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Amino acids, polyols and soluble fibres as sugar replacers in bakery applications: Egg white proteins denaturation controlled by hydrogen bond density of solutions

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ABSTRACT

In this paper we demonstrate that the denaturation behavior, i.e. T_{den}, of egg white proteins in sugar and sugar replacer solutions is explained by the volumetric density of hydrogen bonds in the solutions, i.e. $n_{OH,eff}$. The validity of the presented approach is demonstrated using 18 solutions comprising single compounds as well as 7 ternary/quaternary mixtures. Different classes of plasticizers are used at various concentrations and at various ratio with proteins. Sweet amino acids such as L-proline and glycine are included as novel alternatives to polyols. The experimental data are modelled with the Flory-Huggins (FH) theory for biopolymer melting. For such purpose, solutions are treated as a single solvent, which is described by the effective volume fraction of the solvent $\Phi_{w,eff}$ ($n_{OH,eff}$). Overall, the FH model can well describe the denaturation behavior of egg white proteins in sugar and sugar replacer solutions up to 30% concentration. Deviations from the model become particularly evident at high sugar concentrations (i.e. 50%), which relate to conditions of phase separation in a protein-rich and sugar-rich domain. In such conditions, $\Phi_{w,eff}$ does not reflect the composition of the solvent around the proteins. An elevation in T_{den} is observed due to a reduction in hydrogen bond density in the protein-rich domain. The results indicate that phase separation is driven by both the concentration and the molar volume density of effective hydroxyl groups $N_{OH,s}/v_s$ of the plasticizers or plasticizer mixtures. Finally, the proposed approach can predict key phase-transitions which result in protein network formation in pound cake baking.

1. Introduction

In many bakery products, sugar is a major ingredient with a contribution of up to 30–40% depending on product (van der Sman & Renzetti, 2019). The urgency to provide healthy diets to an increasing world population and the prevalence of pathologies associated to excessive caloric intake (Willett & Rockström, 2019), place the issue of sugar replacement among the most compelling topics in food research. However, the reformulation of sweet bakery products with a substantial reduction in sugars has proven to be difficult due to the multiple functionalities that sugars exert in bakery products, next to simply providing sweetness (Pareyt & Delcour, 2008); (Wilderjans, Luyts, Brijs, & Delcour, 2013), (Mariotti & Lucisano, 2014) (van der Sman & Renzetti, 2019).

In bakery products such as cakes, sugars play important functionalities throughout the different process stages, from mixing to baking and cooling of the final cake product (Wilderjans et al., 2013). Sugar functionality in cakes becomes particularly apparent during the baking process. In fact, biopolymer phase transitions such as starch gelatinization and protein denaturation are affected by sugar content and type, which will determine the resulting structure and texture of cake (Struck, Gundel, Zahn, & Rohm, 2016) (Renzetti and Jurgens, 2016). Therefore, understanding the functionality of sugars and sugar replacers with respect to biopolymers phase transitions is key for defining optimal reformulation strategies.

A protein network is lacking in a cake batter as the presence of fat and high sugar concentrations limits the formation of a gluten network during batter mixing (Hesso et al., 2015) (Poland Meiske, Frang Jones, & M. Jones, 1960). Only during the late stages of baking a protein network is formed, as a result of protein denaturation and subsequent polymerization via SS bonds and hydrophobic interactions (Lambrecht, Deleu, Rombouts, & Delcour, 2018). In particular ovalbumin, the most abundant protein in egg white, has a key role in the protein network formation. In the denaturing ovalbumin, the four free sulfhydryl groups rapidly initiate polymerization through SH-SS

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exchange reactions thereby interconnecting different egg white proteins. This process results in the formation of a continuous network (Wilderjans, Lagrain, Brijs, & Delcour, 2010) (Wilderjans et al., 2013) (Lambrecht, Rombouts, De Ketelaere, & Delcour, 2017), also via promoting the incorporation of egg yolk and wheat proteins (Deleu et al., 2015) (Deleu, Wilderjans, Van Haesendonck, Brijs, & Delcour, 2016). In a cake system such as a pound cake, the denaturation of egg white proteins occurs at higher temperatures than those of pure egg white as high sucrose concentrations can raise the denaturation temperature of egg white protein up to about 13 °C (in 54% sucrose w/w) (Donovan, 1977) and consequently delay their gelation. Changes in the denaturation temperature of egg white proteins affects baking quality, as their gelation contributes to the resistance to collapse and thus to the final cake volume (Wilderjans Kerckhofs, Lagrain, Brijs, Wevers & Delcour, 2010) (Wilderjans, Luyts, Goesaert, Brijs & Delcour, 2010), as well as to crumb texture properties such as springiness and cohesiveness (Deleu et al., 2015).

A scientific approach to product formulation requires that the influence of the ingredient composition on the main chemical-physical changes during processing, such as the denaturation temperature of proteins and the gelatinization of starch, can be quantitatively described on the basis of the product recipe (van der Sman & Renzetti, 2018) (van der Sman & Renzetti, 2019). Recently, it has been shown that mixtures of sugars, polyols and water can be treated as a single effective solvent, when accounting for the different degrees of hydrogen bonding of these compounds with biopolymers (Coppola, Djabourov, & Ferrand, 2012) (van der Sman, 2013); (van der Sman, 2016) (van der Sman & Mauer, 2019). Furthermore, the phase transitions of biopolymers such as proteins and starch can be described by the Flory-Huggins (FH) theory for biopolymer melting for pure biopolymer/water mixtures (van der Sman & Meinders, 2011). The FH model can be extended to include mixtures with sugars and polyols in water when accounting for the density of the effective number of hydroxyl groups available for intermolecular hydrogen bonding $n_{OH.eff}$ (van der Sman, 2016).

The objectives of this study are to determine whether the denaturation behavior of egg white proteins is controlled by the volumetric density of intermolecular hydrogen bonds $n_{OH.eff}$ (also indicated as $\Phi_{w.eff}$) and whether it can be predicted by applying the FH theory. For these purposes, the denaturation of egg white proteins is first studied at various conditions of hydration (i.e. protein:water ratio's). Afterwards, the denaturation behavior of egg white proteins is studied in sugars and sugar replacers solutions at similar conditions by treating the sugars/water mixtures as a single solvent which is characterized by the volumetric density of intermolecular hydrogen bonds, i.e. $n_{OH.eff}$. Complex systems are studied which range from binary to quaternary water/plasticizers mixtures and combine different classes of compounds, at concentrations up to 50%. Among the plasticizers, we also include amino acids such as L-proline and glycine as novel alternative plasticizers (Fermin et al., 2006) (Kweon, Slade, & Levine, 2017) (Ahmad, Samuelsen, Garvik, & Oterhals, 2018) as compared to commercial sugar replacers (i.e. polyols and soluble fibres). Finally, the predictions on egg white protein denaturation in sugar solutions are compared with a recently proposed description of heat-induced protein network formation in a sucrose-rich system such as pound cake (Lambrecht et al., 2018).

2. Theoretical background for quantitative description of egg white protein denaturation

2.1. Effective number of hydroxyl groups available for intermolecular hydrogen bonding

As recently described (van der Sman, 2013), the dry T_g of carbohydrates and polyols is controlled by the effective number of hydroxyl groups, $N_{OH,s}$, available for intermolecular hydrogen bonding. $N_{OH,s}$ differs from the total number of hydroxyl groups in a molecule as it is corrected for intramolecular interactions due to stereochemistry. For classes of molecules such as sugars, polyols and sugar oligomers, $N_{OH,s}$ is inversely proportional to the glass transition temperature of the pure compound via:

$$\frac{1}{2}\frac{T_g - T_{g,w}}{T_g^{\infty} - T_{g,w}} = \left(\frac{1}{2} - \frac{N}{N_{OH,s}}\right) \tag{1}$$

where T_g is the glass transition temperature of the pure compound, $T_{g,w}$ is the glass transition temperature of pure water, T_g^{∞} is the glass transition temperature of a compound of infinite size from a particular class of materials (i.e. $T_g^{\infty} = 450$ K for glucose-oligomers, 448 K for fructose oligomers and 339 K for polyols) and $\frac{N}{N_{OH,s}}$ is the inverse of the number of hydroxyl groups per molecule. The dry T_g of the plasticizers can be used to calculate $N_{OH,s}$ as recently reported for sugars and polyols (van der Sman & Mauer, 2019). For glycine and L-proline the dry T_g are 220 and 250 K, respectively (van der Sman, van den Hoek, & Renzetti, 2020).

For oligosaccharides, the dry $T_{g,s}$ can be computed from their M_n following on the Fox-Flory equation (Fox & Flory, 1950):

$$T_g = T_{g,\infty} - \frac{C}{M_n} \tag{2}$$

with $T_{g,\infty}$ being the T_g at infinite molecular weight (M_w), M_n the number averaged molecular weight and C a constant. The constant C for oligofructoses was reported as 75 kDa (Mensink, Frijlink, Van Der Voort Maarschalk, & Hinrichs, 2015).

2.2. Viscosity of sugar and sugar replacers solutions

The viscosity of water-carbohydrate mixtures has been related to the distance from their glass transitions temperature as described by T_g/T (van Der Sman & Meinders, 2013) (van der Sman & Mauer, 2019). The glass transition temperatures of the sugars and sugar replacers solutions can be calculated using the Couchman-Karasz equation:

$$T_g = \frac{y_w T_{g,w} \Delta C_{p,w} + \sum_i y_{s,i} T_{g,s,i} \Delta C_{p,s,i}}{y_w \Delta C_{p,w} + \sum_i y_{s,i} \Delta C_{p,s,i}} \tag{3}$$

with y_w the mass fraction of water and $y_{s,i}$ the mass fractions of the dissolved plasticizers, $\Delta C_{p,i}$ the change in specific heat at the glass transition temperature, and $T_{g,i}$ the glass transition temperature of the pure compound. The glass transition temperature of water is $T_{g,w} = 139$ K, with $\Delta C_{p,w} = 1.91$ kJ/kg.K (van der Sman, 2016). The value of $\Delta C_{p,s}$ is dependent on the class of plasticizers. For saccharides it holds that $\Delta C_{p,s} = 0.42$ kJ/kg.K, for polyols $\Delta C_{p,s} = 0.85$ kJ/kg.K, for inulins and polydextrose $\Delta C_{p,s} = 0.42$ kJ/kg.K, while for amino acids it was assumed $\Delta C_{p,s} = 0.85$ kJ/kg.K.

Viscosity data of sugar and sugar replacers mixtures in solution can be used to investigate the relation previously reported between viscosity η and T_g/T and to test the extension of such relation to the oligofructoses in this study, following on the computation of their dry T_g from equation (2).

The T_g of binary mixtures of water with polyols or sugars has been also related to the molar averaged (effective) number of hydroxyl groups (van der Sman, 2013):

$$N_{OH.eff} = x_w N_{OH,w} + x_s N_{OH,s} \tag{4}$$

with x_i the mole fraction of component i, for which it holds that $\sum_i x_i = 1$. Given the relation between viscosity and T_g/T, a relation should be found also between viscosity and $N_{OH.eff}$ at a constant temperature. In fact, the viscosity of sugars and polyols solution at room temperature have been related to the volumetric density of effective hydroxyl groups (van der Sman & Mauer, 2019):

$$n_{OH,eff} = \Phi_w \frac{N_{OH,w}}{v_w} + \sum_i \Phi_{s,i} \frac{N_{OH,s,i}}{v_{s,i}} \quad (5)$$

In this study, the $n_{OH,eff}$ of all sugars and sugar replacers solutions is computed according to equation (5). These values are checked against the viscosity data obtained at 20, 40 and 60 °C in order to validate the $N_{OH,s}$ values computed for the oligo-fructoses. The validation of $N_{OH,s}$ values of amino acids has been investigated in a recent study (van der Sman et al., 2020).

2.3. Quantitative description of protein denaturation via Flory-Huggins theory for polymer melting extended with the volumetric density of the effective number of hydroxyl groups

According to the FH equation for biopolymer melting, the denaturation temperature of proteins in a water solution can be described as function of the volume fraction of water (Φ_{water}) present in the system, following the equation (van der Sman, 2016):

$$\frac{1}{T_m} - \frac{1}{T_m^\circ} = \frac{R}{\Delta H_U} \frac{v_U}{v_W} \left[\Phi_{water} - \chi \Phi_{water}^2\right] \quad (6)$$

where T_m (K) is the melting temperature of the protein in the system under consideration, T_m° (K) the melting temperature of the dry protein, ΔH_U (kJ/mol) is the melting enthalpy per mole of the repeat unit of the biopolymer, v_U is the molar volume of the protein repeat unit, v_w is the molar volume of water, χ is the FH solvent-biopolymer interaction parameter and R is the universal gas constant. It should be noted that in the FH theory, the crystalline phase of biopolymers does not absorb water. The volume fraction of the biopolymer in the rubbery state is computed based on its degree of crystallinity ξ (van der Sman & Meinders, 2011).

For describing the denaturation behavior of egg white proteins in the sugars and sugar replacers solutions, the volume fraction of water Φ_{water} in equation (6) is better replaced by the effective volume fraction of the solvent, $\Phi_{w,eff}$, comprising the mixture of water and all dissolved sugars. $\Phi_{w,eff}$ is derived from $n_{OH,eff}$ by rewriting equation (7) according to (van der Sman, 2016):

$$\Phi_{w,eff} = \Phi_w + \sum_i \Phi_{s,i} \frac{N_{OH,s} v_w}{N_{OH,w} v_s} \quad (7)$$

where Φ_w is the volume fraction of water, Φ_s that of the plasticizer and $v_w$and v_s are the molar volume of water and plasticizer, respectively, obtained from the ratio of their molar weight over their mass density. For the mixtures under investigation, it holds that $\Phi_w + \Phi_s = 1 - \Phi_p$, with $\Phi_p$the volume fraction of protein. The volume fraction of components in the mixtures is computed from the mass fraction using the mass density ρ_i of each ingredient (ρ_{water} 1000 kg/m^3; $\rho_{xylitol}$ 1520 kg/m^3; $\rho_{glucose}$ 1540 kg/m^3; $\rho_{sucrose}$ 1550 kg/m^3; ρ_{FOS} 1550 kg/m^3; $\rho_{proline}$ 1370 kg/m^3; $\rho_{glycine}$ 1660 kg/m^3; $\rho_{proteins}$ 1330 kg/m^3).

Following on the description of $\Phi_{w,eff}$, the FH equation (6) is rewritten as (van der Sman, 2016):

$$\frac{1}{T_m} - \frac{1}{T_m^\circ} = \frac{R}{\Delta H_U} \frac{v_U}{v_W} \left[\Phi_{w,eff} - \chi_{eff} \cdot \Phi_{w,eff}^2\right] \quad (8)$$

with the effective interaction parameter equal to (van der Sman, 2016):

$$\chi_{eff} = \chi_0 + (\chi_1 - \chi_0)(1 - \Phi_{w,eff}^2) \quad (9)$$

χ_0 is the interaction parameter of the hydrated biopolymer and equal to 0.5, while χ_1 that of the dry biopolymer, independently of temperature (van der Sman, 2016).

3. Materials and methods

3.1. Materials

Pasteurized fresh hen egg white (91% proteins on dry mass basis) was purchased at a local supermarket. The egg white was then freeze-dried and gently ground in a mortar. Freeze-drying was performed immediately after purchasing, thus limiting conversion of ovalbumin into S-ovalbumin (Deleu et al., 2015) (Donovan & Mapes, 1976).

Nine different plasticizers among sugars, polyols, amino acids and soluble fibres were used in the study. Glucose and sucrose were taken as sugar references. Soluble fibres, glycine, L-proline and xylitol were chosen to have plasticizers from different classes of compounds and providing variations in M_w and in the effective number of hydroxyl groups, $N_{OH,s}$. Glycine, L-proline and xylitol are also of interest as sweeteners. Glucose, sucrose, xylitol, L-proline and glycine were from Sigma-Aldrich (St. Louis, MO, US); Three different fructo-oligosaccharides were used: Frutalose OFP (OFP) and Frutafit CLR (CLR) provided by Sensus (Roosendaal, The Netherlands) and Actilight (FOS) from Tereos (Marckolsheim, France); the polydextrose (PDX) Litesse Ultra was supplied by DuPont (Wilmington, Delaware, US). The average molecular weight of the fructo-oligosaccharides was preliminarily determined from the degree of polymerization of the constituent polysaccharides. Sugar contents were obtained by high performance anion exchange chromatography with pulsed amperometric detection (Dionex DX-500 apparatus fitted with a Dionex PA-100 column and a Dionex PA-100 guard column; BIO-LC system, Dionex, Amsterdam, The Netherlands) (Renzetti, Voogt, Koldeweij, & Meinders, 2012). The peaks were separated with a gradient of NaOH (0.25 M)/sodium acetate (0.65 M) starting with a volume ratio of NaOH/H_2O/sodium acetate of 40/60/0 and ending with a ratio of 1/39/60. Integration of the peaks was performed using Empower software (Waters Co.). The fructo-oligosaccharides varied in molecular weight, with FOS 605 g/mol (M_n = 564), OFP being 725 g/mol (M_n = 626) and CLR 1769 g/mol (M_n = 878), based on DP analysis provided by the suppliers.

3.2. Methods

3.2.1. Solutions of sugars, polyols, amino acids, soluble fibres and mixtures thereof

Twenty-five different solutions were prepared using single compounds as well as mixtures of two and three plasticizers in water. The eighteen solutions comprising single compounds are listed in Table 1. In sweet bakery products, sucrose concentration in the water phase can be as high as 50–60%, while not all sugar replacers may be fully soluble at those high concentrations. For such reason, ranges between 15% and 50% were chosen for the study. A 30% sucrose solution was taken as reference for an intermediate concentration. The seven solutions comprising mixtures of sucrose, xylitol and FOS are listed in Table 2. The

Table 1
Compositions of sugars and sugar replacers in water used for the protein denaturation studies.

Plasticizers	Concentrations (%, w/w)
Sugars	
Glucose	15, 30, 50
Sucrose	15, 30, 50
Polyols	
Xylitol	30
Soluble fibres	
FOS	15, 30, 50
PDX	30
OFP	30, 50
CLR	30
Amino acidsv	
Glycine	15
L-Proline	15, 30, 50

Table 2
Compositions (%, w/w) of ternary and quaternary mixtures of sugars and sugar replacers in water.

#	water	sucrose	xylitol	FOS	OFP	PDX	Solvent $n_{OH,eff}$
1	70	22.5	1.8	5.7			67.0
2	70	15	3.6	11.4			66.9
3	70		7.2		22.8		66.5
4	70	22.5	7.5				67.5
5	70	15	15				67.9
6	70	22.5		7.5			66.9
7	70	15		15			66.5

mixtures were selected as an example of partial or full replacement of a 30% sucrose solution. The specific combinations were chosen to have a $n_{OH,eff}$ of the solvent around that of the 30% sucrose solution (i.e. 67.2). Distilled water was used in all preparations. Solutions were slightly heated (up to ≈ 55 °C) to ensure complete dissolution.

3.2.2. Characterization of the viscosity of sugars and sugar replacers solutions

The viscosity of sugars and sugar replacers solutions were measured at three different temperatures, i.e. 20, 40 and 60 °C, using a DHR2 hybrid rheometer (TA Instruments, New Castle, USA) equipped with a double wall concentric steel cylinder. A shear rate ramp was applied going from 0.01 to 1000s and back to 0.01s. Measurements were performed in duplicates.

3.2.3. Egg white proteins denaturation in water solution and in solutions of sugar and sugar replacers

The denaturation temperature of the egg white proteins was first studied as function of water content by preparing different ratio's between egg white proteins and water. In particular, water solution with egg white mass fractions ranging from 0.1 to 0.9 (w/w) with incremental steps of 0.1 were studied.

Denaturation of egg white proteins was then studied in the sugar and sugar replacer solutions listed in Table 1 by using the same range of egg white mass fractions (0.1–0.9) as for the study with water. Egg white mass fractions of 0.3 and 0.5 were studied for the solution with PDX, OFP, CLR and for the ternary and quaternary mixtures listed in Table 2.

Differential scanning calorimetry (DSC) was used to determine the denaturation behavior of egg white proteins in the different solutions. High volume hermetic stainless steel cups were filled first with the egg white powder and then the solution was added. Cups were closed and stored overnight to allow full hydration of the egg white powder. Tests conducted to assess the effect of hydration time on denaturation profile indicated full hydration at the conditions used in this study. After hydration, samples were then analysed in a DSC Q200 (TA Instruments, New Castle, USA) by first equilibrating at −40 °C for 5 min and then by heating up to 160 °C at a rate of 5 °C/min. The onset of protein denaturation (T_{onset}), peak temperature (T_{peak}) and denaturation enthalpy (ΔH) were determined using the analysis tool available in the Universal Analysis software (TA instruments, New Castle, USA). Experiments were performed in triplicates.

The protein denaturation data (T_{onset} and T_{peak}) derived from the DSC measurements were tested with the extended FH theory to check the validity of $\Phi_{w,eff}$ for various sugars, sugars replacers and mixtures thereof.

4. Results

4.1. Egg white proteins denaturation in water solutions

The denaturation of egg white proteins was studied at different egg white:water ratio's with water mass fractions (wmf) ranging from 0.1 to 0.9. An overview of the denaturation profiles obtained for such ranges is provided in Fig. 1. In diluted solutions (wmf > 0.4) two main melting peaks could be clearly detected at about 65 °C and at 81 °C. These peaks are commonly attributed to ovotransferrin and ovalbumin (Donovan, Mapes, Davis, & Garibaldi, 1975), respectively, being the main fractions with 12% and 54% of total egg white proteins (Deleu et al., 2016). A shoulder was observed between the two main peaks which is attributed to lysozyme and ovomucoid (Donovan et al., 1975).

Decreasing wmf resulted in a significant shift in the peak denaturation temperature (T_{peak}) of ovalbumin for wmf <0.4 ($p < 0.00$). Below such water amount a progressive increase in T_{peak} of the ovalbumin fraction was observed with decreasing water content (Fig. 1A). Instead, T_{peak} of ovotransferrin significantly increased only at wmf <0.3 ($p < 0.00$). Furthermore, T_{peak} of ovalbumin and ovotransferrin merged into one broad peak at wmf of 0.1.

The denaturation onset temperature (T_{onset}) and T_{peak} of the egg white proteins as detected by DSC was plotted as a function of $\Phi_{w,eff}$ (which for pure water solution is then equal to Φ_{water}), as computed from equation (7). The denaturation behavior of ovalbumin in water as obtained from this study was also compared to a similar study from Rao and Labuza (2012)(Fig. 1B), showing good agreement. The elevation of T_{peak} to temperatures of ≈140 °C for extremely low values of $\Phi_{w,eff}$ is consistent with other studies (Bell & Hageman, 1996). The FH prediction of the melting line for T_{onset} and T_{peak}, as derived from equation (8) are also plotted in Fig. 1B. The fitted parameters were $T_{m,0}$, ΔH_U, and the degree of crystallinity ξ (van der Sman & Meinders, 2011). Similarly as to the semi-crystallinity of starch, one has to take into account that crystalline domains will not absorb water at the beginning of the denaturation process. In fact, the denaturation of ovalbumin and plant globulins is associated with a concomitant loss in crystallinity, as observed with combined DSC and X-ray diffraction measurements (Gorinstein, Zemser, Friedman, & Chang, 1995) (Gorinstein, Zemser, & Paredes-López, 1996). The denaturation enthalpy of those proteins increases with increasing crystallinity. The solvent-protein effective interaction parameter χ_{eff} in the FH theory (equations (8) and (9)) is composition dependent and ranges from $\chi_0 = 0.5$, the value for hydrated biopolymers, and $\chi_1 = 0.8$, which is reported for starch and hydrophilic proteins (van der Sman, 2016). Via regression we obtained $T_{m,0} = 418$ (K), $\Delta H_{U,T_{peak}} = 57$ kJ/mol, $\Delta H_{U,T_{onset}} = 52$ kJ/mol and $\xi = 0.7$.

4.2. Viscosity of sugar and sugar replacers solutions in water

The viscosity of the various sugars and sugar replacers solutions in water as well as of mixtures thereof were measured at 20, 40 and 60 °C. The viscosity data were gathered in order to test their correlation with T_g/T, as derived from equation (3) and the measuring temperature. In fact, it has been previously demonstrated that the viscosity of sugars and polyols solutions scales with T_g/T following a master curve (van Der Sman & Meinders, 2013) (van der Sman & Mauer, 2019), which could serve to validate the computation of the dry $T_{g,s}$ obtained from equation (2). Furthermore, the viscosity of sugars and polyols solution at a specific temperature has been also correlated to $n_{OH,eff}$ (van der Sman & Mauer, 2019). Consequently, the viscosity of the sugar and sugar replacers solutions including soluble fibres should also correlate with $n_{OH,eff}$, with $n_{OH,s}$ of the soluble fibres computed from equation (1). The dry $T_{g,s}$ and $N_{OH,s}$ from which T_g/T and $n_{OH,eff}$ of the studied solutions have been computed are reported in Table 3. It is assumed that the soluble fibres used in this study are fully amorphous in the water solutions.

The results obtained indicated that the viscosity of the different solutions highly correlated with T_g/T (Fig. 2A), following the expression of the viscosity master curves recently proposed for sugars and polyols (van der Sman & Mauer, 2019):

$$log\left(\frac{\eta}{\eta_0}\right) = a_1 q + a_2 q^2 + a_3 q^3 \quad (10)$$

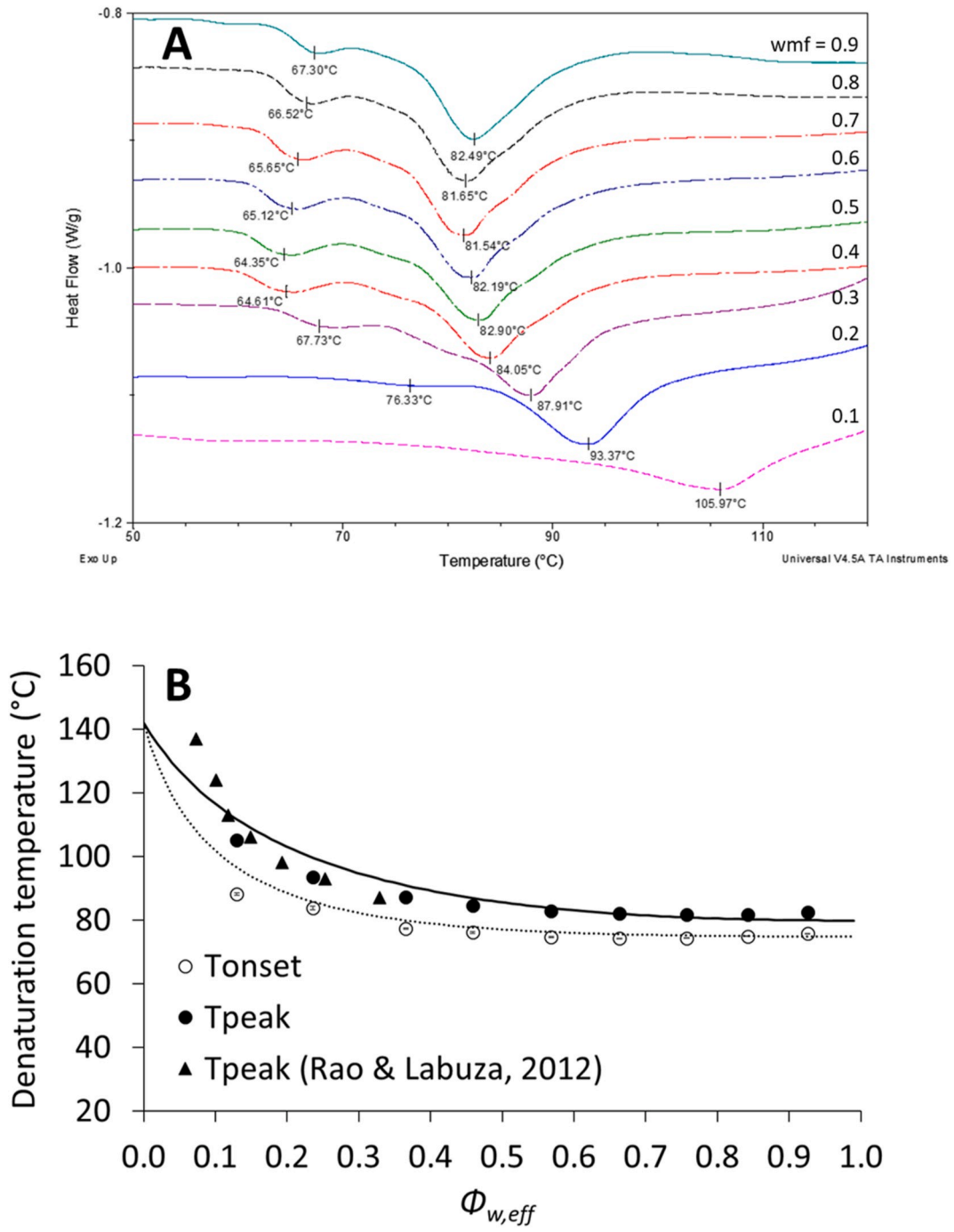

Fig. 1. [A] Denaturation profile of egg white from DSC thermograms for various hydration levels expressed as water mass fractions (wmf). Peaks are attributed to ovotransferrin (64–76 °C) and ovalbumin (81–105 °C) [B] Denaturation T_{onset} and T_{peak} of ovalbumin as described by $\phi_{w,eff}$, following on the application of FH theory for biopolymer melting (solid and dotted lines).

with $\eta_0 = 0.231$ mPas, $q = \frac{T_g}{T} - 0.35$and a_1, a_2, a_3 being fitting coefficients for sugars and polyols solutions in water. The data collected for the water-oligosaccharides mixture fit well in the observed relation ($r^2 = 0.942$ and 0.945 for sugar and polyol master curves, respectively), thus indicating that the estimated dry T_g is in agreement with the presented theory (Fig. 2A). The viscosity of the sugars and sugar replacers solutions at each of the studied temperatures well correlated also with the $n_{OH,eff}$ computed from equation (2) (Fig. 2B), thus validating the $N_{OH,s}$ used for the soluble fibres (Table 3). The $N_{OH,s}$ of amino acids including L-proline and glycine have been recently determined combining the current experimental data with others from literature (van der Sman et al., 2020) and are reported in Table 3.

4.3. Egg white proteins denaturation in 30% solutions of sugars and sugar replacers

The working hypothesis for this study was that the denaturation temperature of egg white proteins in sugar and sugar replacers solutions is modulated by the effective hydrogen bond density of the solution, i.e. $\Phi_{w,eff}$, following on its application in the FH theory for biopolymer melting (van der Sman, 2016). As plasticizers like sugars, polyols and oligosaccharides vary in their size and $N_{OH,s}$ (Table 3), they would alter

Table 3
Chemical-physical characteristics of the investigated compounds.

Compound	M_w (g/mol)	Density (kg/m^3)	T_g (K)	$N_{OH,s}$
Water	18	1000	139	2
Glycine	75	1660	220[a]	2.63
L-Proline	115	1370	250[a]	2.99
Xylitol	152	1520	249	2.94
Glucose	180	1540	306	4
Sucrose	342	1550	336	4.48
FOS	605	1550	315[b]	4.66
OFP	725	1550	328[b]	5.16
CLR	1769	1550	363[b]	7.24
PDX	2160	1550	366[c]	6.16

[a] From van der Sman et al. (2020).
[b] Computed from Flory-Fox equation.
[c] From van der Sman (2019).

the ability of the water solution to act as plasticizer for the proteins. The T_{onset} and T_{peak} of ovalbumin and ovotransferrin would consequently vary according to the master curve obtained in Fig. 1B, based on a shift in denaturation temperature and not in a change in the molecular mechanisms of denaturation. The $\Phi_{w,eff}$ of the different solutions tested in this study were computed by using the dry $T_{g,s}$ and other material properties listed in Table 3, according to equations (1) and (7). The dry $T_{g,s}$ and material properties of sugars and polyols have been previously reported (van der Sman, 2016) (van der Sman & Mauer, 2019).

The T_{onset} and T_{peak} of ovalbumin in 30% sugars, polyol and oligofructose solutions in water are shown in Fig. 3A and B. Similar for the results obtained in pure water solutions, by decreasing the volumetric density of hydrogen bonding $\Phi_{w,eff}$ the denaturation temperature of ovalbumin increases. The data of the ternary mixtures protein/plasticizers/water are well in line with the predictions from the FH theory earlier applied to the binary protein/water solutions. Similarly, the FH model well described the T_{onset} and T_{peak} of ovotransferrin (data not shown). Differences in $N_{OH,s}$ and in molar volumes among the plasticizers tested explains the differences in the volumetric density of hydrogen bonding sites for similar protein:solvent ratio's. In general, the larger the M_w of the plasticizer, the lower the $\Phi_{w,eff}$ at a specific concentration.

The T_{peak} of ovalbumin in aqueous solutions of soluble fibres (PDX, OFP, CLR of Table 1) as well as mixtures of sucrose, xylitol and an oligofructose (Table 2) are shown in Fig. 3C and D, while those in solutions of the amino acids L-proline and glycine are reported in Fig. 4. In all cases, the FH model well describes the denaturation profile, thus indicating that the $\Phi_{w,eff}$ parameter can be broadly applied to different classes of hydrogen bonding molecules, including amino acids, as well as to complex mixtures beyond binary ones.

4.4. Egg white protein denaturation in conditions of phase separation

It has been recently reported that phase separation occurs for ternary systems comprising a biopolymer (protein or starch), water and a plasticizer such as sugars or polyols, resulting in separated biopolymer and plasticizer rich phases (Kawai & Hagura, 2012) (Roudaut & Wallecan, 2015). In particular, the smaller the molecular weight of the plasticizer, the larger the region of plasticizer concentrations showing phase separation (Ubbink, 2016) (van der Sman, 2019). For such reason, the influence of plasticizer concentration (15, 30 and 50% w/w) on egg white protein denaturation at various egg white mass fractions was studied with glucose, sucrose, an oligofructose (FOS) and with L-proline (Fig. 5). In general, the denaturation profile of ovalbumin was well described by $\Phi_{w,eff}$ following on its application in the FH model. However, for glucose, sucrose and FOS at 50% concentration, a deviation of the experimental data from the FH model was observed, particularly for T_{peak}. The deviation was the largest for the 50% glucose solution. By carefully looking at the T_{peak} denaturation profile of ovalbumin at 50% concentration of glucose, sucrose and FOS, it could be noted that in the region where deviation from the FH model is observed, the denaturation temperature remains constant. Furthermore, deviations from the FH predictions seemed to occur at different conditions of $\Phi_{w,eff}$ (Fig. 5). In particular, the value of $\Phi_{w,eff}$ at which deviation were observed followed the order glucose < sucrose < FOS, which resembles the order of M_w. These results could be explained by phase separation, resulting in a constant composition of the protein-rich domain, regardless of the $\Phi_{w,eff}$. This hypothesis will be elaborated more thoroughly in the discussion section.

In order to further elucidate the influence of different plasticizers on phase separation and consequently on the denaturation behavior of egg white proteins, the T_{peak} of ovalbumin obtained at $\Phi_{w,eff}$ of 0.60 (±0.01) were collected for 30% concentrations of sugars, polyols and soluble fibres. A strong correlation was observed when plotting T_{peak} as function of the number of intermolecular hydrogen bonding sites per molar volume of the plasticizers molecule, i.e. $N_{OH,s}/v_s$ (Fig. 6). The larger the $N_{OH,s}/v_s$ of the plasticizer (providing an increase in $\Phi_{w,eff}$ at similar concentrations) the larger the increase in T_{peak} induced by phase separation. The correlation seemed to stand also when the average $N_{OH,s}/v_s$ of the plasticizers mixtures of Table 2 were included in the dataset, which were computed from:

$$\left(\frac{N_{OH,s}}{v_s}\right)_{avg} = \frac{\sum_i \Phi_s \frac{N_{OH,s}}{v_s}}{\sum_i \Phi_s}$$

Based on these results, it is worth exploring further validation of the proposed relation with larger datasets. Phase separation mechanisms in mixtures of biopolymer, water and sugars will be investigated in upcoming papers.

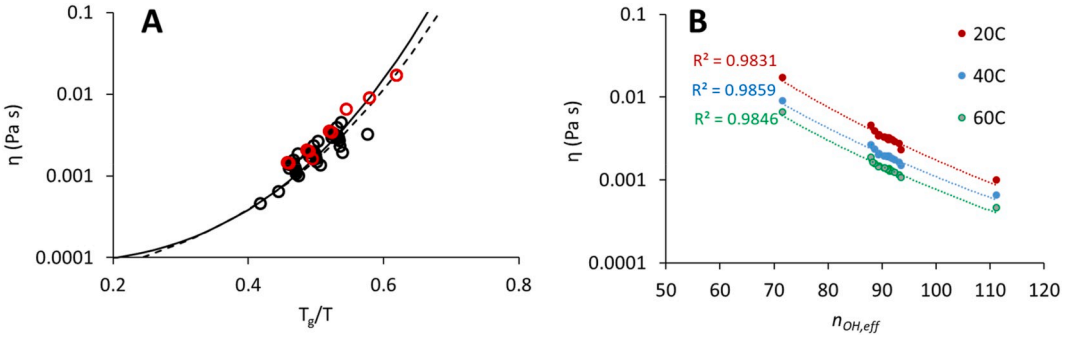

Fig. 2. [A] Viscosity of sugars and sugar replacers solutions obtained at 20, 40 and 60 °C and plotted as function of T_g/T. Black circles indicate the sugars and polyols solutions, while red circles indicate all solutions containing soluble fibres. Solid line and dotted line represent the master curves for sugars and polyols, respectively (van der Sman & Mauer, 2019). [B] Viscosity of sugars and sugar replacers solutions plotted as function of $n_{OH,eff}$ computed from equation (5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

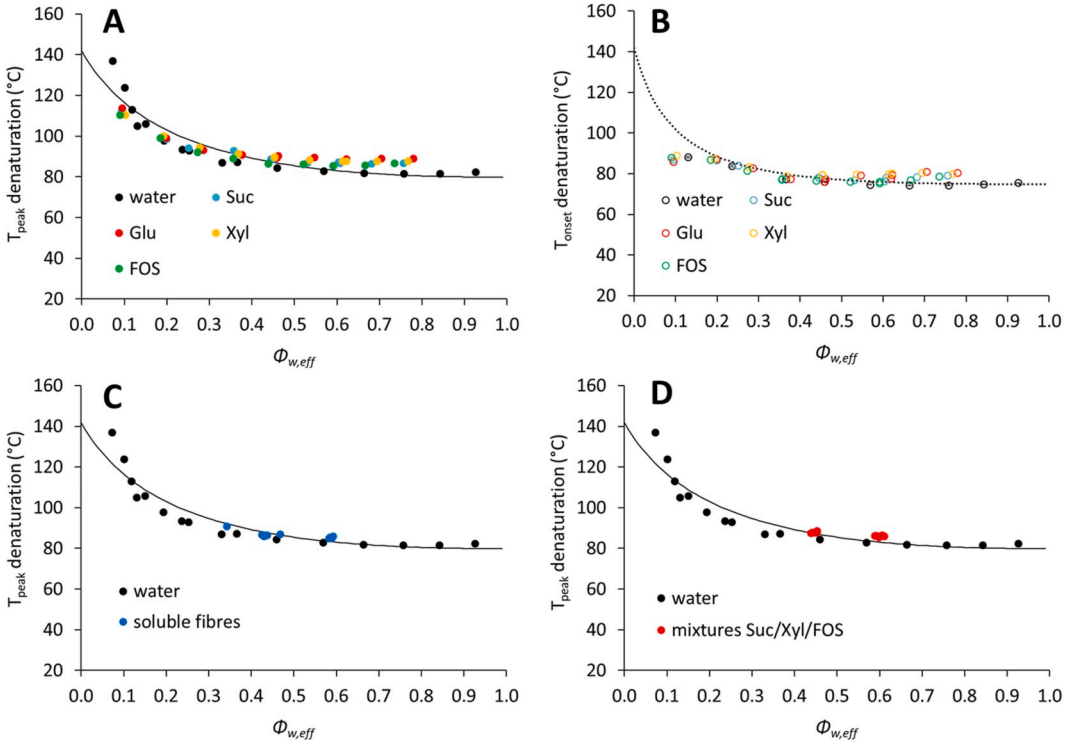

Fig. 3. [A, B] T_{peak} and T_{onset} of ovalbumin denaturation as described by $\phi_{w,eff}$, following on the application of FH theory for 30% (w/w) sugars and sugar replacers solutions in water tested at protein mass fractions ranging from 0.1 to 0.9. [C] T_{peak} of ovalbumin denaturation in 30% solutions of soluble fibres in water (Table 1) tested at protein mass fractions of 0.3 and 0.5. [D] T_{peak} of ovalbumin denaturation in mixtures of sugar replacers solutions in water (Table 2) tested at protein mass fractions of 0.3 and 0.5. The solid lines represent the FH model predictions.

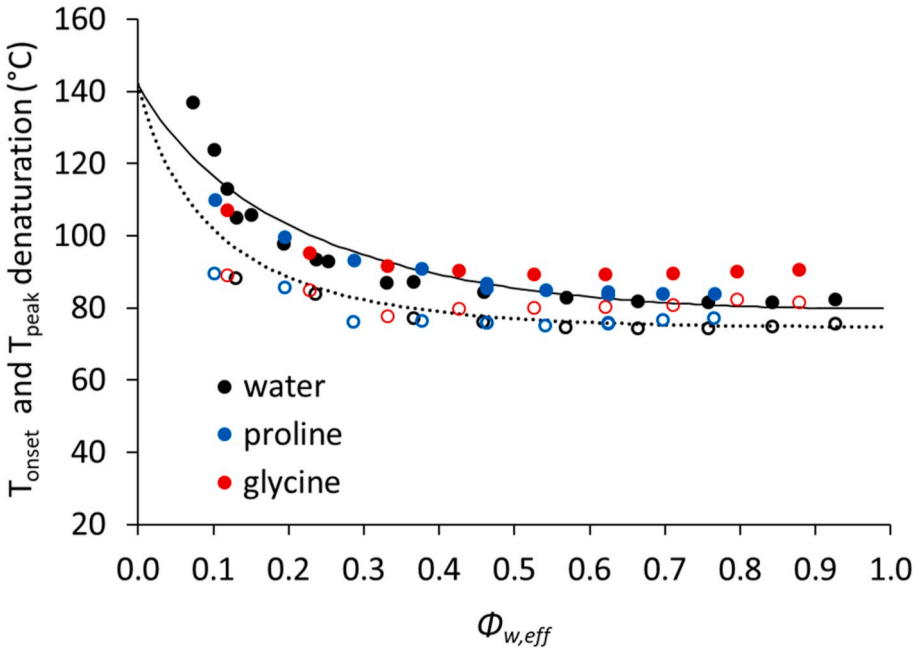

Fig. 4. T_{peak} (filled circles) and T_{onset} (open circles) of ovalbumin denaturation as described by $\phi_{w,eff}$, following on the application of FH theory for 15% (w/w) glycine and 30% (w/w) L-proline solutions in water tested at protein mass fractions ranging from 0.1 to 0.9. The solid line and dotted line represent the FH model predictions for T_{peak} and T_{onset}, respectively.

4.5. Egg white proteins denaturation and protein network formation in the supplemented state diagram of pound cake baking

Denaturation and consequently the aggregation of egg white proteins during heating is important in many food applications. In sugar-rich products such as pound cake, the thermosetting behavior of the batter during baking largely determines the amount of expansion and volume of the cake. Egg white protein coagulation is of particular importance as it coincides with the moment of gas release from the expanding bubbles (Mizukoshi, Maeda, & Amano, 1980), thus contributing to stiffen the gas

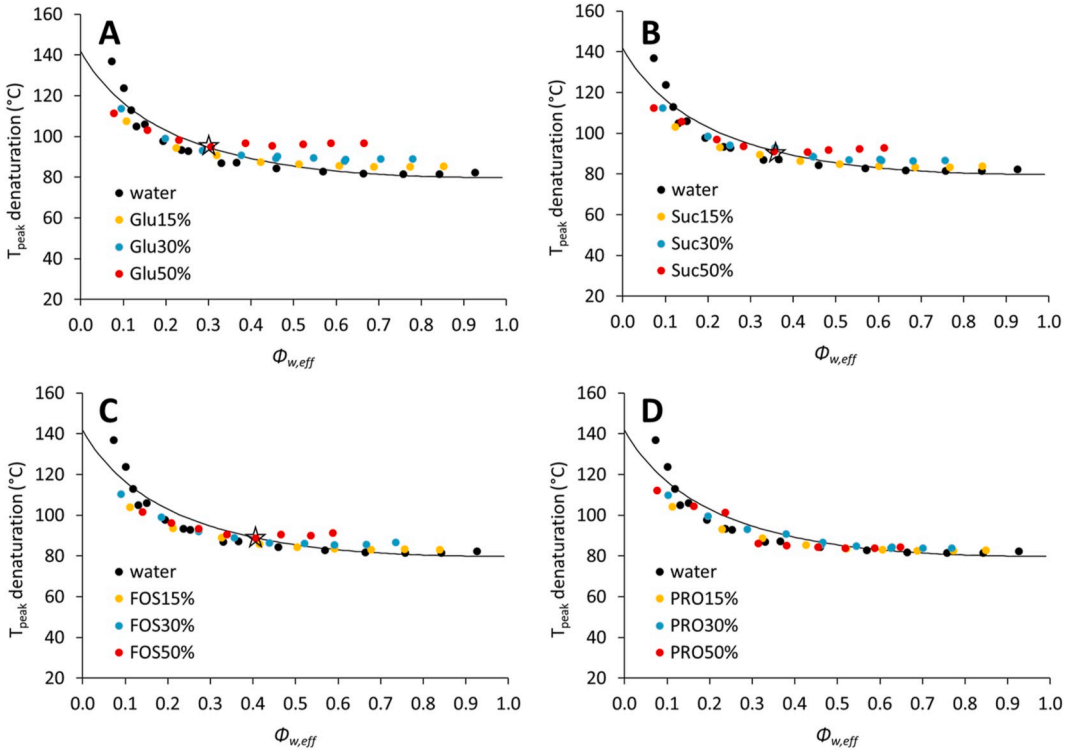

Fig. 5. T_{peak} of ovalbumin denaturation as described by $\phi_{w,eff}$, for 15, 30 and 50% solutions in water of glucose [A], sucrose [B], an oligo-fructose [C] and L-proline [D]. All tests were performed using protein concentrations ranging mass fraction from 0.1 to 0.9. The black star symbol estimates the $\phi_{w,eff}$ condition from which deviation from the FH model is occurring. The solid lines represent the FH model predictions.

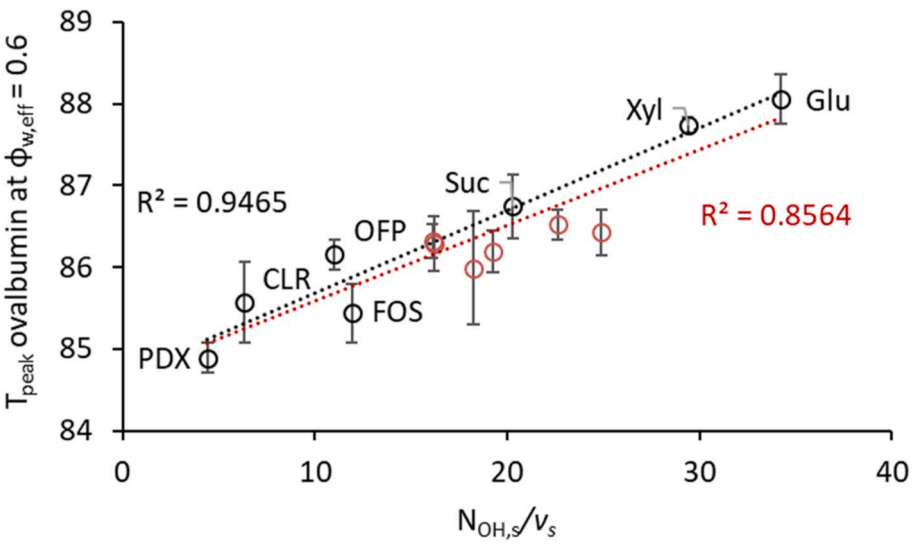

Fig. 6. T_{peak} of ovalbumin denaturation measured at conditions of $\phi_{w,eff} = 0.6$ (±0.01) for 30% sugars and sugar replacers solutions in water. The molar density of the effective number of hydroxyl groups $N_{OH,s}/v_s$ is plotted for the individual plasticizers (black circles) as well as for the mixtures of plasticizers reported in Table 2 (red circles). Correlations are indicated for the individual plasticizers (black dotted line) as well as for the entire dataset (red dotted line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bubble wall and preventing early rupture. For reformulation purposes such as sugar replacement in bakery products, it is worth to compare the predictions of our proposed model of egg white protein denaturation with a recently proposed description of heat-induced protein network formation in pound cake (Lambrecht et al., 2018). For such purpose, the heat-induced denaturation behavior of egg white proteins has been conveniently depicted in the supplemented state diagram together with the thermosetting of gluten (Fig. 7), as compared to the schematic description of protein network formation. The thermosetting of gluten has been recently provided (van der Sman & Renzetti, 2019). The structuring pathway of the cake batter during baking is represented in the supplemented state diagram based on the compositional and temperature profile data from Deleu et al. (2019) (Fig. 7A). The process described in the supplemented state diagram of Fig. 7A indicates ovotransferrin as the first major egg white protein to denature around 70°C, which is preceded by the thermo-setting of gluten proteins slightly above 60°C. Ovalbumin is the last egg white protein to denature, with the structuring pathway of cake during baking crossing the denaturation T_{peak} of ovalbumin at about 85°C. The structuring process described seems in good agreement with the description from Lambrecht et al. (2018) (Fig. 7B), which reported the reduction in protein extractability of cake batter between 75 °C and 81 °C. In such temperature range about 30% of the yolk proteins were incorporated in the protein network (Deleu et al., 2017) by forming SS bonds with the egg white proteins (i.e.

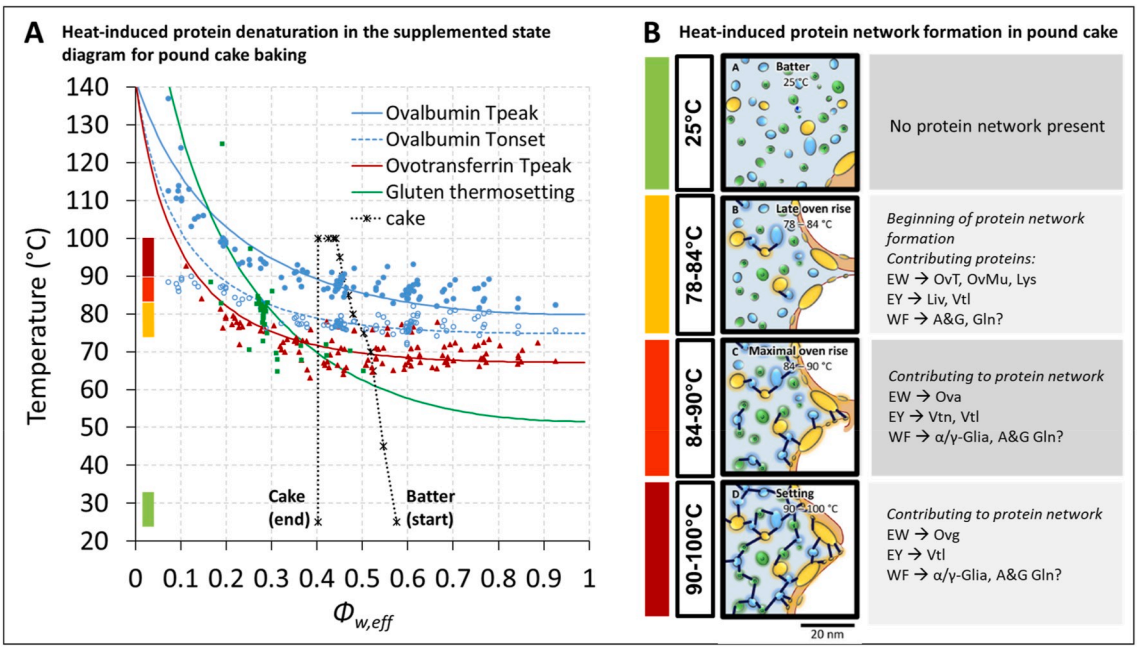

Fig. 7. [A] Supplemented state diagram for thermosetting of gluten and denaturation of egg white proteins as function of $\phi_{w,eff}$. The structuring pathway of pound cake batter is indicated together with the baking phases described by Lambrecht et al. (2018) (color bars for the specific temperature ranges). [B] Schematic representation of protein network formation in pound cake batter/cake prior to and in different phases of baking (adapted from Lambrecht et al. (2018)). The protein source is indicated with colors for egg white [EW, blue], egg yolk [EY, yellow] and wheat flour [WF, green]. The following egg proteins are listed for network formation during the main baking phases (associated with color legends): ovalbumin [Ova], ovotransferrin [OvT], lysozyme [Lys], ovomucoid [OvMu], ovoglobulin [OvG], ovomucin [OvMc], livetin [Liv], phosvitin [Pho], vitellenin [Vtn.]. Wheat flour albumin and globulin [A&G], gliadin [Glia], and glutenin [Gln] are also indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ovotransferrin, ovomucoid and lysozyme). That resulted in about 32% loss in egg white protein extractability between 78 °C and 84 °C (Deleu et al., 2016). Denaturation of ovalbumin resulted in a 51% reduction in the extractability of egg white proteins between 84 °C and 90 °C (Deleu et al., 2016), concomitantly with a loss in egg yolk protein extractability via SS bonds polymerization with ovalbumin (Deleu et al., 2017). The temperature ranges for incorporation of ovalbumin in the protein network are well in agreement with the prediction of its denaturation in the supplemented state diagram of Fig. 7A. It was suggested that also glutenins contribute to the network formation (Lambrecht et al., 2018), but experimental assessment was difficult due to extractability only in reducing conditions (Deleu et al., 2017). The supplemented state diagram for cake baking suggests the contribution of gluten proteins to the initial network formed by egg yolk and egg white proteins, following on its thermosetting profile.

5. Discussion

The hypothesis of this study was that the denaturation profile, i.e. T_{onset} and T_{peak}, of egg white proteins is controlled by the volumetric density of hydrogen bonds $\Phi_{w,eff}$ and that it can be predicted for various sugars and sugar replacers solutions in water, following on its application in the FH theory. The application of the FH model extended with $\Phi_{w,eff}$ would hence provide quantitative guidelines for optimizing sugar replacement in systems where egg white proteins have a major contribution in heat-induced protein networks formation, such as e.g. a pound cake product.

The denaturation of gelatin, gluten, soy and sunflower proteins has been recently related to $\Phi_{w,eff}$ (van der Sman, 2016) (van der Sman & Renzetti, 2019) and analogies were also found for starch gelatinization behavior (van der Sman, 2016) (van der Sman & Mauer, 2019). Hence, both protein denaturation and starch gelatinization can be described as biopolymer melting phenomena (Huson et al., 2011) (van der Sman, 2016) (van der Sman & Mauer, 2019), based on equilibrium between amorphous and crystalline state. From that perspective, protein denaturation is assumed as a thermodynamic process which is entropically driven. Spectroscopic studies conducted with β-lactoglobulin in presence of sugars and polyols demonstrated that sugars and polyols did not affect the reactivity of the Cys121 thiol at room temperature (an indicator of earliest phases in physical and chemical denaturation of β-lactoglobulin) and did not cause changes in the tertiary structure of β-lactoglobulin (Barbiroli et al., 2017). Similarly, spectroscopic studies and molecular dynamics simulations for egg white lysozyme showed that the effect of sugars on protein conformation are relatively weak (Lerbret et al., 2007) (Ionov et al., 2006). In agreement with these studies, the proposed FH model extended with $\Phi_{w,eff}$predicts a shift in denaturation temperature of egg white proteins in presence of sugars and sugar replacers, and not a change in the molecular mechanisms. Nevertheless, protein denaturation is also referred to as a kinetic process involving structural domains with different thermal stabilities (Fessas, Iametti, Schiraldi, & Bonomi, 2001) (Wang et al., 1998), which is influenced by both local and global mobility in the system (Mensink, Frijlink, van der Voort Maarschalk, & Hinrichs, 2017). In literature, the effect of sugars and polyols on protein denaturation has been described in both kinetics as well as thermodynamic terms. For better understanding the denaturation behavior of ovotransferrin and ovalbumin in the conditions of this study, the kinetics aspects are described first, followed by the thermodynamic theories of protein stabilization in sugar-water systems.

The vitrification hypothesis suggests that molecular motions and structural fluctuations of protein residues are arrested in a non-crystalline, glassy state of high viscosity, thereby preventing the denaturation of the embedded protein (Green & Angell, 1989) (Reid & Levine, 1991). Hence, the vitrification theory describes the increase in T_{onset} and T_{peak} observed in the presence of sugars from a kinetic perspective. Protein denaturation as detected by DSC is dependent on scanning rates (Donovan et al., 1975) (Godard, Biebuyck, Daumerie, Naveau, & Mercier, 1978), which suggests the importance of the kinetic aspects. The

heat-induced denaturation rate of ovalbumin follows a first order kinetics, where denaturation is the rate-limiting step due to absence of equilibrium between native and the denatured state (Weijers, Barneveld, Cohen Stuart, & Visschers, 2003). As observed in this study for different sugars, polyols and oligosaccharides, $n_{OH,eff}$ also governs kinetic factors such as viscosity (van Der Sman & Meinders, 2013) (van der Sman & Mauer, 2019) and the glass transition of sugar and polyols in water (van der Sman, 2013), which can play a role in the kinetics of denaturation. A comparison of segmental mobility below and above the glass transition in soy glycinin showed that the protein could be described as a two-phase ordered/disordered structure using basic polymer science principles (Huson et al., 2011). Water plays the role of plasticizer for both the mobile (amorphous) protein segments and for the immobile (crystalline) segments. Consequently, it seems reasonable that both kinetics (related to mobility of amorphous regions) and thermodynamics (related to equilibrium between amorphous and crystalline state) have a relevant contribution to protein stability.

From a thermodynamic perspective, stabilization of proteins in presence of sugars and other cryoprotectants has been recently reviewed by Weng, Stott, and Toner (2019) as described by: (i) the water replacement hypothesis, (ii) the water entrapment hypothesis and (iii) the preferential exclusion/hydration theory. The water replacement hypothesis states that for highly dehydrated systems sugar molecules replace water by forming hydrogen bonds with the protein residues, thus preserving the embedded protein in its native state. Overall, the total number of the hydrogen bonds that the protein forms with the solvent remains unchanged. However, with increasing hydration level, the presence of sugar molecules results in preferential hydration of proteins (preferential exclusion of sugars from the protein surface), thus resulting in increased denaturation temperature due to unfavourable thermodynamic conditions. A double shell structure is suggested to be present, with water intimately surrounding the protein while a layer of sugar molecules extends radially outward. In agreement with the water entrapment hypothesis, which extends to more sugar concentrated conditions, the presence of a hydration shell reduces protein motions, thus stabilizing protein conformation as compared to the pure water system.

As recently observed for starch gelatinization in sugar solutions (van der Sman & Mauer, 2019), these theories focus on the contribution of water and sugar molecules individually, while they neglect the role of the plasticizer (sugar)-water system as a single solvent (Perry & Donald, 2002). Additionally, it has been shown that egg white lysozyme is susceptible to irreversible perturbations of solvent exposed hydrogen bonds (Solomentsev, English, & Mooney, 2010), and that the structural changes constituting the first steps of its unfolding are related to breaking of hydrogen bonding in the solvent exposed β-domain and later in the α -domain (Raskar, Khavnekar, & Hosur, 2016). In agreement with these studies, the approach here presented considers the effective contribution of the solvent as a whole to the hydrogen bond interactions with the protein, as quantified by the $n_{OH,eff}$ parameter. The ability of the solvent to act as plasticizer for the protein is conveniently described by the effective volume fraction of the solvent, i.e. $\Phi_{w,eff}$, which accounts for the volumetric density of hydrogen bonds available for interactions with the proteins present in solution. Hence, the presented theory comprehensively accounts for the ratio of solvent versus protein (solvent quantity), as well as for the intrinsic properties of the solvent (solvent quality). As demonstrated in this study, the approach extends to various plasticizers of different nature such as amino acids, polyols, dextrins and oligofructoses as well as mixtures thereof. Therefore, $\Phi_{w,eff}$ enables a universal application of the FH model for the aims of sugar replacement in food products. A shortcoming of the current model predictions is the assumption of full miscibility between protein and solvent, while phase separation phenomena have been reported for biopolymer-sugar solutions as well as for small plasticizers (Roudaut & Wallecan, 2015) (Ubbink, 2016) (van der Sman, 2019).

Molecular dynamics simulations have shown that the presence of sugars and polyols affect the structure of water, as hydrogen bond interactions between plasticizers and water result in a reduction of bulk water and the formation of a hydration shell (Weng et al., 2019). The dynamics of water in the hydration shell is slower than in the bulk and makes the hydrodynamic volume of the plasticizer larger, thereby increasing its viscosity (van der Sman, 2013) (van der Sman & Mauer, 2019). Hydration being governed by hydrogen bonding, it is not surprising that the hydration number of plasticizers is linked with $n_{OH,eff}$, although it will also depend on plasticizer concentration and temperature (van der Sman, 2013). As confirmed in this study, viscosity is controlled by $n_{OH,eff}$ for different plasticizers types and concentrations at constant temperature, thus further substantiating recent hypothesis that $n_{OH,eff}$ is a measure for the structure of the carbohydrate solutions, which governs the phase transitions of biopolymers (van der Sman, 2013) (van der Sman & Mauer, 2019). Several studies have indicated that solvent (water-sugar) structure can be schematically described by three regions (Kawai & Hagura, 2012) (Towey, Soper, & Dougan, 2012) (Weng, Ziaei, & Elliott, 2016). A dilute aqueous sugar solution in which the bulk water structure is dominating as the average number of hydrogen bonds associated with one water molecule is similar as in pure water. A concentrated sugar solution in which bulk water molecules are completely depleted and the solvent is dominated by the plasticizer as water-water hydrogen bond interactions become rare. An intermediate region between the bulk water and the plasticizer dominant region in which bipercolating clusters of plasticizer and water exist (Lerbret, Bordat, Affouard, Descamps, & Migliardo, 2005) (Heugen et al., 2006) (Lee, Debenedetti, & Errington, 2005). An array of spectroscopic techniques and molecular dynamics simulations provide evidence for the formation of water clusters at intermediate water contents (>10% water) in a range of amorphous systems including carbohydrates (Authelin, Mackenzie, Rasmussen, & Shalaev, 2014). In this region, intermolecular hydrogen bonds between plasticizer molecules increase with increasing plasticizer concentration with concomitant decrease in hydration number (Weng et al., 2016) (Conrad & de Pablo, 1999) (Weng, Chen, Zuo, & Li, 2011).

Based on the reported studies describing changes in structure of carbohydrate solutions and the effect on protein stabilization, we suggest the following interpretation of the denaturation behavior of egg white protein within the conditions of this study. In the conditions of both diluted plasticizer solutions (up to 15%) and highly concentrated protein solutions ($\Phi_{w,eff}<0.3$), the denaturation profile of egg white proteins follows the description of the FH theory based on the $n_{OH,eff}$ of the solvent (Fig. 8A, D). In such conditions, phase separation is rather limited and the denaturation mechanisms described resembles the water replacement theory, where the hydrogen bonding provided by water is replaced by the sugars and sugar replacers, following $\Phi_{w,eff}$. Indeed for egg white lysozyme in dry conditions, molecular dynamics simulations and neutron scattering experiments indicated that sugars such as trehalose form hydrogen bonds with the protein which are significantly larger in number than those formed with water (Lerbret et al., 2012). However, trehalose is able to replace protein hydration water only partially, due to topological constraints on the position of its hydroxyl groups as well as its larger size compared to water. Consequently, hydrogen bonding interactions are found to be weaker than the lysozyme-water ones, slowing down the atomic motions of the protein (Lerbret et al., 2012). These observations seem well in agreement with effect that the $N_{OH,s}/v_s$ (i.e. the molar volume density of effective hydroxyl groups) of the sugars exerts on the quality of the solvent. In concentrated solutions, the replacement of water with sugars further reduces the hydrogen bond density of the solvent, $n_{OH,eff}$, thus further limiting the conformational flexibility of egg white proteins and increasing their thermal stability (Tsai, Udovic, & Neumann, 2001) (Cornicchi, Marconi, Onori, & Paciaroni, 2006).

Depending on the plasticizer type and its concentration, deviation

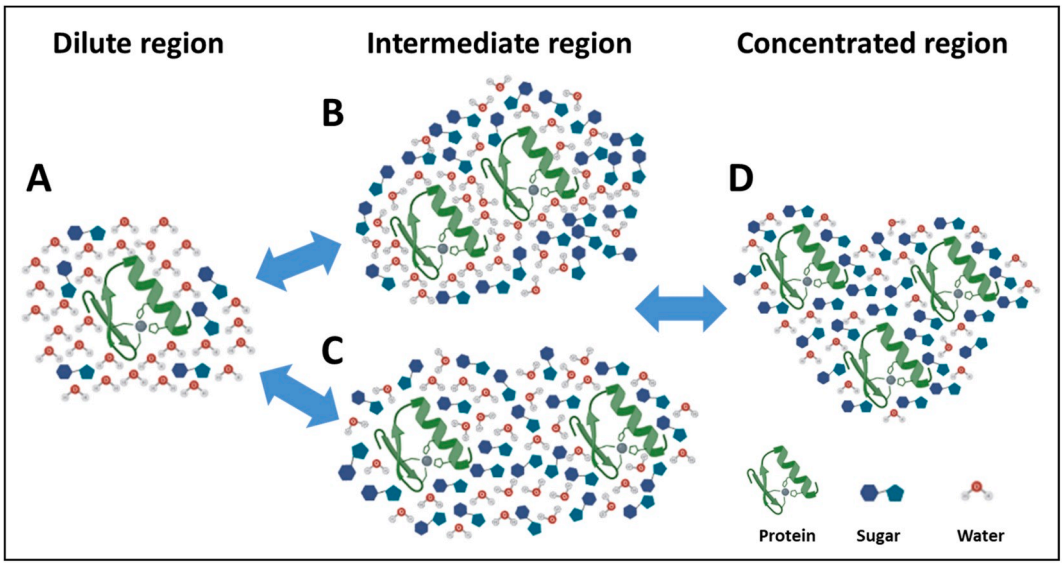

Fig. 8. Schematic representation of structures in mixtures of proteins, plasticizers (i.e. sugars or sugar replacers) and water. In the diluted and concentrated regions (high water:plasticizer ratio and $\phi_{w,eff}<0.3$, respectively), the solvent (i.e. water with plasticizer) and proteins constitute an homogeneous system. The denaturation of protein follows the FH model predictions which is consistent with the water replacement theory [A, D]. In the intermediate region, phase separation occurs which results in a plasticizer-rich domain and a protein-water rich domain [B]. The plasticizer can still interact with the proteins, but is enriched in the dispersed phase. Water is distributed over both the protein-rich phase and the plasticizer-rich phase. The composition of the protein-rich phase does not reflect the computed $\phi_{w,eff}$. This description is consistent with the preferential exclusion theory, resulting in a denaturation temperature higher than what predicted by the FH theory. For sugars, polyols and soluble fibres, phase separation is dependent on type of plasticizer (i.e. $N_{OH,s}/v$) and concentration. For L-proline, no phase separation is assumed [C].

from the FH model prediction occurs as a result of phase separation, which resembles the preferential exclusion theory (Fig. 8C), as proteins will be concentrated in the water clusters. As a result, the plasticizer will contribute less to the intermolecular hydrogen bonds with the protein, as $n_{OH,eff}$ in the water clusters will not reflect the composition of the solvent. In such conditions, $n_{OH,eff}$ is lower than what estimated, consequently increasing denaturation temperature above the FH model predictions. This representation is consistent with evidence collected for egg white lysozyme in disaccharides solutions at intermediate sugar concentrations (i.e. 37-60%). In fact, the analysis of the interactions of sugars with lysozyme via hydrogen bonding suggest that lysozyme is preferentially hydrated (Lerbret et al., 2007) (Lins, Pereira, & Hünenberger, 2004). The disaccharides molecules cluster and move toward the protein, but neither completely expel water from the protein surface nor form hydrogen bonds with the protein (Lins et al., 2004). Consequently, the sugars stiffen the environment experienced by the lysozyme atoms, thereby counteracting the softening of protein vibrational modes upon denaturation (Lerbret et al., 2009). For sugars, polyols, sugar oligomers and mixtures thereof, the larger the average molar volume density of effective hydroxyl groups, i.e. $N_{OH,s}/v$, the larger the increase in denaturation temperature induced by phase separation (Fig. 6). From such standpoint, it is not surprising to find that at intermediate sugar concentrations (37–60%), trehalose (with $N_{OH,s}/v$ of 35) has a stronger influence on the dynamics of water and lysozyme than sucrose (with $N_{OH,s}/v$ of 20) (Lerbret et al., 2007), resulting in a significantly more important shift in denaturation temperature and on the stabilization energy than sucrose (Ionov et al., 2006). Sucrose forms smaller disaccharide clusters than trehalose (Lerbret et al., 2007), which is in agreement with our findings. The suggested relation between $N_{OH,s}/v$ of the plasticizer, phase separation in the protein/water/plasticizer system and the effect on denaturation is a novel element arising from this study which will be worth investigating further in forthcoming papers. Recent studies indicated the influence of plasticizer size on phase separation and antiplasticization region in sugar and polyols mixtures with water and a biopolymer (Ubbink, 2016) (van der Sman, 2019). Furthermore, Sapir and Harries (2015) indicated that exclusion of plasticizers from biopolymer surface is related to microscopic parameters corresponding to biopolymer/plasticizer and solvent/plasticizer interactions, which may be well related to size and molecular density of hydrogen bonding sites. Therefore, these studies seem also supportive of the mechanisms here described.

In the case of L-proline, the transition from dilute to concentrated regions occurs with no deviation from the FH model. Hence, full miscibility of proteins and solvent seems likely and both water and L-proline contribute to hydrogen bonds with the protein as a homogeneous system (Fig. 8C). For a more detailed characterization of the thermodynamic properties of amino acids as plasticizer and humectant, we refer to a separate publication (van der Sman et al., 2020).

Our findings suggest that the FH model can provide a solid basis for a comprehensive description of the influence of sugars (and sugar replacers) on protein denaturation, especially when it is extended to include conditions of phase separation. Phase separation behavior in ternary systems with polysaccharides and polyols has been recently described by an extension of the FH Free-Volume theory by taking into account, among other factors, $\Phi_{w,eff}$ (van der Sman, 2019). A quantitative description of the observed phase separation behavior will be addressed in a forthcoming paper.

Understanding the key phase transitions occurring during heating of bakery products and the compositional factors affecting such transitions provides valuable insights in understanding the influence of reformulation on food structuring processes and consequently on end product quality. In particular, the state diagram of gluten and egg white proteins presented in this study (Fig. 7) gives a quantitative basis to the schematic representation of protein network formation in a complex protein system such as pound cake (Lambrecht et al., 2018). It also allows to predict the effect of sugar and sugar replacers (in terms of both type and concentrations) on protein denaturation behavior from the starting formulation, which will consequently affect protein network formation and cake crumb texture. Hence, such insights can be used as quantitative clues for sugar replacement, such as the optimal combination of polyols, amino acids and oligofructoses in pound cake to match the same transition temperatures depicted in Fig. 7. Additionally, the presented model could also provide insights for finding suitable plant-based proteins for egg replacement in pound cake. For instance, by plotting the

denaturation T_{peak} of soy β-conglycinin (authors' unpublished data) in the supplemented state diagram of Fig. 7, one would learn that it crosses the T_{peak} of ovalbumin around the same temperature at which ovalbumin denatures in the pound cake formulation (Fig. S1).

6. Conclusion

In this paper, we have shown that the volumetric density of effective hydrogen bonds $n_{OH,eff}$ available for intermolecular interactions governs the denaturation behavior of egg white proteins in mixtures of water with polyhydroxy compounds. The results are in agreement with recent studies showing that the same principle applies also to starch gelatinization (van der Sman & Mauer, 2019) as well as to gelatin and soy protein denaturation (van der Sman, 2016) in binary mixtures of water with sugars and polyols. The present study shows the extension of these principles also to soluble fibres and amino acids as well as to complex ternary and quaternary mixtures of sugars and sugar replacers. As recently shown for sugars and polyols (van der Sman & Mauer, 2019), the viscosity of solutions with sugars, polyols, soluble fibres and mixtures thereof at three different temperature conditions correlated with T_g/T as well as $n_{OH,eff}$. For the oligo-fructoses, $N_{OH,s}$ was computed from their $T_{g,dry}$ as obtained by application of the Flory-Fox equation. Hence, T_g (either measured or computed) and viscosity are alternative means to derive $N_{OH,s}$ for these classes of compounds.

The results obtained confirm the recent hypothesis that the interactions between plasticizers such as sugars and sugars replacers and water are controlled by hydrogen bonding, resulting in the formation of a hydration shell which reduces bulk water and affects viscosity of the solution via $n_{OH,eff}$ (van der Sman & Mauer, 2019). The plasticizers-water system can be treated as a single solvent, whose structure controls biopolymer melting. In concentrated protein solutions ($\Phi_{w,eff}$ <0.3) and at high water:plasticizer ratio, $n_{OH,eff}$ provides a quantitative basis to the water replacement theory, where the hydrogen bonding provided by water is partially replaced by the plasticizers. However, at low water:plasticizer ratio phase separation occurs, resulting in a plasticizer-rich and a protein-rich domains, with water partitioning between the two domains. In such conditions, $\Phi_{w,eff}$ does not reflect the composition of the solvent around the proteins and an elevation of the denaturation temperature is observed due to a reduction in hydrogen bond density in the protein-rich domain. This condition resembles the preferential exclusion theory, with phase separation driven by both concentration and the molar volume density of effective hydroxyl groups $N_{OH,s}/v$ of the plasticizer or plasticizer mixtures.

By applying the Flory-Huggins theory for biopolymer melting, we have shown that transition temperatures T_{onset} and T_{peak} can be plotted against $\Phi_{w,eff}$ in the supplemented state diagram, allowing for prediction of egg white proteins denaturation in conditions relevant for food applications such as a pound cake. In a recent review (van der Sman & Renzetti, 2019) and in forthcoming papers, it will be demonstrated that the described principles provide quantitative guidelines for reformulation of pound cake towards sugar reduction, similarly as we recently demonstrated for a biscuit application (van der Sman & Renzetti, 2018).

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.

CRediT authorship contribution statement

Stefano Renzetti: Conceptualization, Formal analysis, Funding acquisition, Visualization, Methodology, Writing - original draft, Writing - review & editing. **Irene A.F. van den Hoek:** Data curation, Investigation. **Ruud G.M. van der Sman:** Methodology, Writing - review & editing, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2020.106034.

Supplementary Fig. S1. Supplemented state diagram for denaturation of egg white ovalbumin, soy glycinin and soy β-conglycinin as function of $\phi_{w,eff}$. The structuring pathway of pound cake batter during baking is indicated.

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