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Functionality of whey proteins covalently modified by allyl isothiocyanate. Part 1 physicochemical and antibacterial properties of native and modified whey proteins at pH 2 to 7

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Functionality of whey proteins covalently modified by the cabbage compound allyl isothiocyanate.

Part 1.

Physicochemical and antibacterial properties of native and modified whey proteins at pH 2 to 7

Highlights

- Whey protein isolate was covalently modified with allyl isothiocyanate
- The adducts showed increased hydrophobicity at pH 2, 4 and 6
- The protein β-sheet content increased
- Interfacial tension was reduced
- Antimicrobial activity of WPI was not significantly influenced

Chemical compounds studied in this article

Allyl isothiocyanate (PubChem CID: 5971), beta-Lactoglobulin (PubChem CID: 142-148)

Abbreviations

ACN, acetonitrile; ALA, α-lactalbumin; ANS, 8-anilinonaphthalene-1-sulfonic acid; AITC, allyl isothiocyanate; ATR-FT-IR, attenuated total reflection Fourier-transform-infrared-spectroscopy; A.U., arbitrary units; BSA, bovine serum albumin; DLS, dynamic light scattering; E'_d, dilatational elastic modulus; E''_d, dilatational viscous modulus; FD, freeze dried; FSD, Fourier self-deconvolution; GC, gas chromatography; IEP, isoelectric point; IFT, indirect Fourier Transform; β-LG, β-lactoglobulin; MIC, Minimum inhibitory concentration; n.tr.b., normalized to transmitted beam; P, protein; RT, retention time; SAXS, small angle X-ray scattering; TFA, trifluoroacetic acid; WPI, whey protein isolate.

Abstract

Whey protein isolate (WPI) (~70 % β-lactoglobulin (β-LG)) is frequently used in foods as natural emulsifying agent; however, at an acidic pH-value its emulsification capacity is strongly reduced. The covalent attachment of natural electrophilic hydrophobic molecules to WPI proteins is a promising method to change the physicochemical properties of WPI in favour of a higher functionality at acidic pH.

In the present study different concentrations of the cabbage compound allyl isothiocyanate (AITC) were covalently bound to WPI, and the related changes in physicochemical properties (charge, aggregation, surface hydrophobicity and secondary structure) were monitored over a wide pH range (pH 2 to 7). Additionally, the antibacterial activity before and after AITC modification against different strains of *S. aureus* and *E. coli* was assessed.

The results showed that both whey proteins β-LG and α-lactalbumin (ALA) were modified by AITC. This modification remained stable during pH adjustment. Unbound AITC was successfully removed by the lyophilization process, which reduced the strong odour of the volatile AITC. The hydrophobicity of the modified WPI increased significantly and the protein´s secondary and tertiary structure was altered. A shift of the isoelectric point towards acidic conditions was observed. The interfacial tension at acidic pH-values 2 and 4 was significantly reduced. These effects were less pronounced at pH 6 and 7. The relatively mild antibacterial effect of native WPI was not significantly influenced by addition of AITC.

Thus, a higher surface activity of the AITC-modified WPI at acidic pH compared to native WPI was evident, mediated by increased hydrophobicity of the WPI as well as increased protein backbone flexibility.

1. Introduction

Commercial whey protein isolate (WPI) is used in various dispersions in food, feed or cosmetic industry due to its foaming and emulsifying capacity (Dickinson, 2001; Gulzar, Lechevalier, Bouhallab, & Croguennec, 2012; Lam, Ricky & Nickerson, 2015). The main protein in WPI is β–lactoglobulin (β-LG) which is a globular amphiphilic molecule comprising 162 amino acids and one sulfhydryl group partially buried. Its surface activity is mainly mediated by adsorbing at an oil/water or air/water interface followed by partial unfolding and alignment of hydrophobic residues towards the oil or air phase (Dickinson, 1999, 2001; Zhai et al., 2011). A smaller fraction of proteins in WPI consists of α-lactalbumin (ALA), which has 123 amino acid residues (Brew, Castellino, Vanaman, & Hill, 1970) and has a lower emulsifying capacity. To further enhance the surface activity of whey proteins like β-LG the physicochemical properties are often changed by inducing high pressure or thermal treatment – leading to partial or complete unfolding of the protein whereupon buried hydrophobic amino acid residues get access to the surface and protein backbone flexibility increases. However, depending on the protein concentration and energy input, the unfolding can subsequently lead to protein aggregation and cross-linking through reactive thiol groups, which then negatively affects the flexibility and surface activity of the protein (Lam & Nickerson, 2015). Another method to modify the surface activity is the esterification of β-LG with alcohols, which increases the hydrophobicity and flexibility of the protein and was found to increase the emulsifying capacity of β-LG at acidic pH (Halpin & Richardson, 1985; Sitohy, Chobert, & Haertlé, 2001).

A novel approach to influence the functionality of proteins is the covalent addition of an electrophilic plant compound (Rade-Kukic, Schmitt, & Rawel, 2011; Keppler et al., 2014a; Keppler, Koudelka, Palani, Tholey, & Schwarz, 2014b). Natural compounds that are known to form covalent adducts with thiols and amines are organosulfur thiosulfinates (from *allium*) (Wilde, Keppler, Palani, & Schwarz, 2016a, 2016b, Wilde et al., 2016c) and thiocyanates (from *brassica*) (Keppler et al., 2014a; Keppler et al., 2014b; Rade-Kukic et al., 2011), as well as α,β-unsaturated aldehydes (from *cinnamomum*) (Björkman, 1973; Kawakishi & Kaneko, 1987; Murthy & Rao, 1986a; Rawel, Kroll, & Schröder, 1998). Such compounds readily interact with deprotonated amine and/ or thiol groups of proteins at elevated pH (> 8).

The isothiocyanate allyl isothiocyanate (AITC) can be found in broccoli, cauliflower, Brussels sprouts, cress and cabbage and is produced by the enzyme myrosinase through hydrolysis of glucosinolate sinigrin upon damaging of plant cells (Fimognari, Turrini, Ferruzzi, Lenzi, &

Hrelia, 2012; Palani et al., 2016). All isothiocyanates have the R-N=C=S functional group in common. The electrophilic C-atom forms thiocarbamoyl adducts with thiols, or thiourea adducts with amines (Nakamura, Kawai, Kitamoto, Osawa, & Kato, 2009). The AITC conjugation is a time, pH-value and concentration dependent reaction: the α-amino group of lysine 1 and ε-amino group residues of leucines of β-LG are primarily reacting with AITC at elevated pH upon deprotonation (Hanschen et al., 2012; Nakamura et al., 2009). The AITC reaction with amino acid residues of β-LG was found to be controllable to a certain degree by changing the protein/AITC ratio, revealing the n-terminal part of the protein as most reactive (Keppler et al., 2014a; Keppler, et al., 2014b).

The covalent modification of milk protein β-LG by AITC was previously described in detail (Keppler et al., 2014a; Keppler et al., 2014b; Rade-Kukic et al., 2011): The addition of AITC to β-LG influenced the proteins folding, structure and consequently its physicochemical properties – for example β-LG was found to interact positive-cooperatively with AITC – resulting in a loosening of the protein folding (Keppler et al., 2014a). A higher flexibility, as well as the attachment of hydrophobic residues could increase the amphiphilic character of whey proteins and may potentially change the emulsifying capacity, especially in acidic environment, where structural rigidity of β-LG reduces its surface activity.

In order to further understand the functionality of AITC-protein complexes, the covalent modification of WPI with AITC and its influence on protein physicochemical properties were studied over a wide pH range (i.e. pH 2, 4, 6 and 7). WPI was chosen instead of pure β-LG as it is commonly used in food industry. Fresh prepared, as well as freeze dried WPI and WPI-AITC conjugates were characterized for their interaction behaviour, followed by analyses of charge (zeta potential), aggregation (dynamic light scattering), surface hydrophobicity (ANS fluorescence), interfacial properties (drop tensiometer) and secondary/tertiary structure (ATR-FT-IR). Additionally, the antibacterial effect of the modified WPI was assessed in comparison to that of unmodified WPI. Effects of the AITC modification on emulsifying capacity of WPI in o/w emulsions at pH 2, 4, 6 and 7 will be described in detail in another study.

2. Materials and Methods

2.1. Materials

All chemicals were of analytical grade: Allyl isothiocyanate (AITC >95 %) and 8-anilino-1-naphthalenesulfonate (ANS) 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% β-LG in dry matter was used for modification.

2.2 Sample preparation

2.2.1. Covalent modification. 25 g L^{-1} WPI was solved in MilliQ water for several hours to assure complete hydration. All denatured proteins were removed by setting the pH-value to 4.6 with 3 M HCl. After 1 hour equilibration the samples were centrifuged (2000 *g, 5 min) to remove precipitated (i.e. denatured) protein. The supernatant was filtered and set to pH 9 using 1 M NaOH. AITC modification was conducted at room temperature in 4 different concentrations: 0.5 g L^{-1}, 1 g L^{-1}, 2 g L^{-1} or 3 g L^{-1} w/w per 25 g L^{-1} WPI (i.e. 0.2 g L^{-1}, 0.4 g L^{-1}, 0.8 g L^{-1} and 1.2 g L^{-1} per 10 g L^{-1} WPI). These concentrations correspond to an approximate molar ratio of 1:3, 1:6.5, 1:13 and 1:20 M proteins per M AITC. The respective amount of AITC was directly added to the protein solution (ca. 10 to 15 minutes after setting the pH-value to 9) and stirred over night at room temperature to assure complete binding as described by Keppler et al., (2014a). The high pH value 9 was chosen to assure deprotonation of free amine and thiol groups, so that a binding reaction with AITC is possible. The addition and binding reaction of AITC directly caused a decrease of the pH-value (to a final pH of ~7 after complete binding); preventing base denaturation of the proteins, which was found to partly occur after 10 h stirring at pH 9 (Boye, Ismail, & Alli, 1996).

2.2.2. Lyophilization. The samples were set to pH 7 and lyophilized to remove any unbound AITC and termed “modified”. Additionally, WPI, which was subjected to the same precipitation process as described above, but without addition of AITC was also freeze dried, to act as reference material as “native” 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. The concentration of remaining AITC in the modified WPI samples after the lyophi lization process was determined by headspace GC as described below. For this 50 g L^{-1} of the modified WPI was

dissolved in 1 ml Milli Q water directly inside the closed GC vials for at least 2 h, before the analysis.

2.3. Analysis of the covalent interaction between AITC and WPI

2.3.1. Analysis of AITC binding by HPLC. 1 g L^{-1} native or AITC-modified freeze dried WPI at pH 7 was dissolved in Milli Q water and analyzed by RP-HPLC as described by Keppler et al. (Keppler, Sönnichsen, Lorenzen & Schwarz, 2014c) using an Agilent 1100 Series HPLC with a diode-array detector and PLRP-S column (300 Å, 8 µm, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA). The injection volume was 20 µl at a flow rate of 1.0 ml/min and a column temperature of 40 °C using eluents A (0.1% (v/v) TFA in water) and B (0.1% TFA (v/v) in ACN). The elution used gradient steps of 35-38% B (1-8 min), 38-42% B (8-16), 42-46% B (16-22 min), 46-100% B (22-22.5 min) and 100-35% B (23-23.5 min). The detection wavelength was 205 nm. Standards and calibrations of pure β-LG AB, as well as ALA and BSA were run.

2.3.2. Analysis of AITC by headspace gas chromatography. Before the lyophilisation process several ml of each modified WPI at pH 7 were put aside for binding analysis in Headspace GC and termed "bound", as they represented the modified WPI. Additionally to these samples, WPI samples with "unbound" AITC were prepared as reference. For this, the WPI supernatant was set to pH 7 instead of pH 9, before addition of the same concentrations of AITC as described above. These samples were not stirred overnight, as at these conditions no binding occurred. The fraction of protein-bound AITC after protein modification was determined mathematically by subtracting the concentration of free AITC in the "bound" sample from concentration of free AITC in the "unbound" sample. Both concentrations were determined by headspace-GC as described by Keppler et al. (2014a). Briefly: All samples were diluted to 10 g L^{-1} protein and 1 ml of the samples were filled into headspace vials (20 ml) and incubated at 65 °C for 15 min prior to injecting 0.2 ml of the sample loop fill from the HP7694 headspace auto sampler to the GC HP 6890 (Agilent Technologies, Böblingen). The GC was equipped with a FID and the column was a DB-1701 column (Agilent J & W, 30 m x 0.321 mm), coated with a low-to mid-polar phase (bonded and cross-linked, 14%-Cyanopropyl-phenyl (-o-methylpolysiloxane)). Oven temperature was programmed from 45 °C, 7 min to 220 °C in 30 °C steps and with 3 min holding time. A split ratio of 2:1 was used and the split flow of the carrier gas was 2 ml/min. A calibration was conducted in triplicate by using different concentrations of AITC dissolved in MilliQ water, without protein addition.

196 **2.4. Surface hydrophobicity by ANS**

197 The hydrophobicity was measured as described in Lam & Nickerson (2015) using the external
198 fluorescence probe 8-anilino-1-naphthalenesulfonate (ANS). Freeze-dried native or AITC-
199 modified WPI (Modification: 0.4 g L^{-1} AITC per 10 g L^{-1} WPI) were adjusted to pH 2, 4, 6 or
200 7 before the final concentration was set to 0.3, 0.4, 0.5 and 0.6 g $L^{-1}$and again controlled for
201 pH-stability. 20 µl of 8 mmol L^{-1} ANS in water, adjusted to the respective pH of the WPI, was
202 added to 1.6 mL protein solution, vortexed and stored in the dark for 5 min. For control,
203 proteins without ANS were used and the blank was ANS in water without protein at the
204 respective pH-value. The fluorescence was measured at 390 nm excitation and 490 nm
205 emission wavelengths, using 2.5 nm slit width for all samples (and additionally a 5 nm slit
206 width for pH 6 and 7 samples) and a 1 cm quartz cuvette with 4 polished sides on a Varian
207 Cary eclipse fluorescence spectrometer (Varian Australia PTY, Ltd.). The net fluorescence
208 intensity was calculated by subtracting the control samples from the protein samples with
209 ANS at each protein concentration. The ANS fluorescence in dependence of the protein
210 concentration gave a straight line. The slope served as hydrophobicity index. All samples
211 were measured in triplicate.
212
213 **2.5. Influence of AITC binding on zeta potential and aggregation**

214 ***2.5.1. Zeta potential and dynamic light scattering***

215 Zeta potential and particle size by dynamic light scattering were measured using a Zetasizer
216 nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). WPI, native or AITC-modified
217 with AITC (Modification: 0.4 g L^{-1} AITC per 10 g L^{-1} WPI) and freeze dried at pH 7 was
218 dissolved in demineralized water for at least 1 h and either measured directly or adjusted to
219 pH 2, 4 or 6 using 1 M HCl. The WPI concentration was set to 10 g L^{-1} and all samples were
220 filtered through a 0.2 µm syringe filter to remove dust. For zeta-potential measurements, the
221 solutions at pH 2, 4, 6 and 7 were filled in clear disposable zeta cells (DTS1060C), which
222 were previously rinsed with ethanol and then cleaned with water. The equilibration time was
223 120 s; the dielectric constant was 78.5 and viscosity 0.8872 cP. To determine the isoelectric
224 point (IEP) of native and modified WPI the pH-value of the solutions was slowly reduced by
225 adding droplets of 0.1 M HCL and confirming the pH-value.

226 For dynamic light scattering (DLS) 1 ml of the filtered WPI solutions at pH 2, 4, 6 or 7 were
227 carefully pipetted into quartz cuvettes with square aperture and 4 clear sides to prevent any
228 foaming. Measurements were conducted using 173 ° backscatter and a refractive index of

1.45 for protein. All measurements were performed at 20 °C and measurements were done at least in triplicate.

2.5.2. Small angle x-ray scattering

SAXS experiments were performed at the BioSAXS Beamline P12 at PETRA III (EMBL/DESY, Hamburg, Germany). The energy of X-ray beam was 10 keV and the beam size 0.1 mm (V) × 0.2 mm (H). Sample to detector distance was 3.1 m and the q-range 0.03 to 4 nm^{-1}. Scattering patterns were measured using Pilatus 2M pixel detector. The samples (of approximately 20 µl volume) were injected into the sample cuvette using an automated liquid handling sample changer. In order to reduce the risk of the radiation damage the sample was moved slightly during the exposure. The temperature was 20 °C. For each measurement, we recorded 20 diffraction patterns originating from the same sample volume, using an exposure time of 0.045 s per frame. Before and after each SAXS measurement from the sample, a signal from H_2O was measured and used for background subtraction. The background-corrected SAXS data were used to calculate one-dimensional scattering curves by angular averaging. The data were corrected for transmitted beam. In order to verify that no artifacts had occurred as a result of radiation damage, all scattering curves for a recorded dataset were compared to a reference curve (typically the first exposure) before being integrated using an automated acquisition and analysis program (Franke, Kikhney, & Svergun, 2012). The range of the reciprocal space vector q was calibrated using diffraction patterns of silver behenate (Blanton, T.N., Barnes, C.L., Lelental, M., 2000).

2.6 Fourier-Transform-Infrared-Spectroscopy (FT-IR) using attenuated total reflection (ATR) measuring cell

The FT-IR measurements were performed with a Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany). The measurements were carried out in the mid-infrared, i.e. in the wave number range 4000-900 cm^{-1}. For the detection a LN-MCT Photovoltaic detector (Bruker Optics), cooled with liquid nitrogen, was used. The spectrometer specific software OPUS Version 65 (Bruker Optics) was applied for recording of the spectra, for monitoring and control of the spectrometer and the thermostat Haake DC 30 (K20) (Thermo Haake, Karlsruhe, Germany). FT-IR was performed using a BioATR-II measuring cell at 25 °C. 180 scans were made per analysis.

10 g L^{-1} modified or unmodified lyophilized WPI at pH 2, 4, 6 or 7 was prepared in demin. water, using 6 M or 1 M HCl, respectively. FT-IR measurements were conducted with these

protein solutions. All samples were corrected for demin. water. Based on the obtained spectra, Fourier self-deconvolution (FSD) was performed with a resolution enhancement factor of 3.0 1/cm and a bandwidth (full width at half height) of 18 1/cm using software OPUS Version 65, over the wavenumber range 1780-1480 cm^{-1}. The resulting bands in the amide I range (1730-1590 cm^{-1}) were integrated for the calculation and quantification of the structural components. In addition, 4^{th} derivatives were calculated from the measured and corrected spectra with the use of 9 smoothing points.

2.7. Determination of the minimum inhibitory concentration

Antimicrobial activity of modified and unmodified WPI, as well as of AITC against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* LMG 10147 and *S. aureus* Mu50 was assessed by a broth microdilution method (Cockerill, 2012). All strains were grown aerobically at 37°C on Tryptic Soy Agar (Oxoid, Drongen, Belgium). Inhibition of bacterial growth was assessed using flat-bottomed 96-well microtiter plates (TPP, Trasadingen, Switzerland). The inoculum was standardized at approx. 5 x 105 colony forming units/ml of Tryptic Soy Broth (Oxoid). Before adding bacteria, the pH of the growth medium was adjusted with 1M HCl. The plates were incubated at 37°C for 24 h and the optical density was determined at 590 nm using a multilabel microtiter plate reader (Envision Xcite, Perkin Elmer LAS, Waltham, MA).

2.8. Statistical analysis

All results were expressed as arithmetic means and standard deviation of replicated analyses. Data were analyzed by two-way ANOVA with Tukey´s multiple comparison test at a 0.05-level of significance. Statistics, as well as all figures were done using GraphPad Prism (version 6.05, GraphPad Software, San Diego, USA).

3. Results & Discussion

3.1. Characterization of the covalent modification

The physicochemical properties of conjugates consisting of allyl isothiocyanate (AITC) and whey protein isolate (WPI) were characterized in detail.

3.1.1. High pressure liquid chromatography. According to HPLC analysis the WPI used for protein modification contained 75.7±1.4 % β-lactoglobulin (β-LG) and 14.7±0.1 % α-lactalbumin (ALA) and < 4 % bovine serum albumin (BSA) and was completely native.

Figure 1 shows the HPLC chromatogram of freeze dried native WPI and WPI modified with different concentrations of AITC. The results indicate that the AITC modification occurred to both genetic variants of β-LG present in WPI (β-LG A and B, retention times (RT) = 19.1 and 19.9 min) to a similar extent, and to a minor extent also to ALA (RT = 10.6 min). The modified ALA (RT = 13.8 min) partly overlapped with the minor peak of residual BSA (RT = 13.7 min). The modified whey proteins became more hydrophobic – reflected in an increase of the retention time (RT > 20 min). For β-LG the occurrence of several new peaks clearly shows that the protein has several binding sites for AITC. It is assumed that the RT of the protein correlates with the number of bound AITC molecules.

According to the chromatogram both proteins, β-LG and ALA, showed a binding reaction with AITC – however, β-LG had multiple binding sites and ALA only approximately 1 binding site per M ALA. A maximum binding of β-LG was found to result in five bound AITC molecules per β-LG molecule (Keppler et al., 2014a; Keppler et al., 2014b). The ALA modification in WPI was expected as AITC was found to react with deprotonated amine- and thiol groups of β-LG, i.e. cysteine, leucine and lysine residues (Keppler et al., 2014a; Keppler et al., 2014b). AITC and other isothiocyanates were found to covalently interact at similar binding sites with various proteins, like mustard 12S protein, soy protein, ovalbumin, legumin, bovine serum albumin (Hernández-Triana, Kroll, Proll, Noack, & Petzke, 1996; Kroll, Noack, Rawel, Kroeck, & Proll, 1994; Kroll, Rawel, Kröck, Proll, & Schnaak, 1994; Murthy & Rao, 1986b; Rade-Kukic et al., 2011; Rawel et al., 1998; Vega-Lugo & Lim, 2009). Following this, the interaction of ALA with AITC as determined in **Figure 1** is consistent with these findings.

A higher hydrophobicity of a protein-AITC conjugate compared to the unmodified protein was also described by Rade-Kukic et al. (Rade-Kukic, Schmitt, & Rawel, 2011). Similarly,

Wilde et al. (2016a) found a higher hydrophobicity of β-LG-allicin and β-LG-diallyl disulfide conjugates using the same HPLC method as described above. The insertion of a hydrophobic molecule in the globular protein tends to reduce its hydrophilic surface patches.

3.1.2. Gas chromatography (GC). By determining the headspace/water equilibrium as described by Keppler et al. (Keppler et al., 2014a) the binding of AITC to WPI can be determined. The concentation of free AITC in the headspace is reduced exactly for the amount of bound AITC in solution, allowing to calculate the bound AITC to a known protein concentration. In a first experiment the stability of the AITC-WPI modification after adjusting to the different pH-values (pH 2, 4, 6 and 7) was controlled and assessed by GC analysis (results not shown). **Table 1** shows the concentration of free and WPI bound AITC after adjusting the samples to pH 7: The results confirm that the AITC concentration in the headspace of the WPI "unbound" samples corresponds to a concentration expected for completely unbound AITC, whereas the "bound" samples showed the reduced amount of AITC after interacting with the protein. The estimated molar concentration of covalently bound AITC increased with increasing addition of AITC to the WPI (i.e.1.19, 1.47, 1.62, 1.85 mM covalently bound after addition of 0.2, 0.4, 0.8 and 1.2 g L^{-1} AITC to 10 g L^{-1} WPI, respectively). Since WPI is a mixture of β-LG and ALA, the bound AITC per mol protein is given as sum of β-LG and ALA.

For freeze-dried WPI ("bound FD") the free AITC concentration was reduced by approximately one or two orders of magnitude (i.e. from $7.90E^{-2}$ g L^{-1} free AITC to $2.11E^{-3}$ g L^{-1} and from 0.249 to $7.75E^{-3}$ g L^{-1}, after addition of 0.2 and 0.4 g L^{-1} AITC to 10 g L^{-1} WPI, and from 0.599 g L^{-1} free AITC to $1.83E^{-2}$ g L^{-1} for 0.8 g L^{-1} AITC addition), confirming that the freeze drying process eleminated most of the unbound fraction of AITC from the sample. The sample with the highest molar ratio of AITC showed only marginal free AITC far below the detection limit, the reason for this is unknown. Removal of unbound allicin from covalently modified WPI by freeze drying or spray drying was reported recently (Wilde et al., 2016b). In accordance with the results from HPLC, an almost complete binding reaction (saturation of binding sites) was visible for the molar ratio 1:24 with n = 3.2 [mol/mol] binding sites. Assuming n = 5 binding sites for β-LG (Keppler et al., 2014a; Keppler et al., 2014b; Rade-Kukic et al., 2011) and n = 1 binding site on ALA (**Figure 1**), the percentual distribution of the proteins in the WPI (i.e. 75 % β-LG, 14 % ALA, < 4 % BSA) allows a maximum of approximately 3.7 to 3.9 binding sites. In summary, it can be concluded that the

increasing addition of AITC to WPI resulted in an increase of AITC molecules covalently bound to WPI proteins – confirming the results shown by HPLC in **Figure 1**. For the following experiments a concentration of 0.4 g L^{-1} AITC was chosen in order to archieve a high β-LG modification rate without using excessive AITC. This concentration also allowed a good removal rate of unbound AITC from the sample and the modification remained stable after adjusting the pH-value to 2, 4, 6 or 7.

3.2. Surface hydrophobicity by fluorescence probe ANS

The influence of AITC modification on the surface hydrophobicity of WPI was further analyzed by using freeze dried native "unmodified" or "modified" WPI with the anionic fluorescence probe ANS (1-anilino-8-naphtalenesulfonate). ANS interacts with hydrophobic patches on the protein surface through hydrophobic or electrostatic interactions (i.e. via ion pairing of the negatively charged sulfonate groups of ANS with positivelely charged amino acids of the protein (histidine, lysine, arginine), stabilized through van der Waals forces (Hawe, Sutter, & Jiskoot, 2007)). The binding of ANS to a protein results in an increase of the ANS fuorescence. Increased ANS binding correlates directly with increased hydrophobicity of a protein.

The hydrophobicity index was higher at pH 2 and 4 (hydrophobicity index ~836 and ~507) compared to pH 6 or 7 (~108 and ~129) (**Figure 2**). As the ANS fluorescence was very low at pH 6 and 7, these measurements were repeated with a higher slit width of 5 nm, but the difference between native and modified protein was still the same as in **Figure 2** (results not shown). The pH-dependent fluorescence intensity of ANS is caused by the positive charge of the proteins at acidic pH (compare zeta potential in **Figure 3**), which favours electrostatic complexes of negatively charged ANS with the protein. Similarly, the low fluorescence at pH 6 and 7 is a result of the negative charge of the WPI. Therefore, a direct comparison of the hydrophobicity index between the acidic and the neutral samples is not possible. However, the AITC-modification increased the ANS binding to the protein at each pH-value tested (hydrophobicity index ~ 919, ~600, ~164 and ~146 for pH 2, 4, 6 and 7 respectively) while the zeta potential of the native and the modified WPI was not significantly different at similar pH-values (with the exception of pH 6, see **Figure 3**). Additionally, the size of the native and modified WPI did not significantly differ at pH 2, 6 and 7 (**Table 2**), so that protein aggregation was not the cause for the differences in the ANS interaction between native and modified WPI. Accordingly, the AITC modification resulted in a higher surface

hydrophobicity of the modified WPI. The increase of the hydrophobicity index at pH 2 is also in accordance with the results from HPLC (**Figure 1**), where both eluents had a pH-value of ~ 2 and the retention time of the modified protein increased with increasing AITC binding. This increase of hydrophobicity could be caused by conformational differences between the native and the modified WPI (**cross ref 3.4.**) and/or be directly mediated due to the incorporation of hydrophobic AITC to the protein.

An intensification of the ANS fluorescence of WPI at acidic pH-value caused by stronger binding effects is consitent with literature (Alizadeh-Pasdar & Li-Chan, 2000; Das & Kinsella, 1989; Rade-Kukic et al., 2011). Further, a higher surface hydrophobicity index for pH 4 of AITC modified β-LG was also described by Rade-Kukic et al., (2011).

3.3 Aggregation and charge analysis

3.3.1. Dynamic light scattering and zeta potential

The aggregation behaviour (based on the hydrodynamic radius (R_H)) of the AITC-modified whey proteins at different pH-values is listed in **Table 2**; the corresponding zeta-potential is visible in **Figure 3**. According to the volume weighted size distribution of the freeze dried native WPI, a main fraction (peak I) comprised proteins with an increasing particle size from 1.55 nm at pH 2 (approx. 1 monomer), 2.13 nm at pH 4 (approx. 1-2 monomers), 2.98 nm at pH 6 and 2.97 nm at pH 7 (both approx. 2 monomers). The percentage distribution of this peak I fraction on the overall particle size was 100 % with the exception at pH 2, where a bimodal size distribution was evident and a peak II fraction (0.32 % volume) with a particle size of 9.44 nm occurred. This size corresponds to aggregates of approximately 250 monomers.

The observed R_H of WPI at pH 2 correlates well with the size of β-LG monomers reported in literature (Majhi et al., 2006; Sawyer & Kontopidis, 2000). The low particle size of the native WPI proteins at pH 2 was expected, since WPI consists mainly of β-LG (~75 %), which is reported to be monomeric at pH 2 (Fogolari et al., 1998). At pH 6 and 7 native β-LG is known to form mostly dimers (Gao et al., 2014; Keppler, Martin, Garamus, & Schwarz, 2015) – explaining the higher particle size of peak I with approximately 2.98 nm.

With the exception of pH 4, the size of AITC-modified WPI was not significantly different from that of native WPI. The main fraction sizes were 1.35 nm, 15.30 nm, 2.03 nm and 2.77 nm at pH 2, 4, 6 and 7, respectively. For the modified WPI, strong protein aggregation was evident at pH 4, with a main fraction percentage of 79 %. A second size fraction (peak II) of

approximately 16 % consisted of aggregates > 150 nm and a third fraction (peak III) of 1 % particles with > 2000 nm size occurred even in the filtered samples. As the samples exhibited turbidity before filtration, larger particles than the pore size of the filter have been excluded. The actual percentage distribution of these large particles is much higher than indicated here.

In contrast to the present results, where no significant size difference between native or modified WPI was evident, we recently reported protein monomerization after AITC modification of pure β-LG at pH 7 (Keppler et al., 2014). However, in the previous study highly purified β-LG was used instead of WPI and the unbound AITC was not removed by lyophilisation, which could be the cause for the differences in the determined size.
The observed aggregation and low solubility of the modified WPI at pH 4 can be explained by its changed IEP after modification: The IEP (zeta potential of ~0 mV) was reduced from pH ~4.99 for native WPI to pH ~4.68 for modified WPI (**Figure 3)**. At pH 2 the zeta potential increased to ~+25 mV for both WPI, and decreased at pH 7 to ~-25 mV. The IEP shift to a more acidic pH-value can be explained by AITC binding to free amine groups – thereby removing some positive charges from the overall charge of the protein. The addition of negative charges to β-LG by phosphorylation or by blocking of positive charges was also found to shift the IEP to more acidic pH-values (Sitohy, Chobert & Hertlé 1995). Pure β-LG was reported to have an IEP of 5.2, and ALA an IEP between 4.1 and 4.8 (Hunt & Dalgleish, 1994). Therefore the results for the determined IEP of the native and the modified WPI are consistent with literature.

3.3.2. Small-Angle–X-Ray Scattering (SAXS)

Experimental SAXS curves for native and modified WPI at pH 2, 4, 6 and 7 **(Figure 4)** were analyzed by applying the Indirect Fourier Transform (IFT) method (Glatter, O. *J. Appl. Cryst.* **1977**, *10*, 415-421.) using software GNOM (Svergun D.I., Semenyuk A.V., Feigin L.A. (1988). Acta Cryst., A44, 244-250). The resulting pair- distance distribution functions (p(r)) are shown as small inserts in **Figure 4**. They describe the paired-set of all distances between electrons in the protein. The maximum possible distance (D_{max}) can be seen by the intersection of the curves with the x-axis. All measurements were executed with 0.5 % and 1 % native and modified WPI at pH 2, 4, 6 and 7 to check the change of the protein structure and interactions among proteins with increasing concentration; however the normalized by protein concentration SAXS curves at intermediate and large q interval were similar for both concentrations.

At pH 2 the native and modified WPI showed almost similar p(r) functions, which almost symmetrical form correlates well with a globular protein (Keppler, Martin, Garamus, & Schwarz, 2015). The slightly higher p(r), as well as R hints at a more unfolded globular structure for the modified protein. At pH 4 the SAXS curves and p(r) functions of the native and the modified WPI differ significantly. Both functions for the native and the modified WPI exhibited an increase in the pair distances hinting at aggregation, but the large R of the modified WPI as well as the small increase at R= 10 nm hints at large elongated aggregates. Precipitated modified WPI was visible at the bottom of the measuring tubes at pH 4, which reveals that even larger protein aggregates were present. In contrast, the native WPI aggregates were still rather spherical. At pH-value 6 the SAXS curves of the native and modified WPI differed primarily in the scattering intensity (I(q)). The corresponding p(r) functions revealed some elongated aggregates at pH 6 for the native WPI, which were not observable for the modified WPI. The pair distance distribution functions of the native and modified WPI at 7 reflect spherical proteins with a higher size compared to the WPI at pH 2.

The described SAXS curves and p(r) functions clearly reflect the size changes of the WPI observed in DLS (Table 2) at the different pH-values. The aggregation of the modified WPI at pH 4 as well as the slight aggregation of the native WPI at pH 6 was caused by the different IEPs of the native and the modified proteins (with the IEP of modified WPI nearer to pH 4 and the native nearer to pH 6). The smaller protein size reflected at pH 2 hints at monomeric structures, whereas the protein sized at pH 7 were rather dimeric or trimeric.

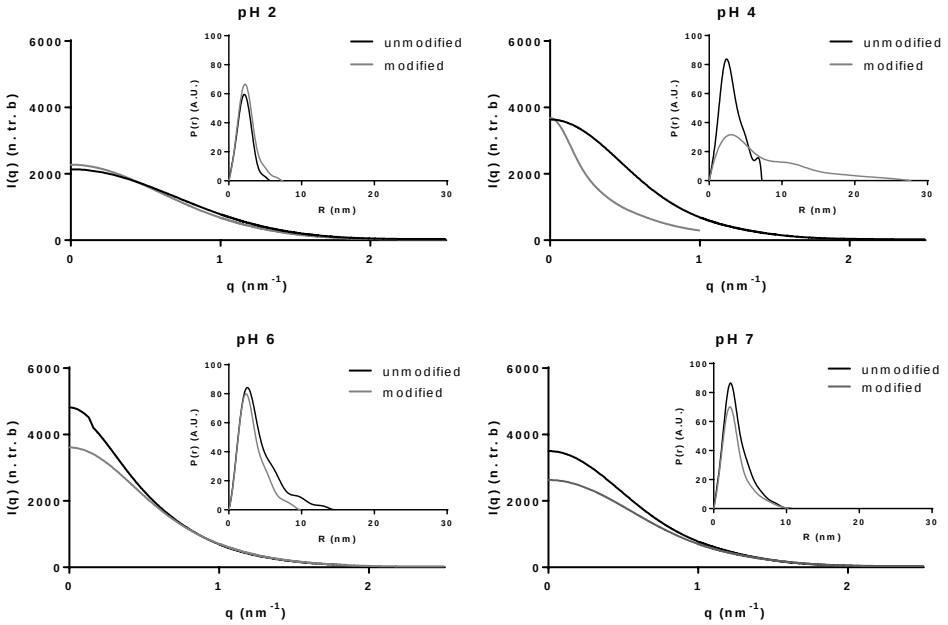

Figure 4 SAXS curves of 0.5 % native and modified WPI at pH 2, 4, 6 and 7. Small inserts show the corresponding p(r) functions. I, intensity; q, scattering vector amplitude; n.tr.b, normalized to transmitted beam.

The SAXS curves were analyzed for the apparent radius of gyration ($^{app}R_g$) [nm], the D_{max} required for the p(r) functions and the possible molecular weight [kDa] of the aggregates (**Table 3**). As expected, the $^{app}R_g$ again reflects the protein aggregation at the respective IEP of the proteins: The native protein exhibited increasing protein sizes in the order pH 2 < pH 7 < pH 4 < pH 6, whereas the $^{app}R_g$ of the modified WPI was in the order pH 2 < pH 7 < pH 6 < pH 4. The D_{max} was almost in similar order of the native and the modified WPI.

Table 3. Apparent radius of gyration, maximum distance, scattering intensity and R_g/R_H ratio for unmodified and modified WPI at pH 2, 4, 6 and 7.

pH value	Sample	Apparent radius of gyration ($^{app}R_g$) [nm]		Maximum distance (D_{max}) [nm]	Scattering intensity I(0) [n. tr.b]		R_g/R_H
pH 2	unmodified	1.69	±0.1[a]	5.8	1788	±4.35[a]	1.08
	modified	1.95	±0.1[a]	7.5	2259	±5.46[b]	1.45
pH 4	unmodified	2.61	±0.1[a]	10	3667	±8.12[a]	1.16
	modified	5.76	±0.60[b]	29	4399	±28.91[b]	0.38
pH 6	unmodified	3.06	±0.1[a]	14.4	4670	±12.49[a]	1.03
	modified	2.44	±0.1[b]	9	3536	±12.87[b]	1.20

pH 7	unmodified	2.36	±0.1[a]	10		3598	±12.10[a]	0.79
	modified	2.19	±0.1[a]	10		2726	±13.83[b]	0.79

492

493 It can be concluded that the native WPI proteins in the present experiment were mostly
494 present as monomers at pH 2, with only some minor dimer fractions. The observed Rg of 1.69
495 nm listed in **Table 3** corresponds well with the Rg of ALA monomers at pH 2 (i.e. acid
496 molten globular structure with 17.2 Å (1.72 nm) (Kataoka, Kuwajima, Tokunaga, & Goto,
497 1997) or ß-LG monomers at pH 2 (i.e.1.52 nm (Verheul, Pedersen, Roefs, & Kruif, 1999).).
498 At pH 4 the size of the native WPI proteins in the present study increased to that of ß-LG or
499 ß-LG/ALA dimers or trimers. The R_g of a dimeric ß-LG A in buffer pH 7 was reported to be
500 between ~2.1 nm (Panick, Malessa, & Winter, 1999) and 2.36 2.36 nm (Verheul, Pedersen,
501 Roefs, & Kruif, 1999), depending on the buffer system used. The high $^{app}R_g$ of native WPI at
502 pH 6 reflects some trimers, and the $^{app}R_g$ at pH 7 corresponds again to dimers. These results
503 are not only in accordance with literature but also verify the results obtained for the R_H by
504 DLS (**Table 2**).

505 The modified WPI has a higher $^{app}R_g$ at acidic pH 2 and 4 compared with the native WPI. The
506 reason could be that the WPI modification results in a more loose protein folding which
507 increases the Rg. Similar finding were reported for ALA, which Rg increased from 1.58 to
508 1.72 nm after forming a molten globule at pH 2 (Kataoka, Kuwajima, Tokunaga, & Goto,
509 1997). A molten globule like structure of the AITC modified ß-LG was suggested at pH 4 by
510 CD measurements (Rade-Kukic, Schmitt, & Rawel, 2011). Additionally to that, the shifted
511 IEP of the modified WPI lowers the solubility at pH 4 and results in aggregation. At pH 6 and
512 7 the $^{app}R_g$ was in the range of ß-LG or ß-LG/ALA dimers, possibly some trimers. The results
513 are again in accordance with the size determination listed in **Table 2**.

514 Both the Rg (determined by SAXS) and the R_H (determined by DLS) are measures of protein
515 dimensions. The R_H reflects the size of a protein compared to a perfect sphere and contains
516 information on protein solvation, whereas the Rg gives information about the shape and
517 structure of a "dry" protein. The Rg/R_H ratio of native WPI was 1.08, 1.16, 1.03 and 0.79 for
518 pH values 2, 4, 6 and 7 respectively. The modified protein exhibited a higher ratio at pH 2 and
519 6 with 1.45 and 1.20. At pH 7 the ratio was similar to that of native WPI (i.e. 0.79). And near
520 the IEP at pH 4 the ratio was surprisingly low with 0.38. For a perfect sphere the Rg/R_H is
521 ~0.77 (Tande, Wagner, Mackay, Hawker, & Jeong, 2001) and a random coil polymer in a

good solvent has ~1.78 (Burchard, 1992). According to this interpretation the WPI modification with AITC resulted in a more lose protein folding at pH 2 and 6. The protein folding and solubility was not influenced at pH 7 and was relatively close to that of a globular object. The low ratio near the IEP was probably caused by the high protein aggregation. Similar observations with a R_g/R_H ratio below 0.77 were suggested to be caused by sample impurities such as aggregates. These aggregates contribute strongly at the low q (large length scale) of the DLS experiments, but not at the q-values (smaller length scale) from SAXS analyses (Moitzi et al., 2011).

3.4. Protein folding (ATR-FT-IR)

3.4.1. Amide I band. Attenuated total reflection (ATR) Fourier transform infrared spectroscopy (FT-IR) was used to analyse the protein folding in water at pH 2, 4, 6 and 7. ATR was chosen due to the stability of the measuring cell at acidic conditions. **Table 4** lists the mean % of distinct peak areas for band frequencies of the amide I region of native and modified WPI, obtained by Fourier self-deconvolution of the measured and corrected spectra. The amide I reflects mainly C=O stretching vibration of the peptide group and can be used to assess the secondary structure of proteins (Pelton & McLean, 2000). For the present study a simple model was used applying group frequency wavenumber around 1686 cm^{-1} for turns and bends (Qi et al., 1997), 1658 cm^{-1} for alpha helical content and 1632 cm^{-1} for antiparallel beta-sheet content (Fang & Dalgleish, 1997)). The spectral peak intensity (positive or negative) correlates with the respective content of the secondary structural element (Ioannou, Donald, & Tromp, 2015). At pH 7 unmodified native WPI had approximately 21.31 % turns and coils, 36.69 % alpha helix content and 42.0 % β-sheet concentration. The secondary structure of native WPI was significantly influenced by the pH-value of the solution – especially at pH 4, where a significant loss of beta-sheet content and increase of alpha helix was evident. Further, a significant loss of coil and turns (1686 cm^{-1}) was also evident with increasing acidity of the solution. The secondary structure of the modified WPI was significantly altered from the native WPI in beta-sheet and alpha-helical content at all pH-values – resulting in a markedly increase of beta-sheet structures in exchange of alpha-helix content. This effect was especially evident at pH 7 and 6, and less so at pH 4 and 2.

According to literature, WPI at pH 7 was found to have 16 % alpha-helix, 33.6 % beta-sheet, 20.9 % turns and 29.6 % unordered structure (Rade-Kukic et al., 2011). Calculated without

555 unordered structure elements this would amount for 21.3 % alpha-helix, 44.8 % beta-sheet
556 and 27.8 % coils. The difference to the present results can be explained by the significantly
557 higher concentration of ALA in the WPI used (26.7 % instead of 4 %). At pH 4 an increase of
558 alpha-helical structure was reported after AITC modification of β-LG in exchange for beta-
559 sheet elements (Rade-Kukic et al., 2011). The increase of alpha-helical content at pH 4 was
560 also evident in the present results. However, in literature the AITC modification resulted in a
561 decrease of beta-strands at pH 7 – probably caused by dimer separation followed by partial
562 collapse of the beta-barrel (Rade-Kukic et al., 2011) - whereas in the present study the beta-
563 sheet content increased significantly with AITC modification. Further, according to DLS no
564 dimer separation was observed for the modified WPI – therefore the beta-barrel structure of β-
565 LG remained intact in the present study. This could be due to different measurement methods
566 applied (ATR-FT-IR versus circular dichroism), as well as due to different WPI used,
567 different AITC modification levels and due to the lyophilization step applied in the present
568 experiments. Additionally, Wilde et al. (2016c) also reported an increase of beta sheet
569 structure following the covalent modification of β-LG thiol group of WPI by allicin.
570
571 ***3.4.1. Amide II and side chain absorption.*** A list of signal maxima shifts in the amide II
572 region obtained after the 4th derivative of the spectra are summarized in **(Table 5**). Generally,
573 a shift from higher to lower wavenumber for the 1550 cm^{-1} band frequency was observed after
574 modification of the WPI at all pH-values tested (i.e. from 1552 cm^{-1} to 1548 cm^{-1} at pH 2,
575 from 1551 cm^{-1} to 1545 cm^{-1} at pH 4, from 1551 cm^{-1} to 1545 cm^{-1} at pH 6 and from 1550 cm^{-1} to 1549 cm^{-1} at pH 7). A shift of amide II signals at 1550 cm^{-1} towards a lower frequency
577 band generally correlates with increased β-sheet structure in proteins (Litvinov, Faizullin,
578 Zuev, & Weisel, 2012), which fits well with the present results of the amide I frequency band.
579 A second signal maximum at 1555 $cm^{-1,}$ at 1557 cm^{-1} or at 1556 cm^{-1} was observed only for
580 the modified WPI at pH 2, 4 and 6 respectively, confirming conformational changes in the
581 amide II region (C-N). The appearance of a positive peak at 1555 cm^{-1} after a covalent
582 modification of a protein amine group was also reported elsewhere (Almeida et al., 2014; Pan,
583 Cui, He, Gao, & Zhang, 2006). It is unclear why this 1555 cm^{-1} signal maximum was not
584 observed in the modified protein at pH 7, since GC and HPLC results (**Table 1** and **Figure 1**)
585 confirm that the AITC modification occurred at all pH-values tested.
586
587 The 1517 cm^{-1} frequency band increased from 1516.93 cm^{-1} at pH 7 to 1517.62 cm^{-1} at pH 6,
588 then decreased again to 1517.59 cm^{-1} at pH 4. At pH 2 the band frequency was lowest with

1515 cm^{-1}. Although WPI is a rather complex sample with several signal overlay, these specific signals could be caused by tyrosine. Tyrosine ring vibration in proteins is known to occur approximately between 1515 cm^{-1} (Reinstädler, Fabian, Backmann, & Naumann, 1996) to 1517 cm^{-1} (Ioannou et al., 2015) and a shift in its wavelength was found to reflect the local environment of tyrosine. It can therefore be used as some kind of senor for solvent accessibility (Barth, 2007; Reinstädler et al., 1996). For example a change towards a lower frequency band was correlated with a more lipophilic environment in a protein (Gaussier, Lavoie, & Subirade, 2003) or reduced solvent exposure (Meersman, Smeller, & Heremans, 2002), which is similar to the frequency shift observed for native WPI at pH 2 in the present study. At pH 7, where the tyrosine frequency of the native WPI was lowest, a beginning deprotonation of the tyrosine residues could be possible (although the pka of the tyrosines in β-LG is around 10) (Barth, 2000). Following this the consecutive signal shifting to a lower wavelength may indicate a more lipophilic microenvironment of tyrosine in native WPI at pH 2 (and to a minor extent also at pH 7), than at pH 4 and 6. These findings are consistent with the results of a higher overall charge at pH 2 and pH 7 (**Figure 3**), resulting in a more rigid globular protein folding especially at pH 2, thereby burying tyrosine in the hydrophobic core of the globular protein and reducing its solvent accessibility. A lower backbone flexibility of whey proteins at pH 2 is also consistent with its reduced emulsifying capacity at this pH-value, hindering protein unfolding and interconnections and is also reflected in the monomerization behaviour observed at this low pH-value (**Table 2**). Interestingly, the described tyrosine frequency shift towards a lower frequency band at pH 2 was no longer evident after protein modification. Here, the tyrosine vibration was lowest at pH 7 with 1515.44 cm^{-1} (showing strong hydrogen bonding) and increased to 1517.01 cm^{-1} at pH 6. A further increase was observed at acidic pH 4 and 2 with 1517.82 cm^{-1} and 1517.81 cm^{-1}, respectively.

In comparison to the native WPI, the AITC modification shifted the posible tyrosine band position towards lower wavenumbers at pH 7 and pH 6 and slightly towards a higher wavelength number at pH 4. A significant increase of the band frequency was observed at pH 2 (increased solvent accessibility). The results at pH 2 and 4 could be evidence for a less rigid protein folding of the modified WPI at these pH-values, increasing the contact of tyrosine with water molecules. This is also partly reflected in the ANS fluorescence (**Figure 2**) which was strongest at pH 2 and 4 after protein modification – suggesting that protein unfolding at these pH-values increased the solvent accessibility of hydrophobic residues– thereby

623 increasing ANS binding to these patches. However, ANS binding was still higher in the
624 modified WPI at pH 6 (although less pronounced) and not significantly at pH 7; therefore the
625 hydrophobicity of the modified WPI was also mediated by the incorporation of hydrophobic
626 AITC molecules itself.
627
628 **3.6. Antibacterial activity**

629 ***3.6.1. Antibacterial acitivty of native and modified WPI.*** Given that the previous results
630 revealed altered physicochemical properties and surface acitvity of the AITC modified WPI, it
631 was of interest to evaluate the growth-inhibitory activity after WPI modification. The
632 minimum inhibitory concentrations (MIC) against different strains of Gram positive
633 bacterium *S. aureus* (Mu50 and LMG10147) and the Gram negative bacterium *E.coli* ATCC
634 8739 were evaluated using lyophilized unmodified or modified WPI (the residual amount of
635 unbound AITC in 1 % modified WPI solutions was below 0.015% (**Table 1)**). The
636 antibacterial activity was tested at pH-values 6 and 7, since at pH-values 2 and 4 the
637 microorganisms did grow poorly at best.
638 At pH-value 7 native WPI showed a mild effect on the growth of *E.coli* ATCC 8739 (0.68
639 A.U. to 0.41 A.U. reduction) and against *S. aureus* Mu50 (0.49 A.U. to 0.34 A.U.) at WPI
640 concentrations between 0.01 % and 4 % WPI (**Figure 5**).
641
642 The modification of WPI with AITC did not influence the antimicrobial acitivity of the WPI,
643 although this effect was not unequivocally visible for *E.coli* at pH 7. To verify this, the
644 antibacterial effect of WPI modified with 0.4 g L^{-1} AITC (the same modification as used
645 above) or 1.2 g L^{-1} AITC (complete modification) was compared to native WPI (**Figure 6**)
646 against *E. coli* strain ATCC 8739 at pH 7. The low activity of native WPI under these
647 conditions was confirmed (0.88 A.U. to 0.42 A.U. at concentrations between 0.004 % and 4
648 % WPI). In comparison, the activity of the modified WPI (both 0.4 and 1.2 g L^{-1}
649 modifications) against the *E. coli* strain tested was less pronounced than for the native WPI,
650 however this effect was less distinct for the 0.4 g L^{-1} modification.
651
652 Mild growth inhibitory activity of pure β-LG against growth of Gram positive *S. aureus*
653 strains was already reported in literature (Chaneton, Pérez Sáez, J. M., & Bussmann, 2011),
654 which confirms the present results. On the other hand no effect of pure β-LG against resting
655 cells of *Pseudomonas fluorescens*, *Pseudomonas fragi* and *Bacillus subtilis* was observed
656 (Pan et al., 2007). Modifications of β-LG (i.e. acylation or amidation) reportedly increased its

antiviral or antimicrobial effect by incorporation of charged groups, e.g. antiviral properties were reported after acylation of β-LG, caused by incorporation of strong negative charges into the protein (Swart et al. 2009), whereas amidation (conversion of glutamate and aspartate residues to asparaginyl and glutaminyl residues) increased the positive charge of the protein which caused a strong bactericidal effect against resting cells of *Pseudomonas fluorescens*, *Pseudomonas fragi* and *Bacillus subtilis*. A much weaker effect was also found against *E. coli*, *Enterococcus faecalis*, *Salmonella typhimurium* and *Listeria monocytogenes* (Pan et al., 2007).

3.6.2. Antibacterial acitivty of AITC. Although no antimicrobial effect of the protein bound AITC was expected, the residual unbound AITC in the modified WPI (4% modified WPI contained < 0.045 % free AITC) might still have antimicrobial activity. Therefore the activity of pure AITC was evaluated without WPI at pH-values 6 and 7 (**Figure 7**). The results confirm a strong growth-inhibitory effect of AITC against *E. coli ATCC 8739* and *S. aureus Mu50*, and a lesser effect against *S. aureus LMG10147*. The unbound AITC in the modified WPI could therefore potentially have some growth inhibitory effect on *E.coli* and *S. aureus Mu50* when using 4 % protein, however this is not clearly evident in **Figures 5** and **6.**

Similar to our results, a strong antimicrobial effect against a five strain mixture of *E. coli* O157:H7 was reported for pure AITC solutions, although this effect was pH-dependent: the MIC was 50 µl/L at pH 6.5 (0.005 %) and 250 µl/L at pH 7.5 (0.025 %) (Luciano & Holley, 2009). Antimicrobial activity of AITC against *E. coli* strains was also found by Mushantaf et al. (Mushantaf, Blyth, & Templeton, 2012) in water and in refrigerated grounds beef (Muthukumarasamy, Han, & Holley, 2003). An antimicrobial effect of AITC against *S. aureus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* was also reported and the antibacterial mode of actions was suggested to be changed membrane properties of the bacteria, resulting in potassium leakage and propidium iodide uptake (Borges et al., 2015). The underlying mechanism is the interaction of the electrophilic C-atom in the R–N=C=S group of AITC with surface components of the bacteria. Interestingly, the interaction of bacteria membrane with AITC also changed the physicochemical properties of the membane (like increased hydrophobicity) (Borges et al., 2015). As this reaction occurs analogus to the described electrophilic reaction of AITC with whey proteins (i.e. AITC is covalently bound to proteins with its electrophilic C-atom), the protein bound AITC can no longer react with bacteria components.

4. Conclusion

The present findings revealed that the covalent modification of WPI with AITC significantly influenced the physicochemical properties of the WPI, especially at acidic pH: The surface hydrophobicity increased strongly, the secondary structure was altered to more beta-sheet components and signs of protein unfolding were evident. The IEP was shifted and the interfacial tension at the oil/water interface was significantly lower compared to unmodified WPI. The antimicrobial activity of WPI was not significantly influenced by AITC addition. Following this, AITC modification of WPI could be a possible means to increase the surface activity of WPI at acidic pH below the isoelectric point. A high emulsifying capacity of AITC-modified WPI at acidic pH-value is conceivable.

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