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The importance of swelling for *in vitro* gastric digestion of whey protein gels

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ABSTRACT

In this paper we report the importance of swelling on gastric digestion of protein gels, which is rarely recognized in literature. Whey protein gels with NaCl concentrations 0–0.1 M were used as model foods. The Young's modulus, swelling ratio, acid uptake and digestion rate of the gels were measured. Pepsin transport was observed by confocal laser scanning microscopy using green fluorescent protein (GFP). With the increase of NaCl in gels, Young's modulus increased, swelling was reduced and digestion was slower, with a reduction of acid transport and less GFP present both at surface and in the gels. This shows that swelling affects digestion rate by enhancing acid diffusion, but also by modulating the partitioning of pepsin at the food-gastric fluid interface and thereby the total amount of pepsin in the food particle. This perspective on swelling will provide new insight for designing food with specific digestion rate for targeted dietary demands.

1. Introduction

Protein is not only an important macro-nutrient for humans, but also a main contributor to food structure, for example in meat, cheese and eggs. The digestion of protein-rich structured food starts in the stomach by mechanical degradation and the chemical action of pepsin and gastric acid. Food structure is a critical factor in food digestion (Bornhorst & Singh, 2014; Singh, Ye, & Ferrua, 2015). For example, the dense structure of a milk clot slows down the casein hydrolysis rate (Ye, Cui, Dalgleish, & Singh, 2016). Another study showed that the structure of whey protein aggregates gels can be manipulated by varying the k-carrageenan concentration, and a denser gel structure delays digestion and thereby the release of curcumin (Alavi et al., 2018). In addition, Hu et al. (2017) found that the gel structure of xanthan-SPI and carrageenan-SPI gels delay the digestion of soybean protein, which was confirmed by scanning electron microscope.

Regarding the effect of food structure on gastric digestion, it is crucial to understand the mechanisms underlying gastric digestion of protein-rich structured food. Currently, the main consensus is that food structure can affect gastric digestion by its impact on mechanical degradation (via the different resistance to physical breakdown), and on chemical degradation (via the different accessibility for gastric acid and pepsin). During gastric digestion, the activity of pepsin is highly dependent on the pH value, with maximum activity between pH 1.5 and 2.5 (Piper & Fenton, 1965). As shown by Bornhorst et al. (2014), pH gradients exist in the stomach, and the activity of pepsin is dependent on the real time local pH. Usually the pH of a food particle is higher than that of gastric acid, for example cheese pH ~ 6.7 and fasting gastric pH ~ 1.5. In the gastric environment, gastric acid would transport into the particle. During uptake of the gastric acid, the pH gradients exist in one food particle and varying among different kinds of food as well. As found by Mennah-Govela, Bornhorst, and Singh (2015), effective diffusivity of gastric acid into the bolus was greater in brown than in white rice due to differences in buffering capacity of proteins in rice. Thus, the uptake of acid into the food particle is crucial for lowering the (internal) local pH to guarantee pepsin activity. In addition, a synergy between acid diffusion and enzymatic reaction was found with the use of time-lapse synchrotron deep-UV fluorescence microscopy to track *in vitro* digestion of protein gels (Floury et al., 2018). Apart from acid uptake, pepsin diffusion is essential for gastric digestion. Pepsin diffusion in protein gels made with whey protein isolate (WPI) or casein is shown to be dependent on crosslinking density and microstructure and to be one of the important factors on digestion rate (Luo, Borst, Westphal, Boom, & Janssen, 2017; Thévenot, Cauty, Legland, Dupont, & Floury, 2017).

During digestion, erosion by gastric fluid (with acid and enzyme) and dry matter loss take place; in addition to these surface phenomena, transport of acid, enzyme and water occurs. (Somaratne et al., 2020;

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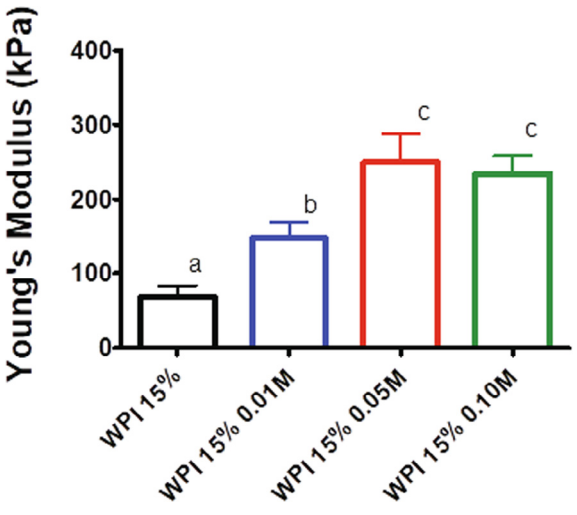

Fig. 1. Young's Modulus of 15% WPI gels with NaCl varying from 0 to 0.1 M; the error bars represent standard deviation, and columns with the same letter did not differ significantly.

van der Sman, Houlder, Cornet, & Janssen, 2019). While the transport of acid and enzyme is mostly towards the inside of the food particle, the water migration could be water uptake from the gastric juice towards the food particle or vice versa; either swelling or shrinking could take place. The swelling behaviour of whey protein gels is pH-sensitive, with a minimum swelling ratio close to the isoelectric point of whey proteins (~5.1) (Betz, Hörmansperger, Fuchs, & Kulozik, 2012). In another study, the swelling of whey protein gels was measured in aqueous solutions with different pH values, e.g. swelling ratio was 24% for pH 2.5 (Oztop, Rosenberg, Rosenberg, McCarthy, & McCarthy, 2010). Thus during gastric digestion, swelling takes place between gastric fluid and food particles, if swelling pressure differences exist between the food products and the surrounding liquid (van der Sman et al., 2019). Swelling pressure is the summation of elastic pressure, mixing pressure, and ionic pressure (Flory & Rehner, 1943; Quesada-Pérez, Maroto-Centeno, Forcada, & Hidalgo-Alvarez, 2011; van der Sman, 2015). As illustrated in Supplementary Fig. 1, when swelling takes place, it is likely that together with the water flow acid and enzyme are transported from gastric fluid to food. Mass transport of acid and enzyme is not only taking place via diffusion, due to a gradient in the concentration, but also via convection, due to the bulk water motion (van der Sman et al., 2019). We hypothesize that water migration is an important factor in gastric digestion. Most digestion studies have focused on the change of dry matter or nutrients, and the effect of swelling on food digestion has not yet been examined specifically.

Therefore this study was conducted to better understand the role of swelling during gastric digestion of solid food. We prepared whey protein gels with different NaCl concentrations at gelation as model systems for protein-rich structured foods and measured their swelling ratio and digestion rate. Finally we proposed how swelling might affect digestion.

2. Materials and methods

2.1. Materials

Pepsin from porcine gastric mucosa (561 activity units/mg) and other chemicals were purchased from Sigma Aldrich, Inc. (St. Louis, USA). Whey Protein Isolate (WPI) was purchased from Davisco Food International, Inc. (Le Sueur, USA), with protein content of 97.9 g/100 g dry solid and ash 1.9 g/100 g dry solid. Green fluorescent protein (GFP, MW = 26.9 kDa) was produced in-house at Wageningen University & Research, with the method as described by Nolles et al. (2015). Milli-Q water (resistivity 18.2 MΩcm at 25 °C, Merck Millipore, Billerica, USA) was used in all experiments.

2.2. Preparation of protein gel and simulated gastric fluid

WPI solution and gel were prepared as described previously (Deng et al., 2020). WPI was dissolved in water (15 wt%), or in NaCl solution (15 wt%) with concentrations of 0, 0.01 M, 0.05 M and 0.1 M at neutral pH. Due to the ash content in whey protein isolate powder there is approximately 0.03 M salt present in WPI gels before adding extra salt. The protein solutions were stirred at room temperature for at least 2 h. To prepare the gels, the solutions were centrifuged at 1000 rpm for 10 min to eliminate air bubbles, and were poured into Teflon tubes with a diameter of 20 mm and then heated in a 90 °C water bath for 30 min with rotating. After that, the Teflon tubes were immediately cooled in an ice-water bath. The gels were stored within the sealed Teflon tubes at 4 °C, 1–5 days prior to use.

Simulated gastric fluid (SGF) was prepared with HCl and NaCl (pH 2.0, I = 154 mM) (Kong & Singh, 2008). Pepsin was added to achieve activity at 2000 activity units/mL, as recommended by Minekus et al. (2014) and Brodkorb et al. (2019). For the control group, no pepsin was added to the SGF.

2.3. Young's modulus

Gels were sliced with a gel slicer into cylinders of 5 mm in height and 20 mm in diameter. Compression tests were performed with an Texture Analyzer (Instron Corp. 5564, USA) using a static load cell of 2000 N. Gel samples were compressed to 80% of their initial height between two parallel plates at a constant deformation rate of 1 mm/s. Measurements were performed at 20 °C in triplicate. As described in Urbonaite, de Jongh, van der Linden, and Pouvreau (2014), the Hencky's strain (ε_h) and true stress (σ(t), Pa) are defined as:

$$\varepsilon_h = |ln\frac{h(t)}{h_0}| \quad (1)$$

$$A(t) = \frac{h_0}{h(t)} \bullet A_0 \quad (2)$$

$$\sigma(t) = \frac{F(t)}{A(t)} \quad (3)$$

$$E = \frac{d\sigma}{d\varepsilon_h} \quad (4)$$

where h_0 is the initial height of the gel slice and $h(t)$ is the height of gel after being compressed for time t. With an assumption that volume of gel remained the same during deformation, the contact surface area (A(t), m^2) at time t can be calculated with Eq. (2), where A_0 is the initial contact surface area of the gel slice. F(t) is the measured force (with unit N). Young's modulus (E, Pa) is defined as the slope from the linear part of stress over strain curve within region 0.05 and 0.15 as Eq. (4).

2.4. In vitro gastric digestion

The *in vitro* gastric digestion was initiated by putting a slice of gel into 30 mL SGF in a tube with a diameter of 35 mm. The gel slices were in the same shape as described in Section 2.3. The digestion was performed at 37 °C in a water-jacket compartment stirring at 100 rpm.

2.5. Determination of free amino acid groups

At different time points during *in vitro* digestion, the supernatant was withdrawn and heated by a pre-heated Eppendorf thermomixer at 90 °C, 850 rpm for 5 min to deactivate pepsin. To track the extent of digestion, we measured the free amino groups in the supernatant by the o-phthaldialdehyde (OPA) method, as described previously (Deng et al.,

2020). Briefly, the OPA reagent was prepared and kept in the dark. A DU 720 spectrophotometer (Beckman Coulter Inc., Pasadena, USA) was set at 340 nm blanked with 1.5 mL OPA reagent and 0.2 mL Milli-Q water. For the calibration curve, 0.2 mL L-serine standard solutions (0–200 mg/L) were added to 1.5 mL OPA reagent in a cuvette, and mixed by pipetting for 5 s. The mixtures were measured with the spectrophotometer after standing for 3 min. The same procedure was applied to samples from the digestion experiments.

2.6. Mass change

After 120 min digestion as described in Section 2.3, the remaining gel was removed from the system and dried with tissue paper to remove excess liquid, and then weighed. The mass change of gels was calculated as:

$$Mass\ change(\%) = \frac{m_{120} - m_0}{m_0} \times 100\% \quad (5)$$

where m_{120} represents the weight of the gel after t = 120 min digestion and m_0 is the initial weight of the gel.

2.7. Swelling property

To measure the swelling property of the gels, we performed same procedure as described in Section 2.3 but using SGF without pepsin. During 120 min incubation, the gel slices were taken out of the SGF and dried with tissue paper to remove excess liquid, and then weighed. The swelling ratio was calculated using Eq. (6).

$$Swelling\ ratio(\%) = \frac{m_t - m_0}{m_0} \times 100\% \quad (6)$$

where m_t represents the weight of the gel after t min incubation and m_0 is the initial weight of the gel.

2.8. Acid uptake

Under the same conditions as described in Section 2.3, by using SGF both with and without pepsin, pH of the supernatant was recorded during 120 min incubation. The pH change of SGF can reflect acid uptake by the gels. We calculated the amount of $[H^+]$ uptake by gel during incubation and the acid uptake by protein after 120 min incubation with Eqs. (7) and (8):

$$\Delta[H^+]/gram\ Gel = \frac{10^{-pH_0} - 10^{-pH_{120}}}{m_{Gel}} \quad (7)$$

$$Acid\ uptake\ (mol/kg\ protein) = \frac{10^{-pH_0} - 10^{-pH_{120}}}{m_{Gel} \times c(protein)} \times V_{SGF} \quad (8)$$

pH_{120} and pH_0 represent pH of the supernatant at 120 min and time 0; m_{Gel} (g) represents the initial weight of the gel slice; c(protein) is the protein concentration in the gels (0.15 g protein per g gel); V_{SGF} is the volume of incubating SGF (30 mL).

2.9. Green fluorescent protein transport

The diffusivity of green fluorescent protein (GFP) in the gels was shown to be comparable to that of pepsin (Luo et al., 2017). In our study GFP was used to represent pepsin. GFP solution was prepared with 0.32 μmol/L GFP and 154 mM NaCl at neutral pH (because of the pH-dependency of the used fluorophore). A slice of the gel was soaked in 10 mL of the GFP solution, with a shaking speed of 50 rpm at room temperature. The gel slices were in the same shape as described in Section 2.3. After 1.5 h, the gel slices were taken out of the solutions and were cut. A narrow slice was obtained by cutting from middle of the original gel slice as illustrated in Supplementary Fig. 2. Firstly, a narrow slice (~2 mm) was obtained by cutting from middle of the original gel slice. After that, the middle narrow slice was laid down on a glass slide. Subsequently, the region (2 × 2 mm^2) as drawn in green in Supplementary Fig. 2 was measured by a confocal laser scanning microscopy (CLSM) to obtain the fluorescent intensity. The excitation wavelength was 488 nm. Fluorescence intensity was recorded through a 177 μm pinhole using a 515 nm filter. The acquired images were loaded into Image J and the green fluorescent intensity at every pixel was extracted. The same measurement was done to measure the gels under control condition using a 154 mM NaCl solution without GFP. Because of the pH dependency of the swelling properties, we measured the swelling behaviours of gels in the GFP solution (0.32 μmol/L GFP and 154 mM at neutral pH) under the set-up of GFP experiment by the same way as described in Section 2.6.

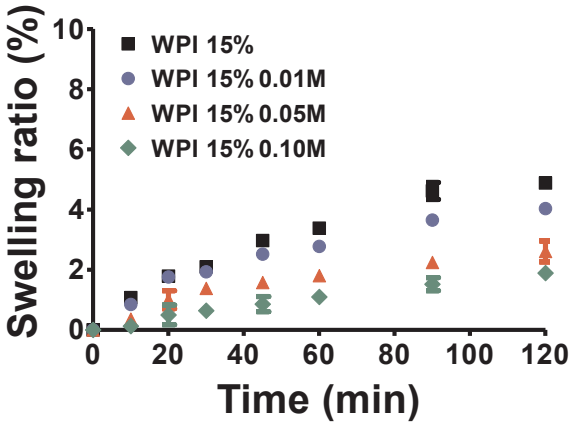

Fig. 2. Swelling ratio of 15% WPI gels with NaCl varying from 0 to 0.1 M in SGF without pepsin; the error bars represent standard deviation.

2.10. Statistical analysis

Independent-samples t-tests and analysis of variance (ANOVA) tests followed by a post hoc LSD test were performed with SPSS statistics software (IBM SPSS Statistics for Windows, Version 25, IBM Corp). Effects were considered statistically significant at $p < 0.05$. The expressions 'value ± value' represent 'mean ± standard deviation'.

3. Result and discussion

3.1. Characterization of the protein gels

The Young's modulus of the gels reflects their hardness and is shown in Fig. 1. With increasing of NaCl concentration from 0 to 0.05 M, Young's modulus of gels increased significantly ($p < 0.05$, *t*-test) from 68.15 ± 16.05 kPa to 249.3 ± 38.34 kPa, indicating the hardness of the gels was enhanced. No significant difference was found between the gel with NaCl 0.05 M and 0.1 M ($p = 0.47$, *t*-test). The observation that Young's modulus stops increasing at a certain salt concentration is in accordance with the results from another study (Urbonaite et al., 2016). The presence of NaCl can create coarse gels with dense protein aggregation and capillary water in between aggregations, this is because the electrostatic repulsion is reduced and protein aggregation is facilitated. As a result, the protein aggregation yields thicker strands, and thereby an increase in hardness. On the other hand, the protein aggregation in strands could reduce the availability of protein, and therefore enlarge the pore size of the protein gel, resulting in decreased hardness of the gel. This increase of pore size is reported in the SEM images of Urbonaite et al. (2016). This explains that the gel with 0.1 M NaCl (likely with larger pore size) did not show higher Young's modulus than the gel with 0.05 M NaCl. Moreover, we would expect a decrease of Young's modulus in gels with higher NaCl concentration.

The swelling properties of the gels were measured by the wet mass

change during 2 h incubation in the SGF without pepsin. During the incubation, it need to be noted that there was hardly no solid loss of the gels, which has also been shown in previous studies (Luo, Boom, & Janssen, 2015). The swelling behaviour of WPI gels with NaCl 0–0.1 M in SGF without pepsin is shown in Fig. 2. All the gels swelled during the 120 min incubation, and less swelling took place with the increase of NaCl concentration (0–0.1 M) in the gel. The difference in swelling ratio among the gels is likely driven by the difference in swelling pressure between the gels and SGF. In the current study, even though the protein concentration in all the gels and SGF was the same, addition of NaCl increased the hardness (Young's modulus). This is likely the reason for the observed decrease of the swelling ratio. Moreover, this observation can also be explained by Flory–Rehner theory; among all the gels with an increase of Young's modulus, elastic pressure increased and mixing pressure increased, which in total resulted in the decrease of swelling ratio (van der Sman, 2015).

Although the swelling of the gels in SGF is a dynamic and complex process, our results show that the swelling properties of gels can be manipulated through varying structure, for example by using different amounts of NaCl at gelation.

3.2. Protein gel digestion

To evaluate the digestion rate of the WPI gels with 0–0.1 M NaCl, we measured the free amino groups in the supernatant. With an increase of NaCl concentrations in the gel, the free amino groups released slower from the gel slice to the SGF (Fig. 3a). The mass loss after 120 min digestion (Fig. 3b) was comparable with Fig. 3a, showing the order of degree of digestion rate: 0 M > 0.01 M > 0.05 M ≈ 0.1 M. Combining this information with Young's modulus of the gels, it suggests that the increase of hardness was approximately in line with the decrease in the rate of digestion. Thus, the food particles with higher hardness are more resistant to be digested, and this has been shown from many other studies. (Guo, Ye, Lad, Dalgleish, & Singh, 2014; Hu et al., 2017).

These results combined with the swelling results from Section 3.1 show that faster digestion (faster release of peptides) took place under higher swelling conditions. The gel with 0.1 M NaCl is an exception: compared with the gel with 0.05 M NaCl, there tended to be less swelling (t_{120min}, $p = 0.051$, *t*-test), but a similar amount of free amino groups was released (t_{120min}, $p = 0.312$, *t*-test). This might be due to the presence of larger pore sizes in the coarse gel with 0.1 M NaCl as discussed in section Section 3.1; the larger pore sizes might improve the accessibility of acid and/or pepsin, so as to partly compensate the effect of less swelling. Overall we observed that digestion of gels was faster when more swelling took place. The potential mechanism could be that the swelling behaviour of gels subsequently affected the accessibility of acid and pepsin during the digestion. This is further discussed in Sections 3.3 and 3.4.

3.3. Acid uptake

To track the acid uptake of the WPI gels, we recorded the pH in the supernatant; and the concentration change of H^+ (in the supernatant) per gram of gel was calculated under control (without pepsin, Fig. 4a) and digestion conditions (with pepsin, Fig. 4b). Under the control condition, the pH change indicates the H^+ transport from the supernatant to gel slice, since the solid loss of the gels was negligible. The pH of supernatant and acid uptake increased over time in all samples (Fig. 4a). The highest acid uptake was found with 0 M NaCl; with the addition of NaCl in the gels, smaller acid uptake was found in the supernatant. After 120 min, [H^+] uptake was significantly decreased from

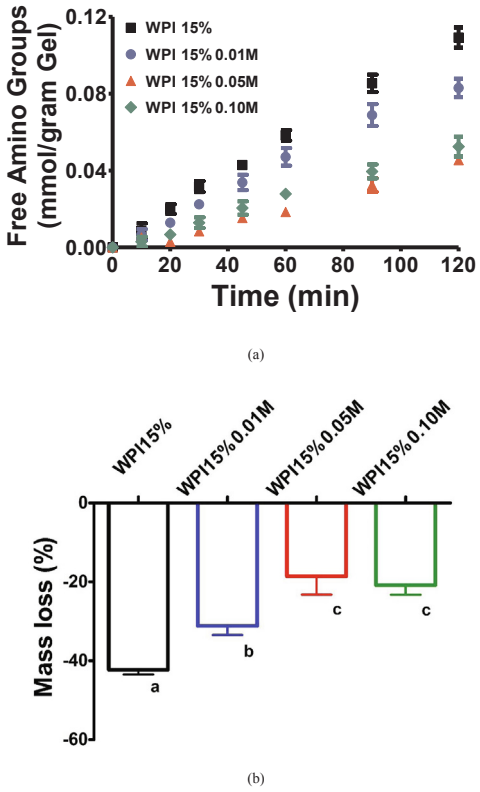

Fig. 3. Free amino groups in the SGF during 120 min digestion (a) and mass loss after 120 min digestion (b) of 15% WPI gels with NaCl varying from 0 to 0.1 M; the error bars represent standard deviation, and columns with the same letter did not differ significantly.

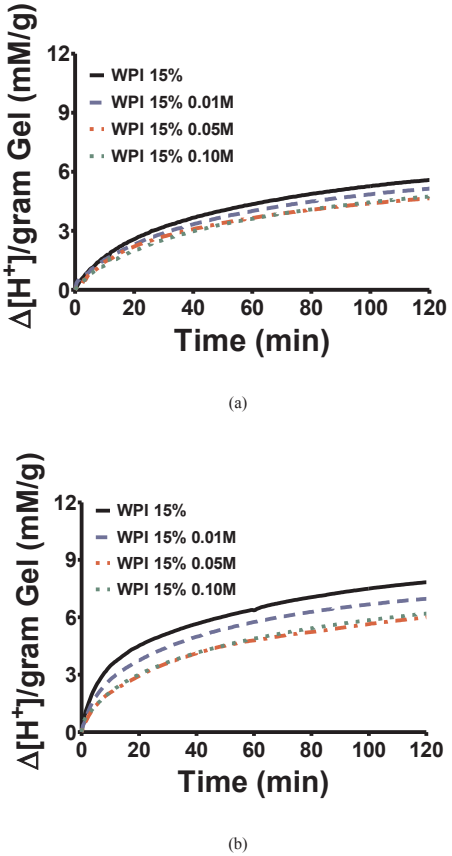

Fig. 4. Acid uptake of per gram of protein gels during 120 min incubation under control condition (a) and digestion condition (b).

5.7 ± 0.1 mM/g gel without NaCl to 4.7 ± 0.1 mM/g gel with 0.1 M NaCl ($p < 0.05$, *t*-test). Thus, the acid moved slower with decreased swelling ratio, with the increase of Young's modulus of the gels. Moisture uptake was found to be positively related with acid uptake as well in sweet potatoes (Somaratne et al., 2019). Acid and water uptake were not always consistent in some studies (Mennah-Govela & Bornhorst, 2016; Mennah-Govela et al., 2015). This is because there are factors driving acid uptake but not water uptake, for example buffering capacity of food particles.

In our study, after 2 h the acid uptake measured by pH change of the SGF was 0.95 ± 0.01–1.14 ± 0.02 mol/kg protein in the gels. These actual acid uptake values are lower than the demanded amount to reach pH 2. The demanded amount is 1.67 ± 0.1 mol/kg protein as reported previously (Luo, Zhan, Boom, & Janssen, 2018). This shows that in our study all the gels need more acid uptake to reach equilibrium due to the buffering capacity of the protein gels. Therefore, the difference in acid uptake among the gels is likely due to the difference in acid diffusion or convection instead of a difference in buffering capacity.

To further figure out the reason of different acid uptake among gels, we calculated acid convection, which is the amount of acid transporting together with water flow, using Eq. (9).

$$Acid\ convection\,(mol/kg\ protein) = \frac{Swelling\ ratio_{120} \times \rho_{SGF} \times 10^{-pH_{SGF}}}{c\,(protein)} \quad (9)$$

where $swellingratio_{120}$ represents the swelling ratio of the gels after 120 min incubation; ρ_{SGF} is the mass density of SGF (1 kg/L); pH_{SGF} is the pH value of SGF (2); c(protein) is the protein concentration in the gels (0.15 g protein per g gel).

The amount of acid convection is from 0.001 to 0.003 mol/kg protein in gels with 0.1 M NaCl to without NaCl; higher convection of acid took place in the gels with more swelling. The convection is hundreds times lower than the actual measured acid uptake. This indicates that the amount of acid convection is too small to differentiate acid uptake among the gels. Thus, it suggests that the difference of acid uptakes among the gels is because of the different amount of acid diffusion. With the fact that more acid uptake took place in the gels with more swelling, it is likely that swelling could increase the diffusion of acid so as to increase the acid uptake. The acid diffusion coefficient (De) has been shown to be larger in foods with more loosen structure, for example De 1.2×10^{-9} m^2/s in canned red beets and 1.1×10^{-10} m^2/s in raw red beets (Mennah-Govela et al., 2020). Therefore, the potential mechanism might be that swelling increases free space which could enhance the acid diffusion in the gels.

Under digestion condition, the pH of supernatant increased with time and there was more acid uptake compared to the control condition in all the samples (Fig. 4b). The highest increase took place in gel without NaCl ($[H^+]$ uptake of 8.1 ± 0.2 mM/g gel), while the smallest increase was in gel with 0.05 M NaCl ($[H^+]$ uptake of 6.1 ± 0.1 mM/g gel)and 0.1 M ($[H^+]$ uptake of 6.3 ± 0.1 mM/g gel). The acid uptake under digestion conditions is due to the sum effect of the acid uptake by the gels and the cleavage of peptide bonds due to digestion. During protein hydrolysis, when a peptide bond is cleaved, a carboxyl and an amino group are released; in the gastric (acidic) environment, these groups undergo ionization which results in an increase of pH. As shown by Luo et al. (2018), the pH change (or $[H^+]$ uptake) due to hydrolysis is proportional to the degree of hydrolysis of protein gels. Thus, it is to be expected that among all the gels under digestion conditions, the difference in $[H^+]$ uptake is in accordance with digestion results (Fig. 3a). The results show that acid uptake increases when the swelling ratio increases and that gels with more acid uptake show a larger degree of digestion. This confirms our hypothesis that swelling can increase the transport of gastric acid into protein gel particle. It is interesting to know whether there is more enzyme present inside the gel if the swelling is stronger.

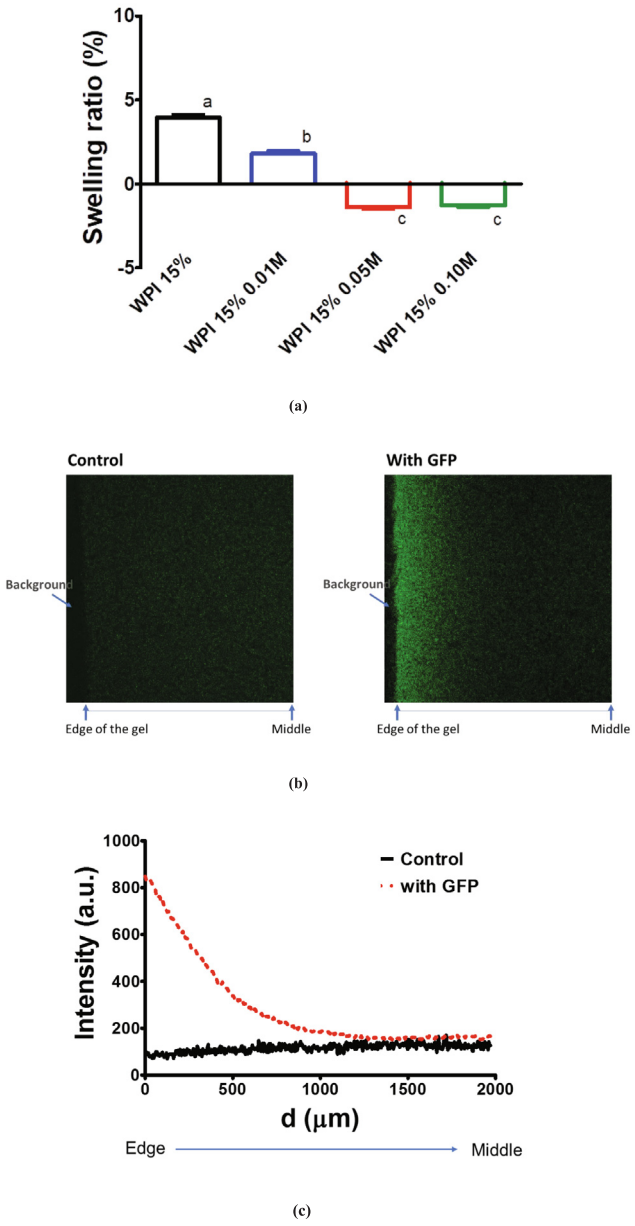

Fig. 5. Swelling ratio of 15% WPI gels with NaCl from 0 to 0.1 M in the GFP solutions (a, the error bars represent standard deviation, and columns with the same letter did not differ significantly), fluorescent images (b) and fluorescent intensity distribution (c) of 15% WPI gels after incubation under control conditions (without GFP) and conditions with GFP.

3.4. Green fluorescent protein transport

Since the diffusivity of green fluorescent protein (GFP) was shown to be similar to that of pepsin in WPI gels by Luo et al. (2017), we used GFP to represent pepsin to explore how swelling would affect pepsin transport. GFP experiment cannot be conducted at the exact same condition (pH, temperature) as gastric digestion due to the limitation of GFP stability. To understand how the swelling properties affect GFP transport, we measured the swelling/shrinking properties of gels under the set-up of GFP experiments (Fig. 5a). With increase of NaCl in the gels, less swelling took place and even turned to shrinking with 0.05 M and 0.1 M NaCl. The trend among the different gels was similar to the swelling results of the gels in SGF (Fig. 2), although the swelling ratio of each gel under these two conditions was not the same. This may be due to pH differences (pH 7.0 for Fig. 5a and pH 2.0 for Fig. 2), in

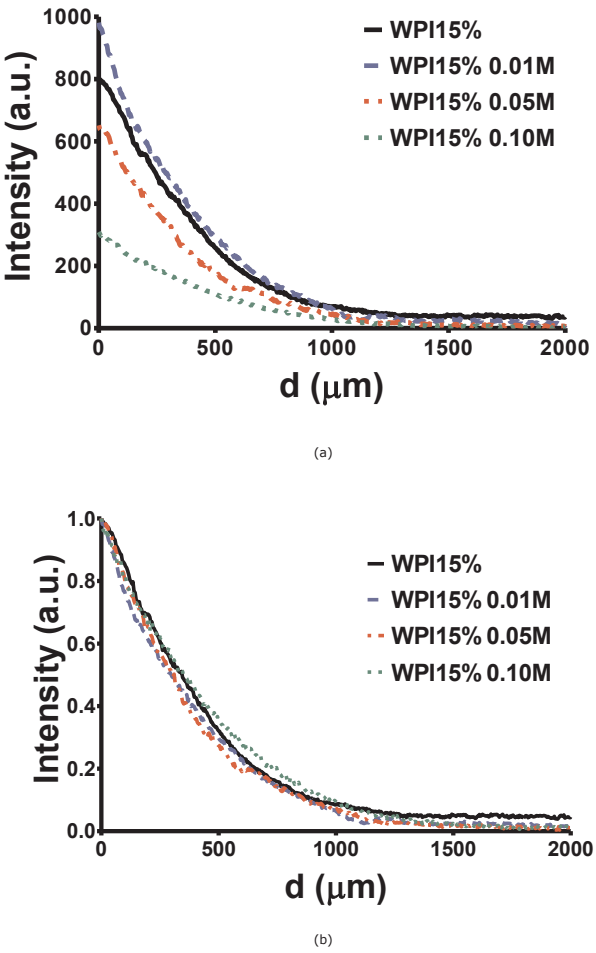

Fig. 6. Fluorescent intensity distribution (a) and relative fluorescent intensity distribution (b) in 15% WPI gels with NaCl from 0 to 0.1 M.

agreement with the finding that swelling behaviour of whey protein gels is pH-sensitive (Betz et al., 2012). The relation between swelling property and GFP transport is further discussed based on the swelling results in Fig. 5a.

The gels were observed with CLSM (Fig. 5b) after soaking in solution with or without GFP (control condition). For the control condition, the homogenous fluorescent signal was visible in the area of the gel, while for the condition with GFP a gradient of the green fluorescent signal present from the edge to the middle. The fluorescent intensity was plotted against the distance from the surface of gels (Fig. 5c). Under control conditions the intensity was very low and consistent in the gel region, while with GFP, fluorescent intensity gradually decreased from the surface till 1 mm depth. This indicates that at this specific excitation wavelength the signal from GFP was dominant, even though whey protein had a small contribution to the fluorescent signal. Besides, the GFP concentration differed depending on the distance from edge of the gel.

To focus on the fluorescent signal from GFP specifically, the fluorescent signal of the gels under control conditions was subtracted from that under conditions with GFP (Fig. 6a). In all the gels, the concentration of GFP was higher near the surface and decreased towards the middle. With increase of Young's Modulus in the gels (with less swelling), the GFP intensity decreased not only in the gels and but obviously also at their boundary.

To compare the transport of GFP in the gels, we corrected the surface intensity as 1, and calculated relative fluorescent intensity from the surface to the middle of gels (Fig. 6b). The decrease curves of GFP intensity from surface to middle of different gels are similar and overlap. This shows that the movement rate of GFP did not change regardless of the different swelling properties among the gels. This is consistent with the finding from a previous study in which the GFP diffusion coefficient does not differ between gel without and with 0.05 M NaCl (Luo et al., 2019). In fact, the higher amount of GFP at the surface and in the gel was observed in gels with higher swelling ratio and digestion rate. The difference in GFP intensity on the surface of gels might be explained by different partitioning of GFP between the protein gel and the gastric fluid. In mass transfer from one phase to the other, the overall mass transfer coefficient is also dependent on the partition coefficient (Wesselingh & Krishna, 2000). Partition coefficients of protein between hydrogel and liquid have been shown to be dependent on pH and ionic strength (Sassi, Shaw, Han, Blanch, & Prausnitz, 1996). As described by van der Sman (2018), a difference in ionic strength induces a difference in the elastic pressure of the swelling pressure. The partitioning coefficient of a solute, defined as the concentration in the gel divided by the concentration in the liquid, decreases with an increase of elastic pressure of the gel.

The results show that more swelling leads to a higher partition coefficient. It is this phenomenon that happens at the interface between the gastric juice and the gel particle that seems to be responsible for the higher GFP concentration inside the gel. If we relate this to pepsin transport towards the gel or food particle, the properties of the gel (or food) might define the partition coefficient of pepsin between the gel (or food) and the gastric fluid.

The results suggest that with more swelling even though the convection/diffusion of pepsin might not be enhanced, more pepsin positioning inside of gel at the surface could increase the pepsin concentration in the gel and thereby the digestion rate. As mentioned above, the GFP experiment that we conducted was not under the same conditions as the digestion experiment (e.g. pH, enzyme concentration), but it does show that the partitioning property of pepsin is crucial for digestion. The factors affecting the partition coefficient of pepsin on food surface are worthwhile to be further explored to better understand gastric digestion.

4. Conclusion

This study pointed out the important role of swelling during digestion. During *in vitro* gastric digestion of WPI gels with different Young' modulus (as a result of gelation with different NaCl concentration 0–0.1 M), the digestion rate, acid uptake and GFP intensity on the surface and inside of the gels were increased with the increase of swelling ratio. This suggests that swelling can increase the acid diffusion to lower the local pH in food particle for gastric digestion. Different swelling properties could modulate the partitioning of pepsin on the food-gastric fluid interface and are crucial for the total amount of pepsin in the food and therefore the digestion rate of food particles. The swelling effect on *in vivo* gastric digestion and on the subsequent digestion phases need to be further explored. Therefore, swelling properties of food particles should be taken into consideration when studying food digestion, and the factors affecting the partitioning property of pepsin need to be further investigated. This will yield better knowledge on the mechanisms of food particle digestion which can provide novel perspectives on designing food with different rates of digestion and nutrient release to satisfy specific target groups.

CRediT authorship contribution statement

Ruoxuan Deng: performed the experiments and analyses and wrote the manuscript; Anja E.M. Janssen, Paul A.M. Smeets and Monica Mars: were involved in the design of the study and data analyses, reviewed all versions of the manuscript; Anja E.M. Janssen: had primary responsibility for the final content of the manuscript; Ruud G.M. van der Sman: reviewed the manuscript and advised on interpretation of the results;

All the authors: read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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