water (o/w) emulsions has gained insights into their physicochemical characteristics and stability behavior under different food processing conditions since last 15 years[1]. Proteins, due to their high surface activity, used to stabilize emulsions, whereas polysaccharides acts as viscosifying agents [2]. The combination of proteins and polysaccharides may provide much better stability to emulsions due to the molecular interactions between them when they carry opposite charges[3]. Whey proteins have high emulsion activity and are used in food industries to stabilize emulsions[4]. Chitosan, a cationic polysaccharide isolated from shrimp waste, is a dietary fibre with good emulsifying properties which depends on the degree of deacetylation and molecular weight. Also, it has water/fat binding capacity[5]. The chitosan coatings improves the stability of protein-coated lipid droplets[6].

Also, chitosan influences the absorption of dietary lipids during human digestion[7, 8]. Rodriguez *et al* reported the formation of floccules at duodenal pH that entraps dietary oil[8].

In our previous studies, the storage-stability of o/w emulsions containing whey protein concentrate (0.2 and 2 w/w %), chitosan (0.1 w/w %) at pH 2 was examined[9]. Also, the effect of droplet size on viscosity of emulsions was reported. The addition of CH to WPC emulsion showed a reduction in droplet sizes and increased viscosity. The stability, viscosity and droplet size of these emulsions was important with respect to the pH change during human digestion.

Until now there are few reports on the behavior of milk protein emulsions during gastrointestinal *in vitro* digestion[10, 11]. Such studies have obtained information on the behavior of different o/w emulsions with respect to physical (temperature, shear) and biochemical (pH, enzymes, bile salts) environments that are relevant to digestion[10, 12]. During digestion, the food remains in stomach for few minutes to hours depending on the nature of food (composition, pH, ionic strength, droplet size, rheological characteristics)[13]. During digestion in stomach, an emulsion due to its peristaltic movement in stomach experience a shear[14]. Hence, it might be important to study the rheological behavior and droplet size changes during gastro duodenal digestion.

Devle *et al* reported the rheological changes in skimmed milk (0.1 % fat) and homogenized full fat milk (3.9 % fat) during an *in vitro* model digestion. Differences among the viscosities of two milk types were reported. This procedure attempted to mimic the human gastrointestinal digestion in the stomach and in the small intestine for 90 min. The degradation, interaction and transit time of the food components and their bioaccessibility might be of importance with regard to digestion.

In the present study, whey protein concentrate and chitosan were used to prepare o/w emulsions containing 1 w/w % rapeseed oil. The emulsions were subjected to rheological measurements using an *ex vivo* gastrointestinal digestion model at pH 2 to 7 for up to total 90 min.
The viscosity changes during gastric and duodenal digestion of emulsions (with/without the addition of HGJ/HDJ) was compared between respective WPC and WPC-CH emulsions. With regard to rheological changes, the droplet sizes of WPC and WPC-CH emulsions during the gastric and duodenal digestion (with/without the addition of HGJ/HDJ) were measured and compared. For a comparison, control emulsions without the addition of HGJ/HDJ were used to observe the effect of gastric/duodenal pH on the emulsions.

MATERIALS AND METHODS

Whey protein concentrate with 80% protein content was obtained from Tine BA, Norway. Chitosan with 75-85% de-acetylation and 200-800 cP viscosity was obtained from Sigma Aldrich (St Louis, MO). Rapeseed oil (Askim Frukt and Bærpresseri AS, Norway) was purchased in a local super market. Glycine, Hydrochloric acid (Reagent grade) and sodium hydroxide were purchased from Sigma Aldrich (St Louis, MO). All the buffers and reagents were prepared in Milli-Q distilled water.

Emulsion preparation

The stock solutions of WPC (10 w/v %) and chitosan (1 w/v %) were prepared in 10 mm Glycine-HCl buffer, pH 2. The chitosan solution was left in cold at 4^{o}C for one night before using for emulsion preparation. Required concentrations of WPC, chitosan stock solutions and RSO were pre-mixed at pH 2 using an ultra-turret (IKA yellow line DI25 basic) at 13000 rpm for 2 min. Then the emulsions were homogenized by a continuous re-circulation (total eight cycles) in a M-120 E Micro-fluidizer (Microfluidics

Int. Corp, Newton, USA) for 2 min and 8-10 kpsi pressure. During the micro-fluidization process the temperature of emulsions was maintained at 4°C. 0.02% (w/v) sodium aside (NaN_3) was added as an antimicrobial agent to the emulsions. The pH of all emulsions was rechecked and set to 2 before the analyses. All emulsions were prepared in duplicates on two different days prior to analysis.

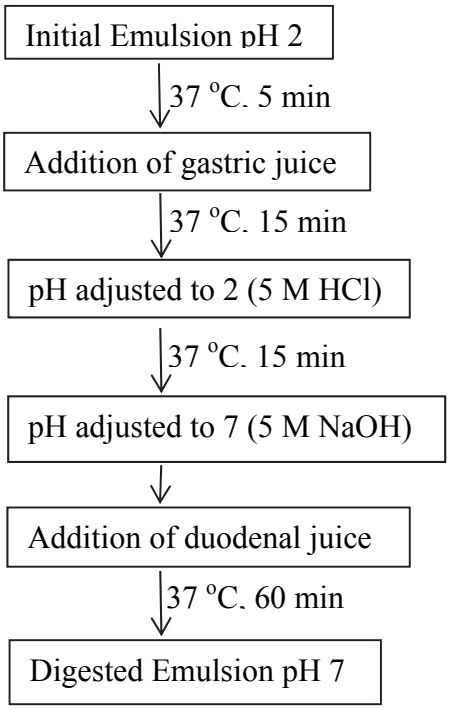

Figure 1. *Ex vivo* digestion flow chart.

Ex vivo digestion

A modified method of digestion, described by Devle *et al*, was performed in two steps using HGJ and HDJ[15]. The *ex vivo* model digestion was developed to mimic the human gastro-intestinal tract, in two steps at 37 °C. (Fig. 1). The gastric phase lasted for 30 min and the duodenal phase lasted for 60 min. The human digestive enzymes were obtained in the active state by collecting HGJ and HDJ according to Holm *et al*[16]. The activities of pepsin, total proteolytic and lipase were 24.3, 11.7 and 700 U/mL, respectively. These assays were described by Ulleberg *et al*[17]. The control (undigested) emulsions were used to observe the effect of pH in the absence of human juices. In the control emulsions, distilled water was added instead of HGJ and HDJ. Two replicate measurements were carried out for each individual emulsion.

Rheological behavior

A Physica MCR 301 rheometer (Paar Physica, Anton Paar, Germany, 2010) was used together with a Titanium CC27 cup and a ST24-2D/2V/2V-30/129 stirrer. The stirrer was positioned with a 0.5 mm clearance from the bottom of the cup. The temperature was controlled by a Peltier and set to 37 °C. The rheometer was set to operate in rotation with a shear rate of 100 s^{-1} corresponding to 100 rpm. The viscosity was calculated by averaging the torque over exactly one revolution. The required quantity of emulsion sample filling in the cup covered the top of the stirrer, and the level increased with dilutions during the experiment.

Droplet size measurements

A Malvern MasterSizer (Malvern Instruments Ltd., Malvern, Worcester, UK) with a measurement range of 0.05 - 5000 µm was used to determine droplet size distribution of different emulsions. The sample was dispersed in Milli-Q distilled water during the size measurements. The mean droplet size was reported as the surface-weighted mean diameter, $D[3;2] = (\Sigma n_i d_i^3 \Sigma n_i d_i^2)$, where n_i is the number of droplets with diameter d_i. The droplet size (µm) values of emulsions, D10, D50 and D90 were also reported.

Statistical analysis

The data series of rheological studies were compared with each other (different series were put together) using analysis of variance (ANOVA) F-Test for significance ($p \leq 0.05$) through Minitab 16 software (Minitab Inc., State College, PA, USA). For all emulsions, (a) the data series of gastric

digestion was compared together with that of duodenal series, (b) Data series were compared among gastric-gastric and duodenal-duodenal levels. The report was a mean and standard deviation (SD) calculated from five readings on an individual sample. Each individual measurement was carried out on two freshly prepared emulsions. For droplet size distribution results (ANOVA, F-Test), all droplet size distribution curves (average of 5 measurements) were compared with each other to check the significance between droplet sizes (D10, D50, D90 and D[3;2]).

RESULTS

Rheological measurements

The viscosity versus time plots of WPC emulsions and WPC-CH emulsions during digestion along with their respective control emulsions (without the addition of human enzymes) are shown in Fig. 2.

Whey protein concentrate emulsions

Addition of HGJ showed a slight increase in viscosity. The viscosity did not change (~4.7 mPas) during and after the adjustment of pH to 2. When pH was adjusted to 7 followed by HDJ addition, the viscosity increased immediately to 5.9 mPas ($p<0.001$) and continued at the same level during the duodenal digestion for 60 min (Fig. 2) It seems that during the complete 90 min digestion, the viscosity of the digested emulsion was almost the same as that of control emulsion without the addition of HGJ and HDJ.

Whey protein concentrate-chitosan emulsions

The complete viscosity profile of WPC-CH emulsion looks similar to that of WPC emulsion at pH 2 (with and without HGJ addition) and pH 7 (with and without HDJ addition). When the pH was adjusted to 7 (with/without HDJ addition) an abrupt increase in viscosity from ~ 5.6 mPas to ~ 25.4 mPas was observed ($p<0.001$). After that a sudden drop in viscosity to ~ 6.4 mPas was observed, that continued till the end of duodenal digestion (Fig. 2). Again, the control emulsion without enzyme addition showed a similar behavior.

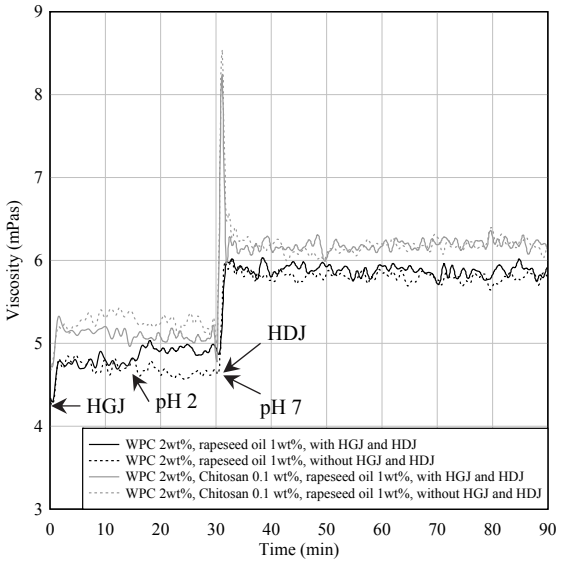

Figure 2. Comparison of viscosity profiles of whey protein concentrate emulsions and whey protein concentrate-chitosan emulsions during *ex vivo* digestion.

Whey protein concentrate emulsions versus whey protein concentrate-chitosan emulsions

For WPC-CH emulsion, the pH adjustment from gastric phase (pH 2) to duodenal phase (pH 7) showed an abrupt increase, but it was not so in WPC emulsions. The viscosity of WPC-CH emulsion (with/without HGJ/HDJ) was somewhat larger than the respective WPC emulsion during the complete 90 min digestion.

Droplet size measurements

For WPC and WPC-CH emulsions, the droplet size distribution measurements were carried out for - (a) the initial emulsion, (b) emulsion (at 30th min) with and without

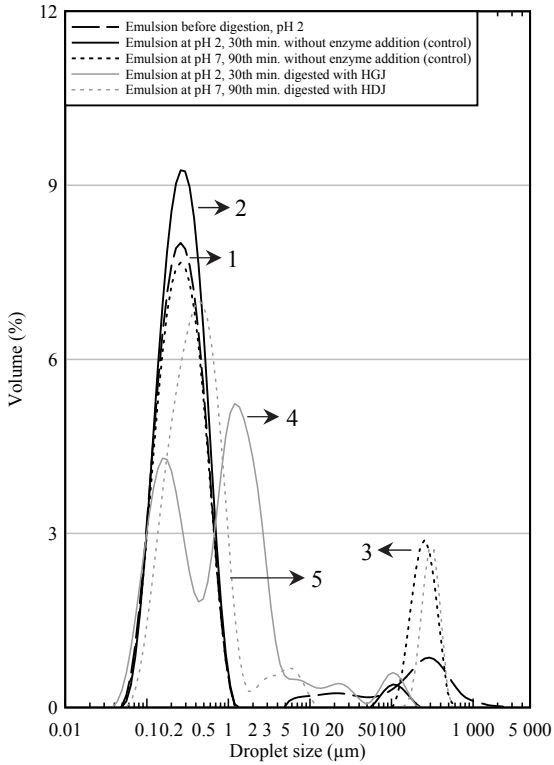

Figure 3. Droplet size distribution curves of whey protein concentrate (WPC) emulsions during *ex vivo* digestion. The curves are numbered according to the emulsion numbers given in Table 1.

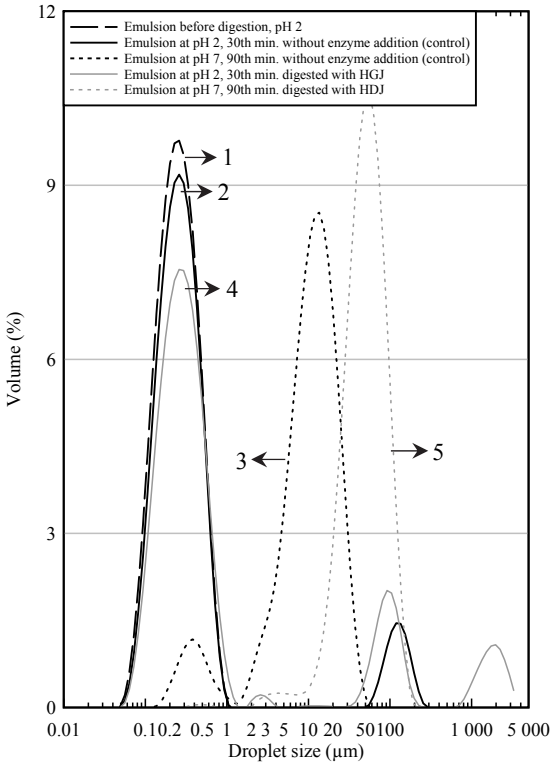

Figure 4. Droplet size distribution curves of whey protein concentrate-chitosan (WPC-CH) emulsions during *ex vivo* digestion. The curves are numbered according to the emulsion numbers given in Table 2.

Table. 1. Droplet size values of whey protein concentrate (WPC) emulsions at pH 2 and 7 during *ex vivo* digestion.

	Emulsion	Droplet size values (µm)			
		D10	D50	D90	D[3;2]
1	Initial, pH 2	0.12	0.29	10.50	0.24
2	No HGJ, pH 2	0.12	0.27	0.62	0.23
3	No HGJ/HDJ, pH 7	0.12	0.29	200.00	0.25
4	HGJ addition, pH 2	0.12	0.80	3.32	0.31
5	HDJ addition, pH 7	0.24	228.00	378.00	0.76

Table. 2. Droplet size values of whey protein concentrate-chitosan (WPC-CH) emulsions at pH 2 and 7 during *ex vivo* digestion.

	Emulsion	Droplet size values (µm)			
		D10	D50	D90	D[3;2]
1	Initial, pH 2	0.12	0.25	0.51	0.21
2	No HGJ, pH 2	0.12	0.26	0.58	0.22
3	No HGJ/HDJ, pH 7	0.71	9.79	22.70	2.30
4	HGJ addition, pH 2	0.13	0.29	1.12	0.25
5	HDJ addition, pH 7	22.90	51.3	101.00	37.10

added HGJ, and (c) emulsion (at 90th min) with and without added HDJ. During the successive digestion, as the digestion proceeds from gastric to duodenal phase, the changes in droplet sizes corresponds to an unknown fraction of total lipid content (<1w/w%). There were observed changes in the droplet volumes in the size distribution range.

Whey protein concentrate emulsions

The droplet size distribution curves of WPC emulsions during digestion with/without the addition of HGJ/HDJ are shown in Fig. 3. The droplet size (µm) values of emulsions, during digestion are shown in Table 1. The size distribution plots indicate that all observations were bimodal, including their respective controls except for the emulsion digested with HGJ

(multimodal). The initial (undigested) emulsions and the control emulsions, at both pH values 2 and 7 seemed to be almost similar.

Initially, the emulsions at pH 2 contained a large volume of smaller droplets having different diameters (d=0.05 – 1.2 μm) and a smaller volume of droplets in the range 90 – 900 μm. It was a significant change ($p<0.01$) in the size distributions and the D [3;2] values when the initial emulsions passed through gastric and then duodenal digestion (Table 1). The volume of smaller sized droplets were reduced as the digestion proceeds from gastric to duodenal phase. At pH 7, during the end of complete digestion (90^{th} min), droplet size distributions in the range 150 – 500 μm were observed (Table 1).

Whey Protein concentrate-Chitosan emulsions

The droplet size (μm) values of emulsions, during digestion are shown in Table 2. The droplet size distributions of emulsions at pH 2 and pH 7 with and without enzyme addition during rheology measurements are shown in Fig. 4.

The initial undigested emulsion contained smaller sized droplets (d=0.05 – 1 μm) showed unimodal size distribution. The gastric digested emulsion was multimodal. At the end of gastric digestion (pH 2, 30 min), an increase in the volume of droplet sizes was observed (unimodal). (Fig. 4). A significant increase ($p < 0.001$) in the droplet size distributions (d= 9-200 μm) and D [3;2] at the end of duodenal digestion was evident as compared to initial emulsion (Table 2). The corresponding control emulsion without enzyme addition at pH 7 also showed a large volume of increased droplet size (d=2 -50 μm).

Whey protein concentrate emulsions versus Whey protein concentrate -Chitosan emulsions

A comparison of droplet size distributions among the respective WPC emulsions versus WPC-CH emulsions with respect to pH 2 (HGJ digestion) and pH 7 (HDJ digestion) was done. At pH 7, among WPC and WPC-CH emulsions without enzyme (HGJ/HDJ) addition, a significant difference ($p<0.01$) was observed. For WPC-CH emulsion, the volume of lower sized droplets (d=0.05-1 μm) was reduced with a corresponding increase in volume of larger droplets (d=2-50 μm). However, there was a fraction of larger droplets (d=120 – 500 μm) present in the WPC emulsion. The emulsions at pH 7 at the end of digestion (90^{th} min) showed differences, WPC-CH emulsions showed a decrease in droplet size values (Table 1 and 2) as compared to WPC emulsion.

DISCUSSION

At pH 2, both WPC and WPC-CH emulsions, with and without the addition of HGJ showed low viscosities, since there might be little droplet aggregation by the conditions in gastric compartment. The droplet aggregation increases the viscosity of emulsion[18]. The increase in the viscosity of WPC and WPC-CH emulsions when the pH was adjusted to 7 (duodenal phase) might be due to the progressive floc dispersion under a constant shear flow[19]. The viscosity of WPC emulsions was somewhat low in comparison with WPC-CH emulsions at pH 2 and 7.

The isoelectric points of WPC and CH are ~5.1 and ~6.5, respectively[20, 21]. Considering the emulsion at gastric pH (pH 2), CH does not have much effect on the viscosity and droplet size. Since WPC and CH have positive surface charges at pH 2, the adsorption of CH molecules might be lesser on to the cationic droplet surfaces and this might be due to electrostatic repulsion. During duodenal digestion (pH 7), a sudden increase in the viscosity of WPC-CH emulsion was evident. Again, the viscosity decreased, but becomes stable until the end

of digestion at pH 7. But the viscosity of WPC-CH emulsion was slightly higher than that of WPC emulsion during duodenal digestion (pH 7). This might be due to the droplet aggregation caused by charge neutralization and bridging flocculation. Therefore, it was an observed increase in droplet size during duodenal digestion (WPC and CH have opposite charges at pH 7). Again, the flocculated droplets (pH 7) might have a higher viscosity than the same concentration of unflocculated droplets (pH 2) because the water molecules trapped within the flocs increase the effective volume fraction of the particles. Further, the unfolding of the structure of whey proteins at pH 2 during the digestion and thereby changes in hydrophobicity might influence the viscosity variations of WPC emulsion. The nature and strength of interactions between WPC and CH at pH 7 might be important during digestion and droplet size variations.

Though, the size measurements here are applicable for a fraction of total lipids due to digestion, it was noticed that, during digestion as the emulsion was passed through gastric to duodenal phase, volume of larger droplets increased with a corresponding reduction in the volume of smaller sized droplets. The initial emulsion droplets are supposed to be smaller when digested in gastric and duodenal phases, but they tended to coalesce and become larger in size. The presence of more than a peak in size distribution plots might indicate that there was coalescence in addition to flocculation. Similar effects have been observed in oil in-water emulsions prepared with whey protein hydrolysis[12].

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