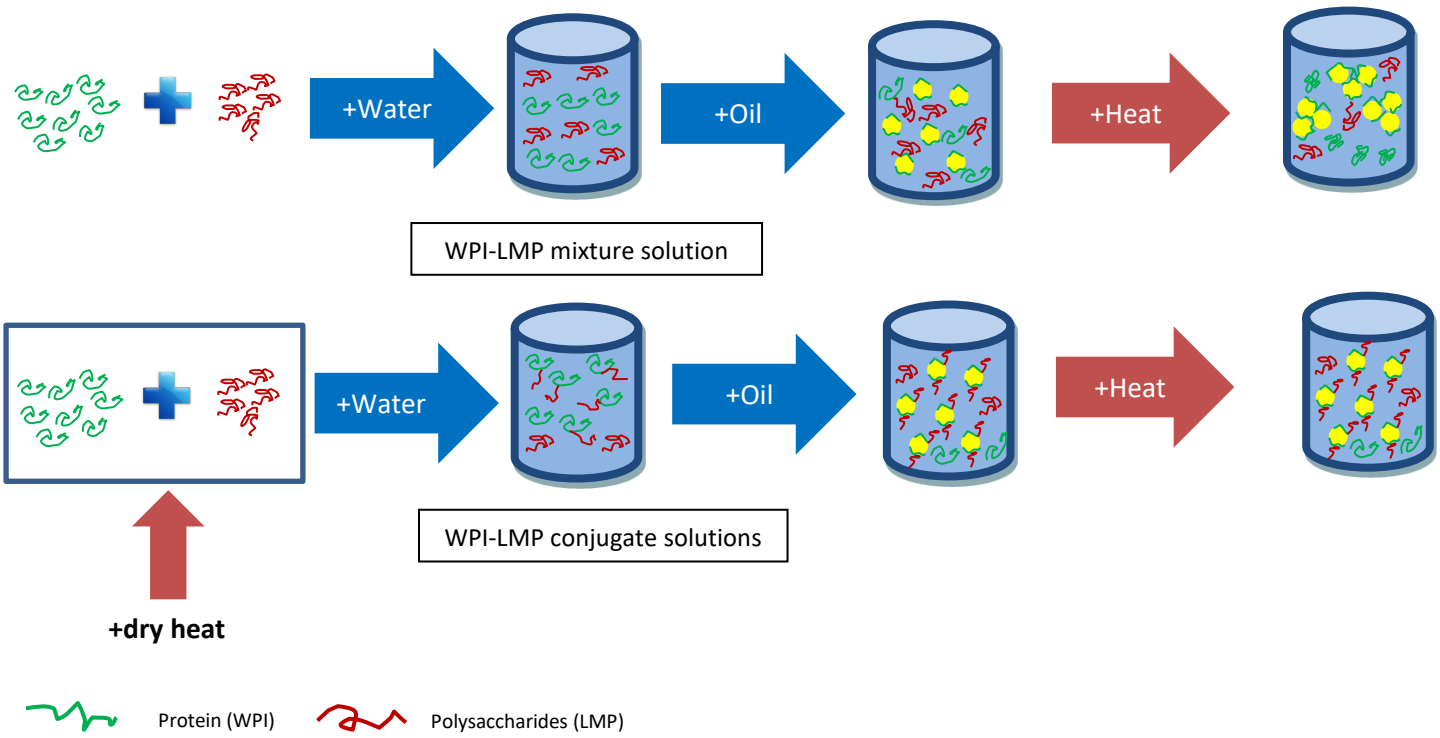
Improved heat stability of protein solutions and O/W emulsions upon dry heat treatment of whey protein isolate in the presence of low-methoxyl pectin

Arima Diah Setiowati[1], LienVermeir[1], Jose Martins[2], Bruno De Meulenaer[3], Paul Van der Meeren[1]

[1]Particle and Interfacial Technology group, Department of Applied Analytical and Physical Chemistry, Faculty of Bioscience Engineering, Ghent University,Coupure Links 653, 9000, Gent, Belgium

[2]NMR and Structure Analysis Unit, Department of Organic Chemistry, Faculty of Sciences, Ghent University, Krijgslaan 281 S4, B-9000 Ghent, Belgium

[3]Research Group Food Chemistry and Human Nutrition, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University,Coupure Links 653, 9000, Gent, Belgium



Abstract

Whereas Whey Protein Isolate may be used as effective emulsifier, it suffers from limited stability upon heating. In this research, the effect of combining Whey Protein Isolate (WPI) with Low Methoxyl Pectin (LMP) on the heat stability of WPI was investigated. This was accomplished either by simple mixing or by conjugate formation by dry heat treatment. WPI-LMP mixtures and conjugates were prepared at a WPI to LMP ratio of 1:0, 4:1, 2:1, and 1:1. Conjugates were prepared by means of dry heat treatment at a temperature of 60^{o}C and 74% relative humidity by incubation for up to 16 days.

The pH effect and brown color development of the WPI-LMP conjugates upon incubation was monitored. SDS-Page, free amino group determination, as well as diffusion coefficient analysis, all confirmed the formation of new compounds with high molecular weight. The heat stability of the conjugates was then tested and compared to the native WPI. Upon 2 minutes of heating at 80 ^{o}C and pH 6.5, the solubility of WPI was reduced by approximately 50% due to thermal denaturation and subsequent aggregation. However, dry heat treatment of the WPI-LMP mixtures highly improved the heat stability of WPI: as the incubation time was increased, the residual protein solubility upon heating of the WPI became higher. Upon 16 days of incubation, the protein solubility of the heated WPI-LMP conjugates was comparable to that of the conjugates before heating. Considering the emulsifying properties, it was found that WPI-LMP conjugates produced smaller oil droplet compared to either native WPI, dry heated WPI, or mixtures of WPI-LMP which were not subjected to conjugation by dry heat treatment. Heating the emulsions at 80 ^{o}C for 10 and 20 minutes revealed that WPI-LMP conjugates stabilized emulsions exhibited excellent stability towards heat: whereas pronounced aggregation and gelation occurred in emulsions stabilized by WPI or mixtures with LMP, the conjugate stabilized emulsions retained their original viscosity and particle size.

Keywords: Whey Protein Isolate, Low Methoxyl Pectin, Dry heat treatment, Conjugate, Heat Stability, Emulsion.

Introduction

Whey Protein Isolate (WPI) is known to have excellent emulsifying properties. However, this dairy protein source exhibits a low stability towards heat and is very susceptible to heat denaturation [1]which can lead to partial protein denaturation and aggregation, thus affecting its emulsifying properties [2]. As heat processing is commonly encountered in dairy industry for improving the safety and shelf life of foods [3], the limited heat stability of WPI becomes a limiting factor for the application of WPI on an industrial scale[4]. Several researches on improving the heat stability of WPI have been performed using different

approaches. Chemical methods [5, 6], enzymatic modification [7, 8], electrostatic interactions [9, 10], and conjugation by heat [11] are among the methods that have been used. Some of the methods showed a successful improvement of the heat stability and emulsifying properties of WPI [12-15]. Nevertheless, among the methods mentioned, chemical methods involve chemicals which sometimes are not compatible for food application. On the other hand, electrostatic interaction has a narrow range of optimum pH and is sensitive to the presence of salts which limits its application [16]. Conjugation by heat is a promising method as it does not involve any chemical addition. This method relies on the addition of another biopolymer to be conjugated to the WPI.

It is known that polysaccharides have a good stabilizing activity [17]. It was stated that the presence of a biopolymer can influence or modify the functional properties of other biopolymers when they are mixed [10]. Thus, combining the properties of WPI and polysaccharides can be a good way to modify the properties of WPI resulting in a molecule with improved functionality [18]. It has been reported that the presence of polysaccharides enables WPI to minimize structural losses during heating due to the steric force obtained from the polysaccharides [13, 16, 19].

Heat induced conjugation of WPI and polysaccharides results in hybrid molecules which are linked together by covalent bonds. This type of interaction is found to be more heat stable than electrostatic interaction [18, 20]. Conjugation of WPI and polysaccharides by heat can be performed either in wet state [12, 21] or in dry state [15, 22, 23]. The latter is more preferable since it is easier to handle dry matter and also microbial contamination leading to an unstable product which can occur in the wet state can be avoided [24]. As this method does not require any chemical addition, it can be a promising replacement for synthetic surfactants in food applications [25], whereby it is suitable for the production of “clean-label” emulsions. Another advantage is that the dry heat treatment does not significantly alter the native-like behavior of the protein while wet heat treatment causes important structural changes resulting in a specific denatured β-Lactoglobulin monomer, which is covalently associated via the free thiol group [26].

Conjugation of WPI and polysaccharides through dry heating can be performed by exposing the dry mixture of WPI and polysaccharides to heat in an atmosphere of controlled relative humidity for a certain period of time. Depending on the type of the protein and polysaccharides used, the incubation can take from hours to days. It is suggested that upon dry heating, Maillard type reactions are responsible for the formation of the conjugates. Conjugation of protein and pectin is basically based on the Amadori arrangement which is part of the Maillard type reaction [24]. Hereby, the terminal and side chain amine groups of the protein are linked to the reducing end of the polysaccharides, resulting in a conjugate of

protein and polysaccharides [27]. However, it has to be kept in mind that the advanced stage of the Maillard reaction is undesirable since it can lead to a loss of solubility, and hence a reduction of the functionality of the WPI [11, 28]. Therefore, the challenge is to obtain WPI-polysaccharide conjugates without any loss of functionality of the WPI during heat treatment [29].

The purpose of this research was to improve the heat stability of WPI by grafting Low Methoxyl Pectin (LMP) to the WPI via dry heat treatment. Pectin was chosen due to its abundant availability which makes it a cheap source of polysaccharides. Furthermore, polysaccharides are more desirable than oligosaccharides and monosaccharides because they are less prone to advanced stages of Maillard reaction that lead to loss of the protein solubility [28]. The WPI was mixed with the LMP at various ratios and dry heating was performed at 60 ^{o}C and 74% RH. Various experiments were performed to confirm the formation of WPI-LMP conjugates and to study the heat stability of the obtained conjugates.

Materials and Methods

1. Materials

The WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contained approximately 92.6% protein, whereby 85% of the protein is β-Lactoglobulin [30]. The Low Methoxyl Pectin (LMP) (UnipectinOB700) was obtained from Cargill (Ghent, Belgium) and contained 89.6% of dry matter. The LMP was used without further purification. Oil in water emulsions were prepared using sunflower oil purchased from a local supermarket as the oil phase.

2. WPI-LMP conjugate preparation

Conjugates were prepared from a 5% (w/v) protein solution and 1 % (w/v) LM Pectin solution. A correction for the protein content and the dry matter was taken into account during the calculation of the WPI and LMP needed. The WPI and LMP were dissolved in distilled water and the pH of the solutions was adjusted to 7 with 1 N HCl to avoid formation of ionic complexes that might form at lower pH during mixing. Both solutions were kept overnight in a refrigerator before mixing. The solutions were then mixed at four different ratios i.e. 1:0, 4:1, 2:1, and 1:1 (on weight basis) and frozen prior to freeze drying.

The frozen samples were lyophilized (Alpha 1-2 LD plus, Christ) to remove all the water and obtain dry products. The freeze dried products were then incubated at a temperature of 60^{o}C for 16 days in a desicator containing saturated NaCl solution to keep the relative humidity at 74%[31]. During incubation, sampling was done at day 4, 8, and 16.

3. pH and absorbance measurement

A 2 mg/ml WPI-LMP conjugate solution was prepared by dissolving the conjugates in distilled water. The pH and brown color development of the solutions were then measured without further dilution. The pH was measured using a Hanna H 4222 pH meter**,** while the brown color development was measured at 420 nm [32]using a UV-1600 PC, spectrophotometer (VWR).

4. SDS Page Analysis

The SDS-Page analysis was performed under reducing conditions in the presence of mercaptoethanol. The running gel and stacking gel contained 15% and 4 % of polyacrylamide. Conjugates were diluted in a 20 mM phosphate buffer (pH 6.5) to a concentration of 1 mg protein/ml. The solutions were then diluted in Laemlli buffer which contained mercaptoethanol to obtain a concentration of 0.5 mg/ml. These diluted solutions were then heated at 90^{o}C, followed by centrifugation at 10000 rpm for 5 minutes. Subsequently, 20 µl of the solution was injected in to the gel. The electrophoresis was performed at 160 V for at least 1 hour and 15 minutes. The gel was subsequently stained using Coomassie blue to visualize the protein.

5. High resolution pfg-NMR diffusometry

High-resolution pulsed field gradient (pfg) NMR diffusion analysis was performed with a Bruker Avance III spectrometer operating at a ^{1}H frequency of 500.13 MHz and equipped with a 5 mm DIFF30 gradient probe with a maximum gradient strength of 18 T/m. Pfg-NMR experiments were performed at room temperature using a monopolar (single) stimulated echo pulse sequence. The samples (650 µL) were filled in 5 mm diameter glass NMR tubes (Armar Chemicals, Switzerland) and were measured upon varying the gradient strength up to 12 T/m, while keeping the gradient duration (δ) constant at 1 ms and the diffusion delay (Δ) fixed at 100 ms. The (non-conjugate) powders of WPI (dry heated for 16 days) and LMP were dissolved in 5 mM sodium acetate (in D_2O) to obtain a concentration of 10 mg/mL, whereas WPI-LMP conjugates were dissolved in 5 mM sodium acetate to obtain a concentration of 20 mg/mL.

Regarding the non-conjugate WPI (WPI dry heated for 16 days) and LMP samples, the obtained experimental echo attenuation ratio (I/I_o) with up to 95% decay as a function of gradient strength was fitted by Eq. 1a and Matlab 7.5.0.342 (R2007b) software (The Mathworks). Hereby, a mass-weighted lognormal distribution of diffusion coefficients was assumed.

$$\left(\frac{I}{I_0}\right)_{expt} = \int_0^{\infty} P_v(D_i) \cdot \frac{I}{I_0}(D_i) \cdot \, dD_i \tag{1a}$$

$$\frac{I}{I_0}(D_i) = \exp\left(-D_i \cdot \gamma^2 \cdot G^2 \cdot \delta^2 \cdot \left(\Delta - \frac{\delta}{3}\right)\right) \tag{1b}$$

$$P_v(D_i) = \frac{1}{\sqrt{2\pi} \cdot D_i \cdot \ln \sigma_g} \cdot \exp\left(-\frac{\left(ln(D_i) - ln\left(D_g\right)\right)^2}{2 \cdot \left(\ln \sigma_g\right)^2}\right) \tag{1c}$$

$$D_a = D_g \cdot \exp\left(\frac{\left(\ln \sigma_g\right)^2}{2}\right) \tag{1d}$$

$$\sigma = \sqrt{{D_a}^2 \cdot \left(exp\left(\left(\ln \sigma_g\right)^2\right) - 1\right)} \tag{1e}$$

Protein and pectin samples may be characterized by a molecular mass range and hence, a polydisperse population of diffusion coefficients with a certain probability P_v. In our calculation, P_v was assumed to follow by a lognormal mass-weighted diffusion coefficient distribution (Eq. 1c). The geometric mean diffusion coefficient (D_g) and geometric standard deviation (σ_g) were converted to the arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ) of the lognormal mass-weighted diffusion coefficient distribution using Eq. 1d and Eq. 1e.

The degree of molecular interaction can be evaluated upon decomposing the WPI diffusion signal obtained from the WPI-LMP conjugate sample $\left[\frac{I}{I_0}\right]_{WPI,conjug.}$ into a freeWPI fraction (i.e. the experimentally obtained non-conjugate WPI signal $\left[\frac{I}{I_0}\right]_{WPI,free}$ and a bound WPI fraction. The bound fraction can be determined upon estimating the diffusion signal of the reacted WPI $\left[\frac{I}{I_0}\right]_{WPI,bound}$ using Eq. 2 and Matlab 7.5.0.342 (R2007b software, The Mathworks). As the molar mass of pectin is much larger as compared to WPI, the molar mass of the conjugate is mostly determined by the pectin. Hence, the bound fraction can be determined assuming the experimentally obtained LMP diffusion signal to be a good approximation of the LMP-bound WPI diffusion signal as written down in Eq. 3 using the Solver add-in (Microsoft Excel 2010). A higher value of the coefficient of determination was obtained upon fitting Eq. 1 to the conjugate LMP signal $\left[\frac{I}{I_0}\right]_{LMP,conjug.}$ (R^2=0.999 in Table 3.1) as compared to the non-conjugate LMP (R^2=0.996) as recorded at 3.2-4.2 ppm. Therefore, the former signal was used in Eq. 3, for which D_a and σ amounted to $5.4 \cdot 10^{-11}$ m^2/s and $15 \cdot 10^{-11}$ m^2/s.

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = \varphi_{free} \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{WPI,bound} \quad (2)$$

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = \varphi_{(1-\varphi_{bound})} \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{LMP,conjug.} \quad (3)$$

6. TNBS Analysis for Free Amino Group Determination

TNBS analysis measures the amount of free amino groups in the sample. This analysis was carried out to find the degree of graft reaction. By knowing the available amino groups in the sample, the approximate amount of amino groups that are cross linked with the polysaccharide during dry heat incubation can be estimated and hence the degree of the graft reaction can be obtained.

The analysis was performed based on the method developed by Adler-Nissen [33]. All samples, blank, and standards were dissolved in 1% SDS solution prior to analysis. 0.25 ml of each sample was brought into a test tube. Subsequently, 2 ml of phosphate buffer (0.2125 M, pH 8.2±0.02) and 2 ml of a 0.1% TNBS solution were added. The test tube was shaken with a vortex and placed in a water bath at 50°C for 60 minutes. The water bath should be covered to avoid light penetrating into the test tubes since light can accelerate the reaction in the blank solution. Afterwards, 4 ml of 0.1 N HCl was added to terminate the reaction. The test tubes were then allowed to cool down to room temperature. The absorbance of the samples was read at 340 nm in a Spectrophotometer (UV-1600 PC, VWR). A standard curve was obtained by considering a dilution series of leucine with concentrations up to 2 mM. The amount of available amino groups (mM) was then deduced from the standard curve. The degree of graft reaction was calculated as:

$$degree\ of\ graft\ reaction\ (DG) = \frac{(-NH_2\ in\ mixture(day\ 0) - -NH_2\ in\ conjugates\ (day\ x))(mM)}{-NH_2\ in\ mixture(day\ 0)\ (mM)}\ x\ 100 \quad (4)$$

7. Heat treatment

2 ml of solution containing conjugates (1 % w/v) were prepared for heat treatment. The conjugates were dissolved in Imidazole buffer containing 20mM imidazole, 30 mM NaCl, and 1,5mM NaN_3; the pH of the buffer was adjusted to 6.55 using 1N HCl. The composition and pH were selected to have an electrolyte composition that resembled dairy products [34]. Subsequently, the solutions were heated at 80°C for 2 minutes using a water bath. Samples were placed into cold water after the heat treatment to stop denaturation. Heat treated samples were then put into 2 ml centrifugation tubes and centrifuged (Sigma,

Scientific 1-15 P) at 12000 g for 20 minutes to separate the insoluble aggregates. The supernatant was kept for further analysis.

8. Colorimetric protein determination

A modification and simplification of the Lowry analysis method by Schacterle and Pollack [35] was used. Prior to analysis, samples were diluted in imidazole buffer of pH 6.55 to fit in the range of the standard curve. 1 ml of sample was brought into a test tube in which 1 ml of alkalic Cu-reagent was added. This step was followed by mixing the solution using a vortex and leaving the solution undisturbed for 10 minutes. Subsequently, 4ml of Folin Ciocalteus reagent was added and the tube was turned upside down two times. The solution was heated at 55 ^{o}C for 5 minutes using a waterbath. The heated solution was put in ice-water immediately after heating. Subsequently, the absorbance of the solutions was read at 650 nm against a blank solution in a spectrophotometer (VWR, UV-1600 PC). A series of standard solutions was made by dissolving WPI in Imidazole buffer to a concentration of 0, 40, 80, 120, 160, 200, 240 mg protein/L. A correction for the protein content in WPI should be performed when calculating the amount of WPI needed in the standard solution.

9. Emulsion preparation

Emulsions were initially prepared by dissolving 0.5% of WPI, dry heated WPI, mixture of WPI-LMP, and WPI-LMP conjugates in the aqueous phase. The aqueous phase was kept overnight in the fridge prior to the emulsion preparation to fully hydrate the hydrocolloid. Emulsions containing 10% (w/w) of oil were prepared by adding 10 g of sunflower oil to 90 g of WPI solution.

The mixture was then premixed using an IKA Ultra-turrax TV45 (Janke & Kunkel, Staufen, Germany) at the highest speed (24000 rpm) for 1 minute. This was followed by homogenization using a Microfluidizer110S for 2 minutes at 4 bar of compressed air pressure corresponding to 560 bar of liquid pressure. The coil of the Microfluidizer was immersed in a waterbath set at 55^{o}C. The heat coagulation test was performed by heating the emulsions in an oil bath at 80oC for 20 minutes.

10. Particle Size analysis.

Particle size analysis was performed using a Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK) equipped with a red and blue light source. The refractive index used was 1.47, while the absorbance index was set at 0.01. The sample was added drop wise to the wet sample distribution unit (Malvern Hydro MV)

until an obscuration level between 10 to 20% was obtained. The speed of the stirrer of the dispersion unit was set at 1500 rpm during the measurement.

11. Statistical Analysis.

Two-way ANOVA was performed on the results of protein solubility analysis using SPSS 22 (IBM) at a significance level of 95%.

Results and Discussion

1. Confirmation of Conjugate formation upon incubation of WPI and LMP

Conjugation between protein and pectin is possible by exposing the two components to heat in a controlled environment. When proteins and polysaccharides are mixed and exposed to heat, the Maillard reaction will take place spontaneously. Upon incubation of WPI and LMP, as the Maillard reaction proceeds there will be changes in the environment of the mixture. This can be used as an indicator to monitor the Maillard reaction and to confirm the formation of WPI-LMP conjugates.

a. pH change and brown color development

Figure 3.1 shows the pH of the WPI-LMP conjugates as a function of the incubation time. The initial pH of the WPI and LMP dissolved in distilled water was approximately 6.6 and 5, respectively. However, the pH of the WPI and LMP solutions was adjusted to 7 before mixing to avoid electrostatic interactions. After freeze drying, the pH of the reconstituted dry mixture of WPI-LMP was found to be between 6.4-6.5. During dry heat incubation, the pH of all the conjugates, regardless of the mixing ratio, decreased. During Maillard reaction, primary amino groups will react and intermediate products are generated, which may contain acidic compound [36-38], which can explain the pH depression of the conjugates upon incubation. Overall, the pH decreased mainly in the first 4 days. It was found that there was no significant effect of dry heat treatment in the absence of LMP. In general, the more LMP was present in the mixture the lower the pH of the conjugates became. It was reported before by Liu *et al* [36] that due to Maillard reaction, the pH will decrease linearly with time. However, on systems with higher sugar content and incubated at 90^{o}C the trend was different: the pH decreased fast during the first few hours and upon further incubation it decreased at a slower rate [36]. This trend is similar to the trend of the conjugates with a ratio of 2:1 and 1:1 (Fig 3.1).

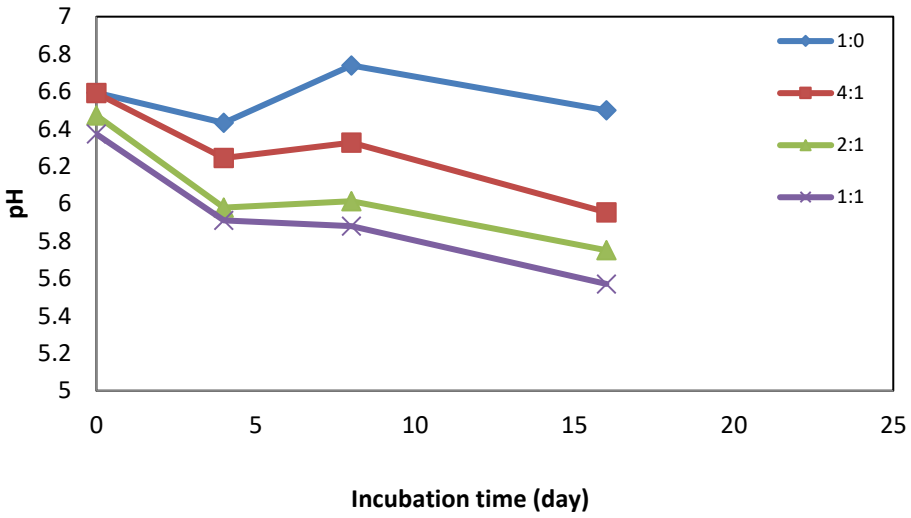


Figure 3.1 pH of the WPI-LMP conjugates as a function of incubation time.

It is known that the Maillard reaction results in brown pigments. Therefore, the progress of the Maillard reaction can be followed by the formation of these pigments in the dry mixture of WPI-LMP. Considering the absorbance at 420 nm as a function of incubation period of the conjugates (Figure 3.2) it can be clearly seen that brown pigment formation became more intensive as the incubation time was extended. In agreement with the pH results, the dry heated WPI (Ratio 1:0) had a stable color and did not undergo development of brown color.

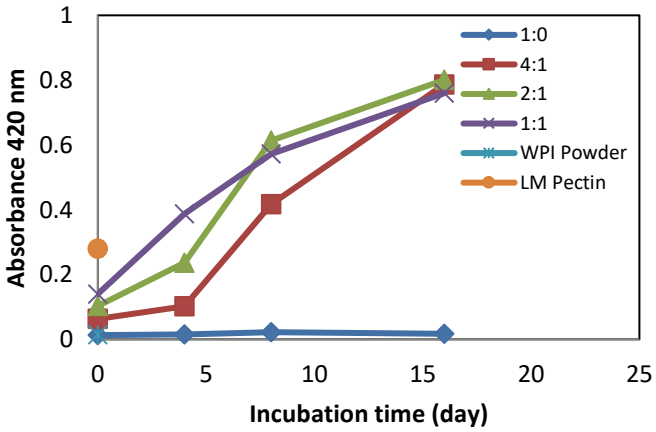


Figure 3.2 Brown color development of WPI-LMP conjugates as a function of incubation time.

In a similar way, the formation of intermediate products during the Maillard reaction can be evaluated from the absorbance at 294 nm. Comparing the absorbance readings at 294 (data not shown) and 420 nm, it was seen that the formation of the intermediate products had the same pattern as the formation of the brown color in the conjugates. This result is in agreement with the finding of Lertittikul *et al.* [39]. It can be clearly seen in Figure 3.2 that the more LMP was present in the conjugates, the higher was the initial rate of brown color formation during incubation. On the other hand, after 16 days all conjugates had a comparable absorbance value. Based on the pH change and brown color development, it can be stated that Maillard reaction took place upon incubation of the WPI-LMP mixture.

b. TNBS Analysis

According to Oliver *et al*. [24], Maillard reaction is a spontaneous and naturally occurring reaction between available amino groups from a protein with reducing sugar entities from polysaccharides, which involves the Amadori rearrangement [11, 40]. Thus, during the Maillard reaction amino groups of the protein will be consumed and as a consequence the availability of the amino groups decreases. This phenomenon can be used as an indicator to observe the Maillard reaction taking place during incubation. By knowing the amount of the free amino groups in the conjugates at different incubation times, the degree of glycosylation or graft reaction during incubation can be estimated.

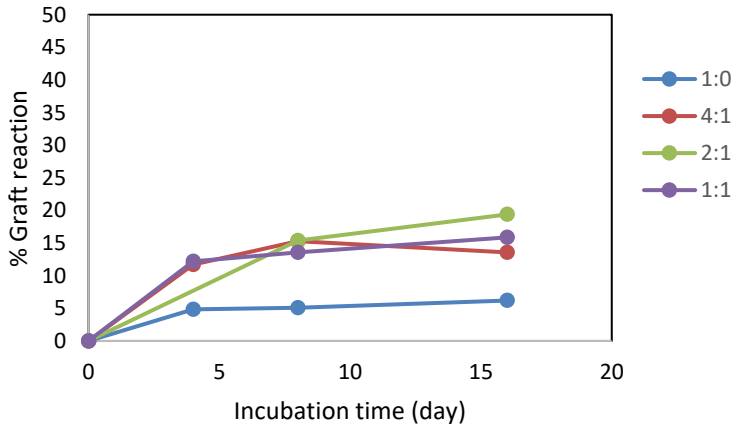


Figure 3.3 Degree of graft reaction of the dry heated WPI (ratio 1:0) and conjugates of WPI-LMP (ratio 4:1, 2:1, and 1:1) during incubation at 60°C and RH of±74%

Figure 3.3 shows that the free amino group content of dry heated WPI was reduced which could be due to protein polymerization upon dry heat treatment. For WPI-pectin conjugates, the free amino group content decreased by about 15 % on the 8th day of the incubation.

From Figure 3.3, it can be depicted that the rate of the graft reaction was most pronounced during the initial period of the incubation, and gradually decreased later on. The latter could be due to the steric hindrance of the LMP which was already attached to WPI. Upon dry heating of BSA with dextran, it was found that a steady state reaction was obtained due to the hindrance from the dextran attached in the BSA [19].

It should be kept in mind that a reduction of the free amino group content in the samples does not necessarily mean an enhancement of the graft reaction. It was reported before that polymerization of protein occurred during incubation of β-Lactoglobulin and dextran at a temperature of 55°C, $a_w$of0.65 and 6:1 weight ratio (dextran to β-Lactoglobulin) [11]. This polymerization reaction may reduce the ability of the free amino groups to react with the TNBS leading to lower absorbance readings at 420 nm. Hence, the reduction of free amino group content observed was probably not solely due to glycosylation of whey protein, but could be the result of protein polymerization as well.

c. SDS-Page analysis

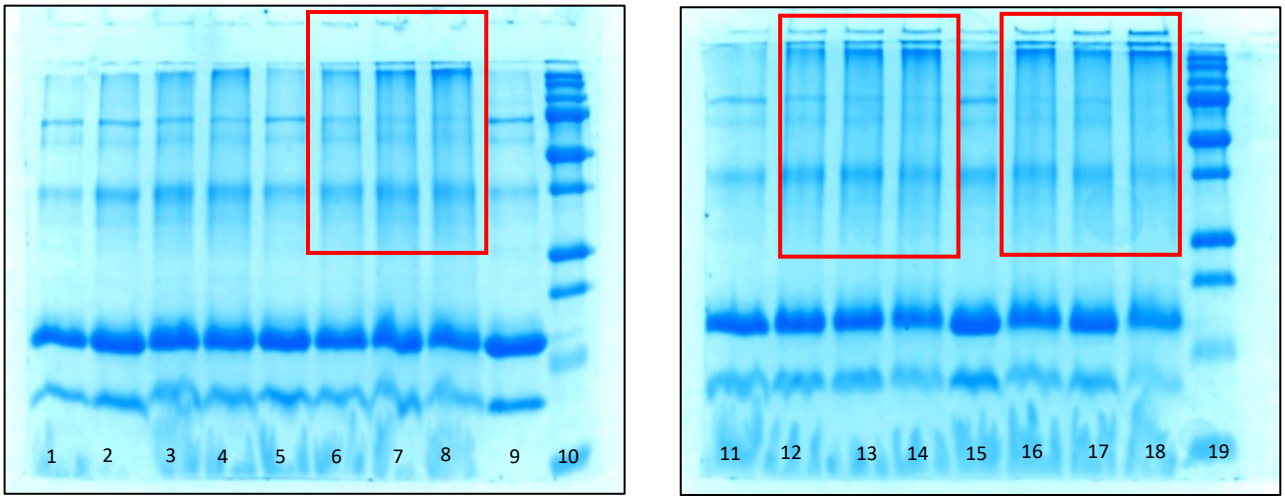


Figure 3.4 SDS PAGE results of WPI and WPI-LM Pectin Conjugates incubated at 60°C in an environment containing saturated NaCl solution, with different ratios of WPI to LM Pectin (Lanes: 1, 2,3,4, Conjugate ratio 1:0; 5,6,7,8,conjugate ratio 4:1; 9, WPI powder; 10, Molecular weight marker; 11, 12, 13, 14, conjugate ratio 2:1; 15,16,17,18, conjugate ratio 1:1) incubated for different periods (Lanes: 1,5,11,15, 0 day; 2,6,12,16, 4 days; 3,7,13,17, 8 days; 4,8,14,18, 16 days)

Figure 3.4 shows the scanned gel from the SDS-Page analysis. For comparison, WPI without any treatment was included as well (Lane 9). The gel profile shows that WPI had two distinct bands, which could be correlated to β-lactoglobulin and α-lactalbumin. In the upper part or higher molecular weight part of the gel it could be seen that there were bands which represented the dimer of β-lactoglobulin and BSA. The molecular weight of β-lactoglobulin, α-lactalbumin, dimer of β-lactoglobulin and BSA is approximately 18.4kDa, 14 kDa, 36.8kDa, and 66.5 kDa, respectively.

In fig 3.4, it can be seen that the mixtures which were only lyophilized but not heat treated (indicated as 0 days) of WPI-LMP for all ratios had the same band pattern as the WPI powder. As the mixtures were incubated, the band for α-lactalbumin seemed to be fading as the incubation period was extended. Furthermore, there were broad bands of high molecular weight compounds in the lanes of the conjugates incubated for 4, 8, and 16 days. The intensity of the band of these high molecular weight compounds increased with the duration of the incubation time. Furthermore, in the injection point of these samples it could also be observed that there was a band representing compounds which were trapped due to their big molecular weight (Lane 12, 13, 14, 15, 16, and 17). In the same lanes there were also compounds trapped in between the stacking and running gel due to their big molecular weight. This leads to the conclusion that the conjugation of WPI and LM Pectin clearly produced compounds with high molecular weight.

Nevertheless, in the dry heated WPI (ratio 1:0) incubated for 4, 8, and 16 days there was also an intensification of the bands of high molecular weight molecules which showed that there was polymerization of the WPI during incubation. It was reported before that dry heating of β-lactoglobulin resulted in compounds with MW higher than the MW of its dimer [11]. This polymerization can be caused by covalent bond formation by either disulfide bonds or other types of covalent bonds [18, 41-43]. Heating will unfold the protein, whereby sulfhydryl containing amino acid groups will be exposed. Particularly, in β-lactoglobulin, sulfhydril-disulfide interchange is initiated when the protein is heated at temperatures between 60-65^{o}C [44]. Since the WPI used is mainly composed of β-lactoglobulin, it is reasonable to compare the behavior of WPI during heating to that of β-lactoglobulin.

Whereas disulfide bonds in the protein aggregates should have been eliminated due to the presence of mercaptoethanol, still protein aggregates with high molecular weight were observed in the lane of the dry heated WPI. This suggests that besides disulfide bonds, other covalent bonds were responsible for the polymerization in the dry heated WPI. Upon dry heating of WPI at 100 ^{o}C and different pH conditions, Gulzar [41] found that at low pH protein aggregation was mainly due to intermolecular disulfide bonds,

while as the pH increased to 6.5, protein aggregation was due to disulfide bonds and covalent bonds other than disulfide bonds. The latter observation is in line with our experiment as the pH of the dry heated WPI was approximately 6.5 (Fig. 3.1).

Due to this phenomenon, it can be questioned whether the high molecular weight compounds observed in the WPI-LMP conjugates were partly because of protein aggregation. In the case of WPI-LMP conjugates (ratio 4:1, 2:1, and 1:1), however, this phenomenon could be minimized or prevented due the presence of LMP. Several authors observed that conjugation of proteins and polysaccharides through Maillard reaction can minimize the structural loss of the protein by protein aggregation during dry heat treatment [19, 45, 46]. In fact, aggregation and Maillard reaction can happen simultaneously and it was reported previously that BSA aggregation upon dry heating can be reduced in the presence of Dextran since it can prevent extra aggregation of BSA [19]. Furthermore, the author suggested that in the presence of Dextran, the amino acids containing the sulfhydryl group responsible for protein aggregation via disulfide bond formation are involved in the Maillard reaction, thus preventing aggregation between proteins. Hence, it is expected that protein aggregation was probably very limited in the presence of LMP.

Whereas it is difficult to use SDS-PAGE results for quantification of the degree of conjugation, still the results can be used as a confirmation of the formation of high molecular weight conjugates during dry heat incubation of WPI and LMP. This result is in agreement with the results of TNBS analysis, the results of pH change and color development analysis which indicated that Maillard reaction already occurred during the first 4 days of the incubation. In addition, SDS-PAGE analysis showed that there was protein aggregation during dry heat treatment of WPI alone which is thought to involve the consumption of free amino groups. This finding was supported by the fact that TNBS analysis showed a reduction of free amino group content of the WPI upon dry heat treatment of WPI.

d. Diffusion analysis via NMR analysis

Further analysis to confirm the formation of conjugates was performed using high resolution pfg-NMR diffusometry. This measurement is based on the diffusivity measurement of the targeted compound. The analysis was performed on WPI-LMP conjugates with a ratio of 1:1 which were incubated for 16 days. In order to know the diffusion behavior of WPI and LMP alone, diffusion NMR was performed on solutions of LMP separately as well as lyophilized WPI which was incubated for 16 days.

Figure 3.5 shows the 1D ^{1}H spectra of the WPI-LMP conjugates of ratio 1:1 which was incubated for 16 days. By comparing this spectrum to the spectra of WPI and of LMP, it became obvious that the

signals observed at 0.3-1.0 ppm and 2.5-3.3 ppm belong to the WPI, while the spectrum at 3.2-4.2 ppm and 5.2-5.5 ppm belonged to the LMP. The other sharp resonances observed represented the H_2O and the Sodium Acetate added upon sample preparation. By fitting equation 1a, the arithmetic mean diffusion coefficient and the arithmetic standard deviation of each signal were obtained (Table 3.1). There was one major signal (0.3-1.0 ppm) and one minor peak (2.5-3.3 ppm) detected for WPI and both had approximately the same diffusion coefficient ($6.9\cdot 10^{-11} m^2/s$) and a narrow distribution ($\pm 1\cdot 10^{-11} m^2/s$) (Table 3.1). LMP has a high molecular weight of approximately several hundred kDa [47] and is characterized by a broad range of molecular weights. This explains the broad diffusion coefficient distribution exhibited by LMP. On the other hand, WPI has a narrow molecular weight distribution, thus possessing a narrow diffusion coefficient distribution.

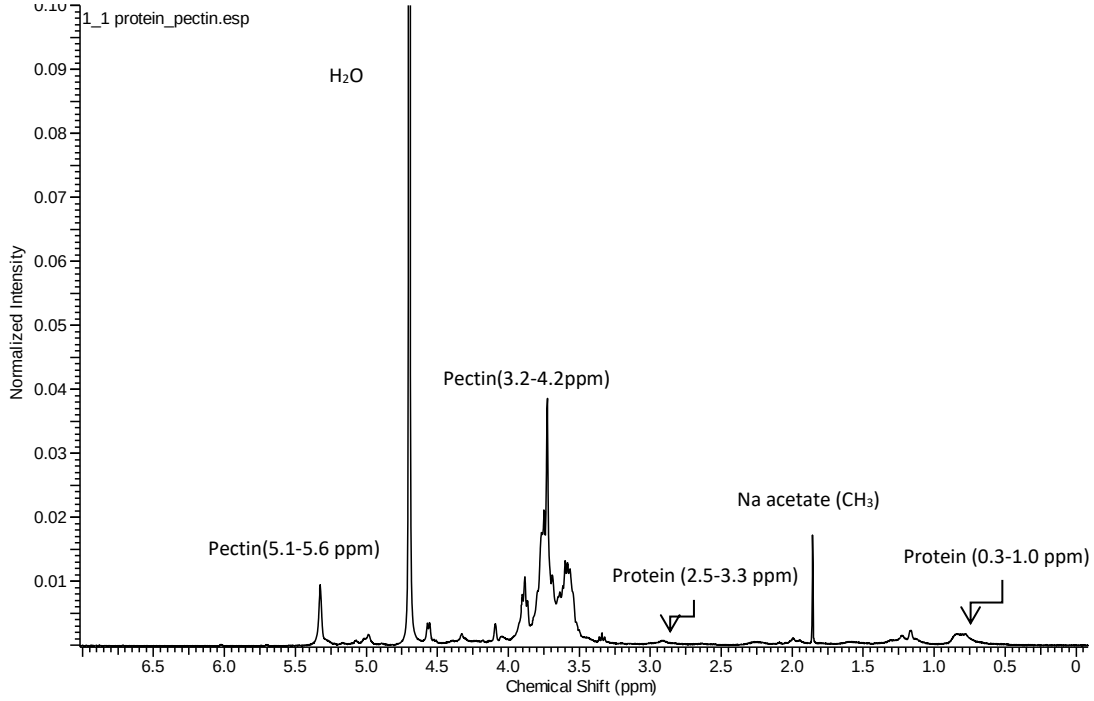


Figure 3.5 1D ^1H spectrum of WPI-LMP conjugates (ratio 1:1) upon dry heat incubation for 16 days

Upon incubation of WPI and LMP, the interaction of LMP and WPI lead to an increase in molecular weight. Thus a change in the average diffusion coefficient as well as in the distribution width will be observed (Table 3.1). It was noticed that conjugated WPI acquired a similar diffusion behavior as LMP molecules. On the other hand, the diffusion of conjugated LMP was not significantly affected by the molecular interaction.

Whereas it was expected that interaction between two compounds would result in a lower diffusion coefficient, Table 3.1 showed that the arithmetic mean diffusion coefficient of WPI increased slightly to 8.8 x 10^{-11} m^2/s upon incubation of WPI and LMP, accompanied by a broader distribution width whose value increased substantially from 1 x $10^{-11}m^2/s$ (narrow) to 14.1 x 10^{-11} m^2/s (broad). This phenomenon is due to the fact that the distribution width of WPI in the presence of pectin increased significantly (Fig 3.7). In fact, for a constant geometric mean, the arithmetic mean increases with increasing distribution width.

Table 3.1 Arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ) value of WPI, LMP, and WPI-LMP conjugates (Day 16) obtained upon fitting Eq. 1 to the diffusion signal of the WPI and LMP contributions in the non-conjugated and conjugate samples.

NMR signal (ppm)	WPI		LMP		WPI-LMP	
	D_a (m^2/s)	σ (m^2/s)	D_a (m^2/s)	σ (m^2/s)	D_a (m^2/s)	σ (m^2/s)
0.3-1	$6.9\cdot10^{-11}$	$1.0\cdot10^{-11}$	-	-	$8.8\cdot10^{-11}$	$14.1\cdot10^{-11}$
2.5-3.3	$6.8\cdot10^{-11}$	$0.9\cdot10^{-11}$	-	-	-	-
3.2-4.2	-	-	$6.7\cdot10^{-11}$	$28.2\cdot10^{-11}$	$5.4\cdot10^{-11}$	$15.0\cdot10^{-11}$
5.1-5.6	-	-	$3.9\cdot10^{-11}$	$9.5\cdot10^{-11}$	$3.3\cdot10^{-11}$	$8.8\cdot10^{-11}$

From the calculation of the decomposition of the protein signal of the mixture into the WPI signal without pectin and the pectin signal of the mixture according to equation 3 (Figure 3.6), it was found that approximately 52 to 59 % of the WPI did not react with the LMP. Whereas the former value was obtained from a least squares approach on the measured I/I_o values, the latter was obtained when minimizing the sum of squared differences based on $\ln (I/I_o)$. From the TNBS results, it was found that approximately 15% of the amino groups were lost upon incubation for 16 days. It means that 15% of the primary amino groups in the WPI were no longer free which could be due to the complex formation through Maillard reaction. On the other hand, the NMR diffusion results indicated that 41 to 48 % of the WPI interacted with LMP. The pronounced difference between the TNBS and NMR results follows logically from the fact that whey proteins contain several amino groups per molecule: as an example, β-lactoglobulin, the most abundant whey protein, contains 15 lysine residues. Hence, a major part of the whey proteins can become conjugated to polysaccharides, despite of only a small reduction in free amino group content.

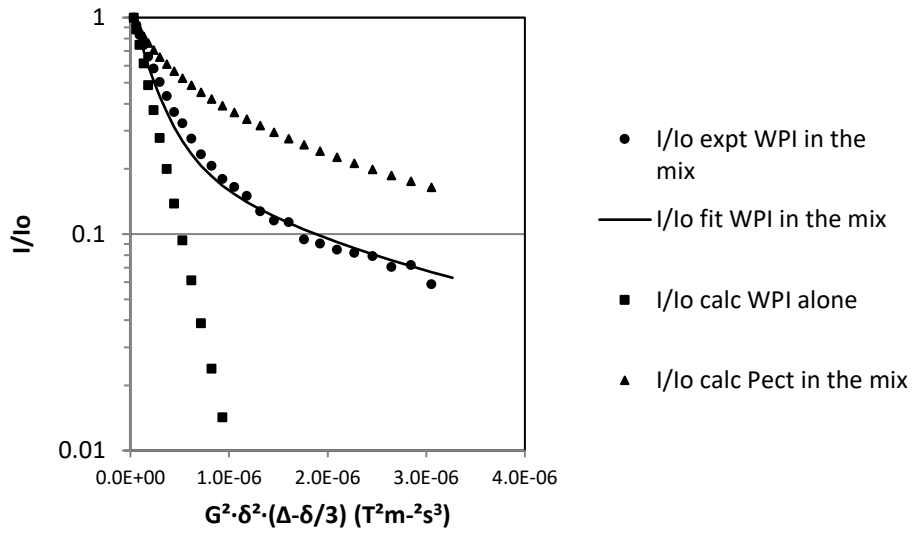


Figure 3.6 Decomposition of protein diffusion signal with pectin (WPI-LMP conjugates ratio 1:1, incubated for 16 days) into the calculated protein diffusion signal without pectin (Lyophilized WPI incubated for 16 days) and the calculated pectin signal with protein pectin (WPI-LMP conjugates ratio 1:1, incubated for 16 days)

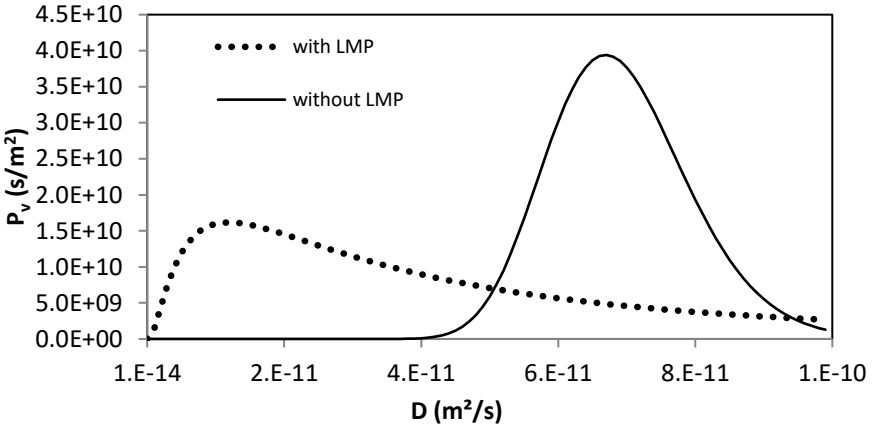


Figure 3.7 Lognormal mass-weighted diffusion coefficient distribution of the protein signals upon dry heat treatment in the absence and presence of LMP

2. Heat stability of WPI-LMP Conjugates

The functional properties and structure of proteins can change due to heat treatment. Loss of solubility, structural unfolding, and heat induced aggregation, are some of the consequences from the

changed structure of proteins due to heat [48]. Thus, the loss of solubility can be used as an indicator of protein stability against heat. In particular, the loss of solubility of proteins leads to a subsequent loss of their functionality [28].

In this research, the heat stability of protein was evaluated based on the solubility of protein before and after heating. Hereby, a high protein solubility after heat treatment was desired as it can broaden the applications of WPI in food applications. The heat stability experiment was conducted at 80°C since at this temperature WPI undergoes irreversible denaturation.

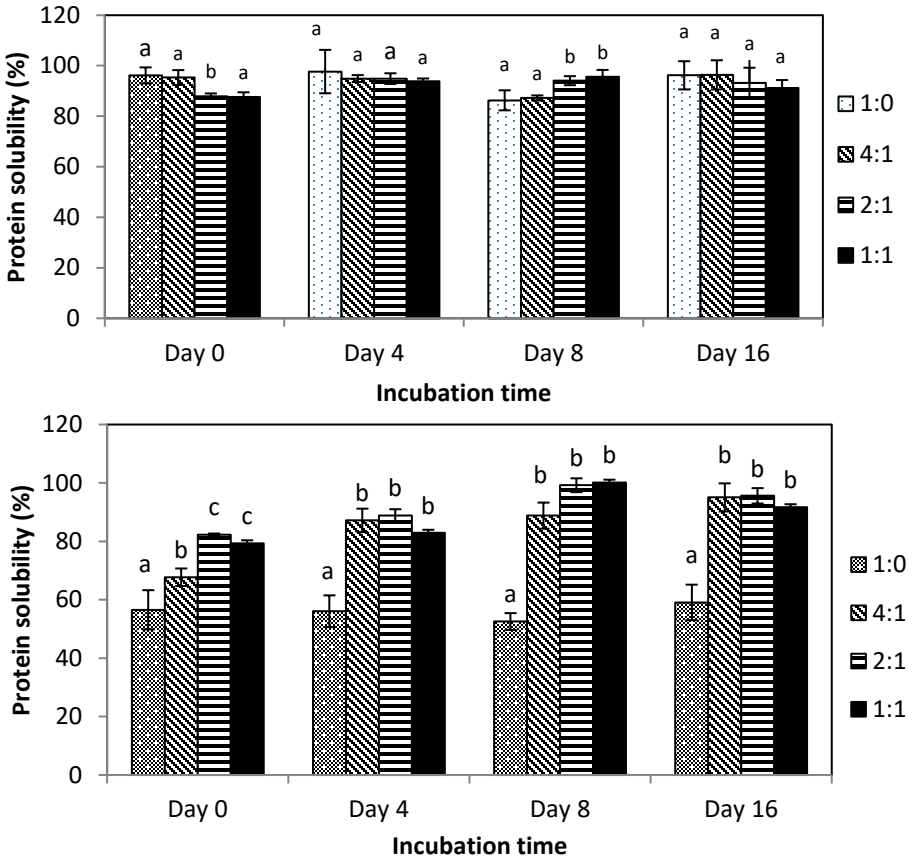


Figure 3.8 Protein solubility (%) and standard deviation bar of WPI alone (Ratio 1:0 Day 0), mixture of WPI-LMP (Day 0), and conjugate of WPI-LMP (Day 4, 8, 16) before (top) and after heat treatment (bottom) at 80°C and pH 6.5 for 2 minutes. Sample with the same alphabet within the same group (Days) indicates no statistical different between them.

In Figure 3.8, it can be seen that mixtures of protein and polysaccharides (WPI-LMP conjugate day 0) at all ratios had a protein solubility of about 90%. Statistically, there was no significant effect of WPI-LMP

ratio on the solubility of the protein, while the duration of the incubation period had a significant effect. Furthermore, two-way ANOVA revealed that there was a significant interaction between ratio and incubation day on the solubility of unheated protein. This means that difference between means of the protein solubility depends on the combination of WPI-LMP ratio and incubation time (Figure 3.8).

Both pH and temperature are among the factors that have an impact on the solubility of proteins. The solubility of proteins is generally reported to be minimum at its IEP and higher both above and below the IEP because of mutual charge repulsion [49, 50]. In this experiment, the conjugates were diluted at pH 6.5 in the presence of 30 mM of salt. It was reported before that whey protein also had low heat stability at pH values around 6.8 to 7, especially in the presence of salt [49, 51, 52]. Thus, it was expected that at this pH the effect of conjugation between WPI and LMP in improving the heat stability of WPI could be observed.

In Figure 3.8, it can be observed that by applying heat, the solubility of the protein was generally reduced. Without addition of pectin, the protein solubility was significantly lower. Statistical analysis showed that both WPI-LMP ratio and incubation time had a significant influence on the residual solubility of the protein upon heating. There was also significant interaction between the effect of WPI-LMP ratio and incubation time on the protein solubility after heat treatment (Figure 3.8).

After heat treatment, the protein solubility of WPI-LMP ratio 1:0 at all incubation time points (WPI only) was significantly lower than that of WPI-LMP mixtures and WPI-LMP conjugates. Heating for 2 minutes at 80^{o}C reduced the protein solubility of WPI by almost half. The heat stability of the dry heated WPI did not improve as the incubation time was prolonged. Without incubation, it was obtained that WPI-LMP ratio 2:1 and 1:1 had comparable protein solubility and both had significantly higher protein solubility than that of WPI-LMP ratio 4:1. Whereas the presence of free or weakly complexed polysaccharides (unincubated) was reported to adversely affect the functionality of the protein [18], a clear beneficial effect of the electrostatic interaction at pH 6.5 is observed in our experiments, which is proportional to the pectin content of the mixtures. The mixture showed to have better heat stability than the native WPI. This could be due to the protective effect that came from the presence of LMP. Additionally, electrostatic interaction between WPI and LMP might be present in the mixture upon sample preparation which also contributed to the protection of WPI against heat induced aggregation.

When the mixtures were incubated for 4 days, it was found that the residual protein solubility after heating of WPI-LMP conjugates of ratio 4:1, 2:1, and 1:1 was not significantly different. The same trend

was obtained for WPI-LMP conjugates incubated for 8 and 16 days. Regarding the incubation time, these results can be linked to the degree of graft reaction of the conjugates. In general, the results showed that conjugates with a higher degree of graft reaction possessed a better heat stability. Therefore, it can be stated that conjugation plays an important role in the improvement of the heat stability of whey proteins. The heat stability analysis results showed that the solubility of the protein could be improved by adding LMP and was further improved upon incubating the mixture of WPI-LMP.

During heat treatment, a change in hydrophobic, electric, and structural properties can generate changes in solubility and functionality of proteins. The mechanism of how conjugates can exhibit heat stability is still being an interesting research topic. The stabilizing effect of LMP towards heat induced WPI aggregation is suggested to be due to the steric repulsive forces provided by LMP. Conjugation of protein and polysaccharides will combine the surface-active properties coming from the hydrophobic parts of proteins and the steric stabilization properties of the hydrophilic groups coming from the polysaccharides [16]. These hydrophilic groups help improving the solubility of WPI. Hereby, conjugates can minimize the exposure of reactive sites of the protein during heat treatment inhibiting interaction between unfolded proteins which can lead to aggregation [53, 54]. Our results are in agreement with the findings of Jimenez-Castano *et al* [43] in which β-lactogblobulin which was incubated with Dextran at a temperature of 60^{o}C and an Aw 0.44 for 4 days obtained better a thermal stability, even at its IEP.

As mentioned before, it is possible for polymerization to occur during dry heat incubation of WPI. However, the results of the experiments showed that dry heated WPI did not improve the heat stability of WPI. This means that, even if polymerization of protein occurred during incubation of WPI-LMP, the high stability of WPI against heat observed was certainly due to the formation of WPI-LMP conjugates instead of protein polymers/aggregates.

3. Emulsifying activity and heat stability.

The emulsifying activity of the conjugates (ratio 2:1 Day 8) was compared to that of a mixture of WPI-LMP (Ratio 2:1), Native WPI, and dry heated WPI (ratio 1:0 Day 8). The results can be observed in Figure 3.9.

WPI and dry heated WPI stabilized emulsions had a comparable droplet size distribution, characterized by a volume-weighted average diameter of 0.90 and 0.89 µm, respectively. Considering the mixture and the conjugates of WPI-LMP, it was found that combination of LMP with WPI was able to produce smaller particle sizes. Despite of the lower protein content (i.e. 0.50% of WPI in WPI stabilized emulsions versus 0.33% of WPI in WPI-LMP conjugate stabilized emulsions), the smallest droplet size (0.61 µm) was

obtained from emulsions stabilized with the conjugates. Therefore, it was confirmed that besides improving the heat stability of the WPI, the conjugation of WPI and LMP also improved the emulsifying activity of the WPI. During the production of the emulsions, it is possible that the WPI-LMP conjugates could rapidly rearrange on the surface of the oil droplet and cover the surface. Hereby, the hydrophobic groups of the protein are anchored in the oil phase, while the LMP conjugated to the protein provides electrosteric stabilization of the oil droplets. Covalently bound LMP has a better effect on the emulsifying properties of WPI than free LMP [18]. Whereas incubation of WPI in the presence of LMP was seen to improve the emulsifying activity of WPI, incubation in the absence of LMP did not have any impact on the emulsifying activity of WPI.

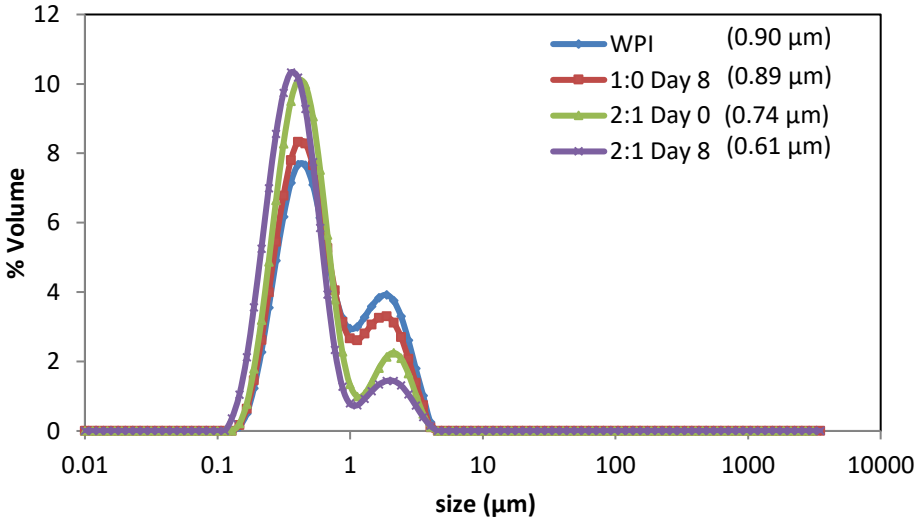


Figure 3.9 Particle size distribution and their respective $d_{4,3}$ (µm) of 10% (o/w) emulsions stabilized by 0.5% WPI, dry heated WPI (ratio 1:0 Day 8), mixture of WPI-LMP (ratio 2:1), and WPI-LMP conjugates (ratio 2:1 Day 8) prepared at pH 6.5

The effectiveness of pectin with a low degree of methyl esterification to improve the emulsifying activity of WPI was recently also reported by Schmidt *et al.* [55]. The authors compared the emulsifying properties of WPI and citrus pectin conjugates as affected by the degree of esterification of the citrus pectin and revealed that citrus pectin with low degree of methyl esterification gave the highest conjugation yield and smallest droplet size. The finding is in agreement with our result in which the presence of conjugated LMP to WPI improved the emulsifying activity of WPI resulting in a smaller droplet size.

In a last part of the research, the heat stability of the emulsions stabilized with the conjugates was investigated in order to check if the heat stabilizing properties of LMP remain preserved in an o/w emulsion.

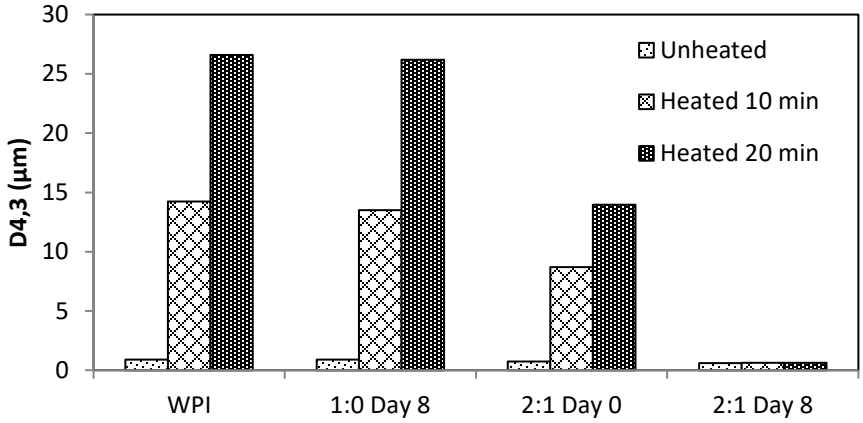


Figure 3.10 Volume-weighted mean diameter ($D_{4.3}$)(µm) of emulsions stabilized by WPI, dry heated WPI (8 days), WPI-LMP mixture, and WPI-LMP conjugates of ratio 2:1 (8 days) before and after heating at 80°C for 10 and 20 minutes at pH 6.5

Upon heating at 80°C for 10 and 20 minutes, emulsions stabilized by WPI and dry heated WPI underwent severe flocculation due to the denaturation and subsequent aggregation of the WPI (Fig 3.10). In this phenomenon, WPI acts as a glue in between the aggregated droplets [57]. Flocculation of the oil droplets was confirmed by measuring the particle size of the heated emulsions with predilution in SDS solution prior to the particle size measurement (data not shown). By using this method, the oil droplet size obtained after 10 minutes of heating became comparable to that of the emulsions before heating. Upon longer heat treatment (20 min) the droplet size obtained using the pre-dilution in SDS solution was still higher than that before heating. This showed that the aggregates could not be completely broken down by dilution in SDS solution or it could be a sign that coalescence occurred in the heated emulsions.

The results included in Fig.3.10 imply that dry heat treatment of WPI did not improve the heat stability of the WPI stabilized emulsions. Moreover, Fig. 3.10 clearly shows that LMP addition as such was not sufficient to obtain heat stable WPI stabilized o/w emulsion. Only upon dry heat incubation, an effective heat stabilization was observed: there was almost no change in the droplet size distribution of WPI-LMP conjugate stabilized emulsions after heating at 80°C. Due to the fact that WPI, dry heated WPI and WPI-LMP mixtures showed a poor stability against heat, both in solutions and emulsions, it can be concluded

that either mixing with LMP (without dry heating) or dry heat treatment (in the absence of LMP) was not sufficient to improve the heat stability of WPI-stabilized emulsions. Hence, these results clearly indicate that the high heat stability exhibited by the emulsions was due to the presence of WPI-LMP conjugates and was not due to the presence of free WPI, polymerized WPI (dry heated WPI) or free pectin. The heat stabilizing activity of the WPI-LMP conjugates was thought to be due to the steric repulsion provided by the LMP attached to the WPI. Upon heating, this steric repulsion is expected to effectively prevent aggregation of thermally unfolded whey proteins, and hence prevent the aggregation of protein-coated emulsion droplets.

Within the studied time frame (i.e. 4, 8, 16 days) no significant effect of incubation time could be observed. The effect of incubation time (degree of conjugation) on the heat stability of emulsions will be further explored in future research. Anyway, the current results indicate that a shorter dry heat treatment time may be sufficient, which is clearly an important aspect for the possible valorization of this technology to prepare more heat-stable emulsifiers.

Conclusion

Upon incubation of WPI and LMP, the Maillard reaction took place resulting in compounds with a higher molecular weight (WPI-LMP conjugates). The conjugates were characterized a better heat stability compared to the native WPI and to mixtures of WPI-LMP. The longer was the incubation time, the higher was the degree of the graft reaction obtained in the conjugates which resulted in a higher heat stability of the WPI. Protein polymerization was observed in the dry heated WPI. Even though it is presumed that polymerization could also take place during incubation of WPI and LMP, the heat stabilizing effect of the dry heat treated mixtures was clearly shown to be due to the presence of conjugates and not to protein polymers. Besides improving the heat stability of WPI, conjugation of WPI and LMP also improved the emulsifying activity of WPI: WPI-LMP conjugates produced smaller oil droplets than native WPI, dry heated WPI, and mixture of WPI-LMP. Moreover, the conjugate strongly increased the heat stability of WPI complexes. Overall, our results indicate that dry heat treatment of protein-pectin mixtures is a promising procedure to improve the protein's functional properties.

4. Acknowledgement

This research is supported by the fund from Indonesian Endowment Fund for Education (LPDP Indonesia).

References

[1] S. Jovanović, M. Barać, O. Maćej, Whey proteins-properties and possibility of application, Mljekarstvo, 55 (2005) 215-233.
[2] V. Raikos, Effect of heat treatment on milk protein functionality at emulsion interfaces. A review, Food Hydrocolloids, 24 (2010) 259-265.
[3] H.B. Wijayanti, N. Bansal, H.C. Deeth, Stability of Whey Proteins during Thermal Processing: A Review, Comprehensive Reviews in Food Science and Food Safety, 13 (2014) 1235-1251.
[4] M. Dissanayake, L. Ramchandran, O.N. Donkor, T. Vasiljevic, Denaturation of whey proteins as a function of heat, pH and protein concentration, International Dairy Journal, 31 (2013) 93-99.
[5] K. Demetriades, D. Julian McClements, Influence of sodium dodecyl sulfate on the physicochemical properties of whey protein-stabilized emulsions, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 161 (2000) 391-400.
[6] M.A. Hoffmann, P.J. van Mil, Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds, Journal of Agricultural and Food Chemistry, 45 (1997) 2942-2948.
[7] J.M. Chobert, C. Bertrand-Harb, M.G. Nicolas, Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin, Journal of Agricultural and Food Chemistry, 36 (1988) 883-892.
[8] C. Gauche, J.T.C. Vieira, P.J. Ogliari, M.T. Bordignon-Luiz, Crosslinking of milk whey proteins by transglutaminase, Process Biochemistry, 43 (2008) 788-794.
[9] O.G. Jones, E.A. Decker, D.J. McClements, Formation of biopolymer particles by thermal treatment of β-lactoglobulin–pectin complexes, Food Hydrocolloids, 23 (2009) 1312-1321.
[10] T. Harnsilawat, R. Pongsawatmanit, D. McClements, Characterization of β-lactoglobulin–sodium alginate interactions in aqueous solutions: a calorimetry, light scattering, electrophoretic mobility and solubility study, Food Hydrocolloids, 20 (2006) 577-585.
[11] L. Jiménez-Castaño, M. Villamiel, P.J. Martín-Álvarez, A. Olano, R. López-Fandiño, Effect of the dry-heating conditions on the glycosylation of β-lactoglobulin with dextran through the Maillard reaction, Food Hydrocolloids, 19 (2005) 831-837.
[12] N.G. Diftis, T.A. Pirzas, V.D. Kiosseoglou, Emulsifying properties of gelatin conjugated to pectin under alkaline conditions, Journal of the Science of Food and Agriculture, 85 (2005) 804-808.
[13] E. Dickinson, M.G. Semenova, Emulsifying properties of covalent protein—dextran hybrids, Colloids and Surfaces, 64 (1992) 299-310.
[14] B. Vardhanabhuti, E. Allen Foegeding, Effects of dextran sulfate, NaCl, and initial protein concentration on thermal stability of β-lactoglobulin and α-lactalbumin at neutral pH, Food Hydrocolloids, 22 (2008) 752-762.
[15] A. Kato, Y. Sasaki, R. Furuta, K. Kobayashi, Functional protein–polysaccharide conjugate prepared by controlled dry-heating of ovalbumin–dextran mixtures, Agricultural and Biological Chemistry, 54 (1990) 107-112.
[16] E. Dickinson, Protein-polysaccharide interactions in food colloids, SPECIAL PUBLICATION-ROYAL SOCIETY OF CHEMISTRY, 113 (1993) 77-77.
[17] E. Dickinson, Hydrocolloids as emulsifiers and emulsion stabilizers, Food Hydrocolloids, 23 (2009) 1473-1482.
[18] E. Dickinson, V.B. Galazka, Emulsion stabilization by ionic and covalent complexes of β-lactoglobulin with polysaccharides, Food Hydrocolloids, 5 (1991) 281-296.
[19] S. Xia, Y. Li, Q. Zhao, J. Li, Q. Xia, X. Zhang, Q. Huang, Probing Conformational Change of Bovine Serum Albumin–Dextran Conjugates under Controlled Dry Heating, Journal of Agricultural and Food Chemistry, 63 (2015) 4080-4086.
[20] G. Stainsby, Proteinaceous gelling systems and their complexes with polysaccharides, Food Chemistry, 6 (1980) 3-14.

[21] D. Zhu, S. Damodaran, J.A. Lucey, Physicochemical and Emulsifying Properties of Whey Protein Isolate (WPI)−Dextran Conjugates Produced in Aqueous Solution, Journal of Agricultural and Food Chemistry, 58 (2010) 2988-2994.
[22] N. Diftis, V. Kiosseoglou, Physicochemical properties of dry-heated soy protein isolate–dextran mixtures, Food Chemistry, 96 (2006) 228-233.
[23] M.S. Martinez-Alvarenga, E.Y. Martinez-Rodriguez, L.E. Garcia-Amezquita, G.I. Olivas, P.B. Zamudio-Flores, C.H. Acosta-Muniz, D.R. Sepulveda, Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate – Maltodextrin conjugates, Food Hydrocolloids, 38 (2014) 110-118.
[24] C.M. Oliver, L.D. Melton, R.A. Stanley, Creating proteins with novel functionality via the Maillard reaction: a review, Critical Reviews in Food Science and Nutrition, 46 (2006) 337-350.
[25] N. Garti, What can nature offer from an emulsifier point of view: trends and progress?, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 152 (1999) 125-146.
[26] F. Morgan, J. Léonil, D. Mollé, S. Bouhallab, Modification of Bovine β-Lactoglobulin by Glycation in a Powdered State or in an Aqueous Solution: Effect on Association Behavior and Protein Conformation, Journal of Agricultural and Food Chemistry, 47 (1999) 83-91.
[27] E. Dickinson, Interfacial structure and stability of food emulsions as affected by protein–polysaccharide interactions, Soft Matter, 4 (2008) 932-942.
[28] A. Kato, Industrial Applications of Maillard-Type Protein-Polysaccharide Conjugates, Food Science and Technology Research, 8 (2002) 193-199.
[29] E. Dickinson, Emulsion Stabilization by Polysaccharides and Protein-Polysaccharides Complexes, in: A.M. Stephen (Ed.) Food Polysaccharides and Their Application, Marcel Dekker, New York, 1995.
[30] P. Van der Meeren, M. El-Bakry, N. Neirynck, P. Noppe, Influence of hydrolysed lecithin addition on protein adsorption and heat stability of a sterilised coffee cream simulant, International Dairy Journal, 15 (2005) 1235-1243.
[31] L. Greenspan, Humidity fixed points of binary saturated aqueous solutions, Journal of Research of the National Bureau of Standards, 81 (1977) 89-96.
[32] W.-W. Sun, S.-J. Yu, X.-A. Zeng, X.-Q. Yang, X. Jia, Properties of whey protein isolate–dextran conjugate prepared using pulsed electric field, Food Research International, 44 (2011) 1052-1058.
[33] J. Adler-Nissen, Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid, Journal of Agricultural and Food Chemistry, 27 (1979) 1256-1262.
[34] S.G. Anema, The effect of chymosin on κ-casein-coated polystyrene latex particles and bovine casein micelles, International Dairy Journal, 7 (1997) 553-558.
[35] G.R. Schacterle, R.L. Pollack, A simplified method for the quantitative assay of small amounts of protein in biologic material, Analytical biochemistry, 51 (1973) 654-655.
[36] S.-C. Liu, D.-J. Yang, S.-Y. Jin, C.-H. Hsu, S.-L. Chen, Kinetics of color development, pH decreasing, and anti-oxidative activity reduction of Maillard reaction in galactose/glycine model systems, Food Chemistry, 108 (2008) 533-541.
[37] S.-L. Chen, S.-Y. Jin, C.-S. Chen, Relative reactivities of glucose and galactose in browning and pyruvaldehyde formation in sugar/glycine model systems, Food Chemistry, 92 (2005) 597-605.
[38] J.M. DeMan, Principles of food chemistry, AVI Publishing Co., Inc.1976.
[39] W. Lertittikul, S. Benjakul, M. Tanaka, Characteristics and antioxidative activity of Maillard reaction products from a porcine plasma protein–glucose model system as influenced by pH, Food Chemistry, 100 (2007) 669-677.
[40] C.A. Dunlap, G.L. Côté, β-Lactoglobulin−Dextran Conjugates: Effect of Polysaccharide Size on Emulsion Stability, Journal of Agricultural and Food Chemistry, 53 (2005) 419-423.
[41] M. Gulzar, S. Bouhallab, R. Jeantet, P. Schuck, T. Croguennec, Influence of pH on the dry heat-induced denaturation/aggregation of whey proteins, Food Chemistry, 129 (2011) 110-116.

[42] H. Enomoto, Y. Hayashi, C. Li, S. Ohki, H. Ohtomo, M. Shiokawa, T. Aoki, Glycation and phosphorylation of α-lactalbumin by dry heating: Effect on protein structure and physiological functions, Journal of Dairy Science, 92 (2009) 3057-3068.
[43] L. Jiménez-Castaño, R. López-Fandiño, A. Olano, M. Villamiel, Study on β-lactoglobulin glycosylation with dextran: effect on solubility and heat stability, Food Chemistry, 93 (2005) 689-695.
[44] S. Prabakaran, S. Damodaran, Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced aggregation, Journal of Agricultural and Food Chemistry, 45 (1997) 4303-4308.
[45] M.J. Spotti, M.J. Martinez, A.M.R. Pilosof, M. Candioti, A.C. Rubiolo, C.R. Carrara, Influence of Maillard conjugation on structural characteristics and rheological properties of whey protein/dextran systems, Food Hydrocolloids, 39 (2014) 223-230.
[46] Y. Sun, S. Hayakawa, K. Izumori, Modification of Ovalbumin with a Rare Ketohexose through the Maillard Reaction: Effect on Protein Structure and Gel Properties, Journal of Agricultural and Food Chemistry, 52 (2004) 1293-1299.
[47] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, W. Pilnik, Enzymic degradation of apple pectins, Carbohydrate Polymers, 2 (1982) 25-33.
[48] U. Einhorn-Stoll, M. Ulbrich, S. Sever, H. Kunzek, Formation of milk protein–pectin conjugates with improved emulsifying properties by controlled dry heating, Food Hydrocolloids, 19 (2005) 329-340.
[49] D.H.G. Pelegrine, C.A. Gasparetto, Whey proteins solubility as function of temperature and pH, LWT - Food Science and Technology, 38 (2005) 77-80.
[50] K. Demetriades, J.N. Coupland, D.J. McClements, Physical Properties of Whey Protein Stabilized Emulsions as Related to pH and NaCl, Journal of Food Science, 62 (1997a) 342-347.
[51] K. Ryan, B. Vardhanabhuti, D. Jaramillo, J. van Zanten, J. Coupland, E. Foegeding, Stability and mechanism of whey protein soluble aggregates thermally treated with salts, Food Hydrocolloids, 27 (2012) 411-420.
[52] K. Demetriades, J.N. Coupland, D.J. McClements, Physicochemical Properties of Whey Protein-Stabilized Emulsions as affected by Heating and Ionic Strength, Journal of Food Science, 62 (1997b) 462-467.
[53] C. Schmitt, C. Sanchez, S. Desobry-Banon, J. Hardy, Structure and technofunctional properties of protein-polysaccharide complexes: a review, Critical Reviews in Food Science and Nutrition, 38 (1998) 689-753.
[54] Y.-W. Shu, S. Sahara, S. Nakamura, A. Kato, Effects of the length of polysaccharide chains on the functional properties of the Maillard-type lysozyme-polysaccharide conjugate, Journal of Agricultural and Food Chemistry, 44 (1996) 2544-2548.
[55] U. Schmidt, V. Pietsch, C. Rentschler, T. Kurz, H.-U. Endreß, H. Schuchmann, Influence of the degree of esterification on the emulsifying performance of conjugates formed between whey protein isolate and citrus pectin, Food Hydrocolloids, 56 (2016) 1-8.
[56] N. Diftis, V. Kiosseoglou, Stability against heat-induced aggregation of emulsions prepared with a dry-heated soy protein isolate–dextran mixture, Food Hydrocolloids, 20 (2006) 787-792.
[57] S.R. Euston, S.R. Finnigan, R.L. Hirst, Aggregation kinetics of heated whey protein-stabilized emulsions, Food Hydrocolloids, 14 (2000) 155-161.