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Functionality of whey proteins covalently modified by allyl isothiocyanate. Part 2: Influence of the protein modification on the surface activity in an O/W system

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ABSTRACT

Allyl isothiocyanate (AITC) is a small electrophilic molecule which can be found in cabbage after degradation of glucosinolates. The covalent attachment of AITC to whey protein isolate (WPI) was previously reported to increase their hydrophobicity and structural flexibility at acidic pH values. It is thus hypothesized, that the o/w interface adsorption behaviour and interfacial structure will be altered. To further understand the effect of the AITC-modification on the emulsifying capacity, adsorption kinetic and interfacial properties of unmodified and modified WPI were investigated at the o/w interface.

The WPI-modification resulted in a significantly increased surface adsorption kinetic and a lower equilibrium interfacial tension at acidic pH values. The ratio of α-lactalbumin (ALA) and β-lactoglobulin (BLG) at the oil droplet surface differed between unmodified and modified WPI (modBLG > ALA+modALA). Several layers of loosely attached proteins were evident on the oil droplet surface in all modified WPI emulsions. The hyperfine coupling (a_N) of the EPR spin probe TB residing at the oil droplet surface reflected an increased hydrophobicity of the modified proteins. A lower order parameter (S) in the lipid phase of the modified WPI emulsions gave evidence of an altered alignment of the modified proteins at the interface, probably sticking into the oil phase.

In conclusion, the present results indicate that the increased flexibility and hydrophobicity of the modified whey proteins, especially of modified BLG, surpass the surface activity of unmodified ALA in acidic pH values.

1. Introduction

Whey proteins are frequently used as emulsifying agents because of their high surface activity and availability. Their surface activity is mediated by the whey proteins adsorbing at the liquid interface where they unfold and form a stabilizing layer against oil droplet coalescence, droplet flocculation or creaming (Dickinson, 1999, 2001; Zhai et al., 2011). The stability of emulsions is dependent on the structural properties of this adsorbed protein layer (Zhai et al., 2011). The protein adsorption and the accompanying structural rearrangements are also strongly dependent on the pH value of the aqueous solution (Audebrand, Ropers, & Riaublanc, 2013; Dickinson, 2001; Lam, Ricky & Nickerson, 2015; Masson & Jost, 1986), because the pH value determines the protein's charge, its solubility, flexibility and aggregation behaviour (Das & Kinsella, 1989; Kim, Cornec, & Narsimhan, 2005).

The predominant protein in whey protein isolate (WPI) is β-lactoglobulin (BLG), wt. 80%, followed by α-lactalbumin (ALA), wt. 20%. At neutral pH values, BLG is the main protein adsorbed at the liquid interface in WPI, because of its flexible structure

Abbreviations: ACN, Acetonitrile; ALA, alpha-lactalbumin; modALA, modified alpha-lactalbumin; AITC, allyl isothiocyanate; a_N, hyperfine coupling; BSA, bovine serum albumin; C_{ini}, initial protein concentration; C_{ser}, protein concentration in serum; DLS, dynamic light scattering; EPR, electron-paramagnetic-resonance; E'_d, dilatational elastic modulus; E''_d, dilatational viscous modulus; FD, freeze dried; IEP, isoelectric point; BLG, β-lactoglobulin; modBLG, modified β-lactoglobulin; γ, interfacial tension; γ0, initial interfacial tension; γeq, equilibrium interfacial tension; S, order parameter; So, oil droplet Surface; SLS, static light scattering; Γ, protein surface adsorption; TB, 4-hydroxy-TEMPO benzoate; TFA, Trifluoracetic acid; τc, rotational correlation time; UF, centrifugal ultrafiltration; V_c, volume continuous phase; V_{oil}, volume oil phase; WPI, whey protein isolate.

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(Shimizu, Saito, & Yamauchi, 1985). The globular BLG loses its intramolecular β-sheet folding in favour of α-helix structures after adsorbing at the liquid interface (Audebrand et al., 2013; Zhai et al., 2010). At acidic pH values, however, ALA adsorption prevails over BLG in WPI, because ALA is in the molten globule state at pH 2. This state is characterized by high structural flexibility and hydrophobicity (Matsumura, Mitsui, Dickinson, & Mori, 1994). The flexibility and hydrophobicity of the protein itself is thus an important factor for its surface activity. Generally, WPI exerts a low emulsifying capacity at acidic pH-values because of the structural rigidity of BLG at these conditions (Keppler & Schwarz, 2017; Shimizu et al., 1985).

Attempts are made to enhance the emulsifying capacity of WPI, especially of BLG, at acidic pH values to expand its versatility in a broad range of food products. One such approach is to increase the hydrophobicity by introducing hydrophobic alcohols through esterification (Halpin & Richardson, 1985) or by covalent addition of polyphenols to proteins (Liu, Sun, Yang, Yuan, & Gao, 2015).

Another approach is the cleavage of intramolecular disulphide bonds of BLG to increase the structural flexibility (Shimizu et al., 1985). We previously found, that the covalent modification of WPI with hydrophobic allyl isothiocyanate (AITC) (for example from *brassica*) resulted in an increased hydrophobicity at pH values 2 to 7 (Keppler et al., 2017) as well as a cleavage of disulphide bonds in BLG (Keppler, Koudelka, Palani, Stuhldreier et al., 2014; Keppler, Koudelka, Palani, Tholey, & Schwarz, 2014). The latter lead to a less rigid folding of the protein only at acidic pH values (Keppler et al., 2017). These alterations of the physicochemical properties of the whey proteins were then found to enhance the emulsifying capacity of a rapeseed oil in water emulsion at acidic pH values only, whereas the creaming stability was enhanced at pH 2 *vs.* pH 7 (Keppler & Schwarz, 2017). The underlying mechanisms of the alterations are complex combinations of several factors (*i.e,.* charge, surface hydrophobicity, flexibility, protein adsorption competition), which in turn are pH-dependent.

In order to better understand the complexity of the underlying mechanisms of the surface properties of modified vs. unmodified WPI in emulsions the systems where investigated using various physicochemical methods characterizing the interfacial adsorption behaviour, the composition of the oil droplet surface and properties of various microenvironments in o/w emulsions.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade: Allyl isothiocyanate (AITC >95%) and 4-hydroxy-TEMPO benzoate (TB) were obtained from Sigma Aldrich (Seelze, Germany), whey protein isolate (WPI) (BiPRO, Davisco Foods International, Inc., Eden Prairie, US) with 97.7% protein and 75% BLG in dry matter was used for modification. Highly standardized rapeseed oil (Canola oil, with the main fatty acids being C18:1 n-9c (59.45%), C18:2 n-6c (18.76%) and C18:3 n-3 (8.34%)) was purchased in the local supermarket.

2.2. Sample preparation

The preparation of the samples is described in detail in the previous paper (Keppler et al., 2017): Briefly, 25 g L^{-1} WPI was solved in Milli-Q water (18 mΩ) for several hours; set to pH 9 and 1 g L^{-1} w/w AITC was added directly. The mixture was stirred over night at room temperature to assure complete binding (Keppler, Koudelka, Palani, Stuhldreier et al., 2014; Keppler, Koudelka, Palani, Tholey et al., 2014). The modification resulted in a decrease of the pH value to ~7, the samples were then lyophilized in order to remove any unbound AITC and termed "modified". In addition, WPI, which was subjected to the same process as described above, but without addition of AITC, was also freeze dried, to act as reference material as "unmodified" WPI. For lyophilisation, all samples were frozen at −20 °C (to prevent cold denaturation) for 24 h and freeze dried using a laboratory freeze dryer (Gamma 1-16 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and then stored in tightly sealed flasks at 0 °C in the dark until further use.

2.3. Determination of the protein composition and modification level by HPLC

Protein analysis in the aqueous phase was conducted by RP-HPLC as described in (Clawin-Rädecker, Kiesner, & Schlimme, 2000; Keppler, Sönnichsen, Lorenzen, & Schwarz, 2014), using an Agilent 1100 Series HPLC with a diode-array detector and PLRP-S column (300 Å, 8 μm, 150 × 4.6 mm, Agilent Technologies, Santa Clara, USA). Standards of pure BLG genetic variant AB and ALA, as well as AITC modified BLG genetic variant AB and ALA were run for calibration. For the latter, however, a superposition with unmodified and modified BSA occurred. The resulting concentration determined for modALA of approximately 4.2 g l^{-1}, was overestimated by factor 2.95. This was determined by calculating the difference of the ALA concentration in the unmodified WPI and the remaining ALA in the modified WPI. The overestimation factor was used in the following to correct the concentration of the modified ALA in the surface adsorption analyses.

2.4. Interfacial properties of modified and unmodified WPI

2.4.1. Interfacial tension measurements

The interfacial tension between stripped rapeseed oil and aqueous protein solutions ("unmodified" or "AITC-modified" WPI (prepared in ultrapure water, adjusted at various pH values (2, 4, 6 or 7) with HCl 0.1 M or NaOH 0.1 M) was measured with a drop tensiometer (Tracker, Teclis, Longessaigne, France) used in rising drop configuration. Rapeseed oil was stripped by direct mixing with magnesium silicate powder (Florisil, Sigma Aldrich) to remove surface-active impurities, as described previously (Berton, Genot, & Ropers, 2011). Interfacial tension measurements were conducted for at least 2 h at room temperature. The interfacial tension (γ) was determined by analysing the profile of the oil drop immersed in the protein solution and using the Laplace equation (Benjamins, Cagna, & Lucassen-Reynders, 1996). The equilibrium interfacial tension (γ_{eq}) was determined by calculating the average interfacial tension of 20 measurements points after 2 h.

In order to quantify the different regimes (*i.e.,* rate constants K (1 s^{-1}) and the percentage contribution of each regime on the interfacial tension decrease), the dynamic interfacial tension kinetic was analyzed with Prism 6 (GraphPad, San Diego, CA) using non-linear regression for a three phase (Fast, Medium and Slow) exponential decay [Eq. (1)].

$$\gamma_{(t)} = \gamma(t)_{Fast} + \gamma(t)_{Medium} + \gamma(t)_{Slow} + \text{Plateau} \quad [1]$$

Where:

$$\gamma(t)_{FAST} = (\gamma_0 - \text{Plateau})\%_{FAST} 0.01 \exp(-K_{FAST} t) \quad [1a]$$

$$\gamma(t)_{SLOW} = (\gamma_0 - \text{Plateau})\%_{SLOW} 0.01 \exp(-K_{SLOW} t) \quad [1b]$$

$$\gamma(t)_{MEDIUM} = (\gamma_0 - \text{Plateau})(100 - \%_{FAST} - \%_{SLOW}) 0.01 \exp(-K_{MEDIUM} t) \quad [1c]$$

Where $\gamma(t)$ is the interfacial tension at time t; γ_0 is the interfacial tension at the start value t = 0; Plateau is the γ at infinite time;