Title

Gelation of a combination of insect and pork proteins as affected by heating temperature and insect:meat ratio

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Abstract

In order to better understand structure formation in hybrid meat products containing insects, viscoelastic properties, protein aggregation and surface hydrophobicity of extracted insect and meat proteins in different insect:meat nitrogen ratios (100:0, 75:25, 50:50, 25:75 and 0:100) at different heating temperatures (from 20 to 80 °C) were studied. During heating, meat proteins showed best gelling properties. This was probably associated with the formation of hydrophobic interactions, as was confirmed by the strong increase in surface hydrophobicity of the meat proteins upon heating. Insect proteins, on the other hand, formed a considerable amount of additional structure during cooling, resulting in gels with high gel strength, although their gel stability was low. As for the mixtures of insect and meat proteins, they showed lower final gel strengths compared to the pure insect and meat protein samples. Furthermore, proteins with molecular weights of 230 and 16 kDa, that aggregated in the pure samples, did not aggregate in the mixtures. Although the mechanism of the latter effect remains to be elucidated, it probably explains the lower gel strength observed in the protein mixtures.

Keywords

Extracted proteins; *Zophobas morio* larvae; Isothermal heating; Viscoelastic properties; SDS-PAGE; Molecular weight distribution; Surface hydrophobicity; Co-gelation

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Abbreviations

ZM: *Zophobas morio* larvae; PS: pork shoulder; S₀: surface hydrophobicity index

1 Introduction

In recent years, the interest for insects as an alternative protein source has grown ([van Huis & Oonincx, 2017](#)). This is due to their favorable nutritional composition ([Rumpold & Schlüter, 2013a](#)) and high feed conversion efficiency ([Rumpold & Schlüter, 2013b](#)). Insect consumption is also considered as an environmentally-friendly alternative for meat consumption ([van Huis & Oonincx, 2017](#)). However, the question remains how to convince Western consumers, who generally maintain an averse attitude towards eating insects ([Looy, Dunkel, & Wood, 2013](#)). One possibility is the incorporation of edible insects into familiar food products, in such a way that the insects are no longer recognizable ([Caparros Megido et al., 2016](#); [Gmuer, Nuessli Guth, Hartmann, & Siegrist, 2016](#); [Hartmann, Shi, Giusto, & Siegrist, 2015](#)). In this respect, processing insects into hybrid meat products, containing both a meat and an insect fraction, may aid in persuading Western consumers to reduce their meat consumption and introduce insects into their diets.

Several properties of processed meat products, including texture and water and fat retention, are determined by meat protein gelation ([X. D. Sun & Holley, 2011](#)). During protein gelation, the liquid protein sol is transformed into a viscoelastic gel. During this gelation process, the proteins first unfold, which is followed by protein aggregation ([Banerjee & Bhattacharya, 2012](#)). If the attractive and repulsive forces between the proteins are well-balanced, the protein aggregation will result in the formation of a three-dimensional network ([Tornberg, 2005](#)). The types of protein-protein interactions that can be formed during aggregation include hydrophobic and electrostatic interactions, as well as disulfide and hydrogen bonds ([Foegeding & Davis, 2011](#)). Different parameters can influence the gel formation, such as heating temperature, pH and ionic strength ([Banerjee & Bhattacharya, 2012](#)).

There is ample literature regarding meat protein gelation (for example [Glorieux, Steen, Paelinck, Foubert, and Fraeye \(2017\)](#); [Liu and Foegeding \(1996\)](#); [Tornberg \(2005\)](#); [Westphalen, Briggs, and Lonergan \(2006\)](#); [Xiong and Blanchard \(1994\)](#)). Insect protein gelation is less well-understood. However, there are some studies which investigated the gelling properties of extracted insect proteins ([Lee, Kim, Ji, & Lee, 2019](#); [Mishyna, Martinez, Chen, & Benjamin, 2019](#); [Mishyna, Martinez, Chen, Davidovich-Pinhas, & Benjamin, 2019](#); [Ndiritu, Kinyuru, Gichuhi, & Kenji, 2019](#); [Purschke et al.,](#)

2018; [Wessels, Azzollini, & Fogliano, 2020](#); [Yi et al., 2013](#); [Zhao, Vazquez-Gutierrez, Johansson, Landberg, & Langton, 2016](#)) and of the proteins in insect flours ([Adebawale, Adebawale, & Oguntokun, 2005](#); [Kim, Setyabrata, Lee, Jones, & Kim, 2017](#); [Kim, Setyabrata, Lee, Jones, & Kim, 2016](#); [Mishyna, Martinez, Chen, & Benjamin, 2019](#); [Mishyna, Martinez, Chen, Davidovich-Pinhas, et al., 2019](#); [Omotoso, 2006, 2018](#); [Osasona & Olaofe, 2010](#); [Torruco-Uco et al., 2018](#)), insect model systems ([Scholliers et al., 2019](#)) and insect-based meat analogues ([Azzollini, Wibisaphira, Lakemond, & Fogliano, 2019](#)). Furthermore, a limited number of research papers studied hybrid meat products ([Choi et al., 2017](#); [Kim et al., 2017](#); [Kim et al., 2016](#); [Park et al., 2017](#); [Walkowiak, 2019](#)) or hybrid model systems ([Scholliers, Steen, & Fraeye, 2020](#)). However, to the best of our knowledge, there is currently no literature regarding gelation of a combination of extracted insect and meat proteins. This would provide more fundamental insights into the interactions between these proteins and how this affects important properties (e.g. gel characteristics) and, eventually, the ability of these proteins to contribute to the structure of hybrid meat products containing insects.

As a better understanding of the thermal gelation of meat and insect proteins, when they are combined, will be important in the development of high-quality hybrid meat products, the aim of the current study is to investigate the effects of different heating temperatures and insect:meat ratios on the gelling properties of meat and insect protein mixtures. To this end, proteins were extracted from pork shoulder and *Zophobas morio* larvae. Through dynamic oscillatory rheology, the gelation of the proteins during heating and the gel properties after heating were studied. Furthermore, SDS-PAGE of non-aggregated proteins at different heating temperatures provided insights into the molecular weight of the proteins in the protein extracts, as well as insight into protein aggregation in function of heating temperature. Through determination of surface hydrophobicity at different heating temperatures, information regarding the formation of hydrophobic interactions was gathered.

2 Materials and methods

2.1 Materials

Z. morio (ZM) larvae were purchased alive and in one large batch from Nusect (Deerlijk, Belgium). The larvae were subsequently sieved, vacuum packed and stored at -18 °C. A large batch of pork

shoulder (PS) was purchased from a local supplier (Norbert Impens, Melle, Belgium). After removing visible connective tissue and fat from the meat, the meat was subsequently chopped, ground through a 4 mm plate, vacuum packed and stored at -18 °C.

2.2 Protein extraction

The methods used for the extraction of proteins from pork shoulder and *Z. morio* larvae were similar, with some slight differences. They were both based on the method described by [Glorieux et al. \(2017\)](#), with some modifications based on [Yi, Van Boekel, and Lakemond \(2017\)](#). The larvae were ground to a paste before extraction, while the meat was already ground beforehand (see section 2.1). Meat was first mixed for 1 minute with 0.05 M Tris-HCl buffer (pH 8), containing 0.6 M NaCl, in a 1:5 meat:buffer ratio using an Ultra-Turrax Homogenizer, model T25 (IKA-Werke, GmbH, Staufen, Germany). The first step in insect protein extraction was similar, but the buffer also contained 0.01 M ascorbic acid, to prevent browning, and a 1:3 insect:buffer ratio was employed. For both meat and insect, the suspensions were shaken overnight at 4 °C, after which they were centrifuged for 30 minutes at 9,508 g (Hettich Universal 320R, Newport Pagnell, UK). The supernatants were then subjected to dialysis against deionized water using dialysis membranes with a molecular weight cut-off of 12 kDa (Dialysis tubing cellulose membrane, Sigma-Aldrich Co., Darmstadt, Germany). After dialysis, the supernatants were frozen and lyophilized, to obtain dry protein extracts. For both the meat protein extracts and the insect protein extracts, lyophilisates from multiple extraction runs were collected and mixed to obtain a homogenous batch, which was stored at -18 °C. The nitrogen content of the lyophilized protein extracts was determined using the Kjeldahl method.

2.3 Preparation of protein solutions

For all analyses, the protein extracts were resuspended in a 0.05 M phosphate buffer (pH 6), containing 0.6 M NaCl. The pH and salt content were chosen in order to mimic conditions in meat products. The protein solutions were then stirred overnight and put in an ultrasonic bath (USR 3/2, Julabo, Seelbach, Germany) for 10 minutes the next morning to ensure optimal solubilization. Solutions with insect:meat nitrogen ratios of 100:0 (100ZM), 75:25 (75ZM:25PS), 50:50 (50ZM:50PS), 25:75 (25ZM:75PS) and 0:100 (100PS) were prepared and studied. There is currently

no standard conversion factor for insect (protein extracts), nor a specific conversion factor for *Z. morio* larvae (protein extracts). Furthermore, conversion factors may be affected by amino acid composition (Lourengo, Barbarino, De-Paula, Pereira, & Marquez, 2002), which can differ depending on insect species and on the proteins that were extracted. Therefore, the protein extract ratios were based directly on the nitrogen content, rather than the (calculated) protein content.

2.4 Dynamic oscillatory rheology

The viscoelastic properties of protein solutions (0.64 % nitrogen concentration), with different insect:meat ratios (see section 2.3), were studied as described by Scholliers et al. (2019). A stress-controlled rheometer (AR 2000ex, TA instruments, New Castle, USA), equipped with an upper-heated plate, a Peltier temperature-control system and cross-hatched parallel plates (diameter 40 mm), was used. For each analysis, a 500 μ m gap was employed and 1.7 mL of protein solution was loaded. To prevent dehydration, the samples were covered with a layer of paraffin oil and a cap was placed over the samples. The protein solutions were successively subjected to a temperature, frequency and stress sweep, during which G' (elasticity modulus), G'' (viscosity modulus), G^* (complex modulus) and δ (phase angle) were measured. The rheological measurements were performed at least in triplicate, each time on a freshly prepared protein solution.

2.4.1 Temperature sweep

Immediately following the loading of the samples, the temperature sweep was initiated. The samples were thereby isothermally heated at 70 or 80 °C during 1 h (phase 1), cooled to 20 °C at a rate of 2 °C/min (phase 2) and kept at 20 °C for 1 h (phase 3). During the temperature sweep, a constant strain (0.025) and frequency (1 Hz) were maintained.

2.4.2 Frequency sweep

A frequency sweep was performed immediately after the temperature sweep, in a frequency range of 0.1-10 Hz (15 measuring points per decade). A constant temperature (20 °C) and oscillatory stress (within the linear viscoelastic region (LVR)) were employed.

2.4.3 Stress sweep

Finally, the samples were subjected to a stress sweep, during which the oscillatory stress was increased from 1 to 15,000 Pa (70 measuring points per decade) at a constant temperature (20 °C) and frequency (1 Hz). Through the stress sweep, the length of the LVR was determined, by calculating the oscillatory stress at which the corresponding G^* differed more than 5 % from the average G^* of the previous 5 measurements.

2.5 Protein aggregation at different heating temperatures

The aggregation of the (combinations of) insect and meat proteins at isothermal heating temperatures between 20 and 80 °C was studied through SDS-PAGE, according to the method described by [Glorieux et al. \(2017\)](#), with some slight modifications. Protein solutions (0.18 % nitrogen concentration) with different insect:meat ratios (see section 2.3) were prepared. Aliquots, containing 1 mL of protein solution, were heated for 1 h in a temperature-controlled water bath (Julabo Seelbach, Germany) at 20, 40, 50, 60, 70 or 80 °C. The samples were then immediately transferred to ice to prevent further protein denaturation and aggregation. After allowing the heated protein solutions to equilibrate at room temperature, they were briefly centrifuged (Centrifuge 54115D, Eppendorf Belgium NV, Aarschot, Belgium), precipitating all aggregated proteins. The supernatants, containing the soluble, non-aggregated proteins, were then subjected to SDS-PAGE analysis. To this end, the supernatants were mixed in a 1:1 ratio with sample buffer, containing 95 % Laemmli Sample buffer and 5 % β -mercaptoethanol (Bio-Rad Laboratories Inc., Hercules, California, USA). These mixtures were then heated at 100 °C for 4 minutes. After they were cooled down, the samples (45 μ l) and a protein standard (Precision Plus Protein^{TM} Unstained Protein Standard, Bio-Rad Laboratories, Inc.) (20 μ l) were loaded on Criterion^{TM} TGX Stain-Free^{TM} precast gels (Bio-Rad Laboratories, Inc.) which were then placed in a Criterion^{TM} Cell Unit (Bio-Rad Laboratories, Inc.). Next, the gels underwent electrophoresis in 2 steps. First, a constant current of 20 mA (25 minutes) and then a constant voltage of 300 V (30 minutes) (PowerPac^{TM} Basic Power Supply, Bio-Rad Laboratories, Inc.) were applied. After electrophoresis, the molecular weight distribution of the proteins in the different samples was

analyzed using a Molecular Imager® Gel Doc™ EZ System and Image Lab™ Software 6.0.1 (Bio-Rad Laboratories, Inc.).

2.6 Surface hydrophobicity

To gain more insight into the amount of hydrophobic interactions that may be formed between the proteins, their surface hydrophobicity was studied using a fluorescent probe, 8-anilino-1-naphthalenesulfonate (ANS), according to the method of [Glorieux et al. \(2017\)](#), with some slight modifications. For each analysis, a fresh protein solution (with different insect:meat ratios (see section 2.3) and containing 0.016 % nitrogen) was prepared. Using this stock solution, diluted protein solutions with concentrations of 0.00016, 0.0008, 0.0016 and 0.008 % nitrogen were made. Of each dilution, 4 mL was then transferred to a test tube, in triplicate for each temperature. Subsequently, the test tubes were heated for 1 h at 20, 30, 40, 50, 60, 70 or 80 °C in a temperature-controlled water bath (Julabo Seelbach, Germany), after which they were put on ice to prevent further protein denaturation and aggregation. After equilibration at room temperature, 20 µl of ANS (8mM in 0.05 M phosphate buffer, pH 7.4) was added to the heated protein solutions and the fluorescence intensity was measured (Shimadzu RF-1501, Kyoto, Japan). Excitation and emission wavelengths of 390 and 480 nm, respectively, were employed, with excitation and emission band widths of 10 nm. For each sample at each temperature, fluorescence intensity was plotted in function of protein concentration and the slope was calculated using linear regression analysis. The slope was used as an index of protein surface hydrophobicity (S_0). This analysis was performed at least in triplicate, each time with a freshly prepared stock solution.

2.7 Statistical analysis

All analyses, except SDS-PAGE, were performed at least in triplicate, with the results being expressed as mean $\pm$ standard error. Using IBM SPSS 25 software, two-way ANOVA was performed on the logarithmic values of the rheological parameters ($G'_{\text{end heating}}$ (at the end of phase 1), $G'_{\text{end temperature sweep}}$ (at the end of phase 2) and length of LVR) to investigate the effect of heating temperature and insect:meat ratio. Two-way ANOVA was also performed on (the absolute values of) the surface hydrophobicity index (S_0) to study the effect of temperature and protein type (insect or meat protein).

For all studied parameters, a significant interaction was determined. These interactions were further interpreted and Tukey's *post hoc* tests were performed to determine significant differences. The applied significance level was $p < 0.05$.

3 Results and discussion

3.1 Nitrogen content of the protein extracts

Nitrogen contents of the insect and meat protein extracts were 8.52 ± 0.05 % and 12.46 ± 0.14 %, respectively. This indicates that the extracts did not purely consist of protein, but also contained other constituents. Since an aqueous solvent was used, it is highly unlikely that the extracts contained fat. The same applies to chitin in the insect protein extract, as chitin is insoluble in regular solvents (e.g. water, mild acidic or basic solutions, etc.) due to its highly aggregated nature ([Roy et al., 2017](#)). [Yi et al. \(2013\)](#), who performed an aqueous extraction on 5 insect species (including *Z. morio* larvae) and who found similar nitrogen contents in their protein extracts, also did not expect chitin to be present in the supernatant. Other constituents of the protein extracts may include moisture, ash and soluble carbohydrates.

3.2 Dynamic oscillatory rheology

3.2.1 Temperature sweep

The temperature sweeps of the insect and meat proteins, in the different insect:meat ratios, are shown in Fig. 1. In all cases, δ remained relatively constant during the isothermal steps (phases 1 and 3), but increased slightly during the cooling step (phase 2). However, as δ stayed well below 45° in all graphs, it can be concluded that gels with predominantly elastic characteristics were formed by all (combinations of) proteins. Regarding the course of G' , a sharp rise occurred at the start of phase 1. This sudden increase in the elastic character of the protein solutions indicates a rapid development of an initial protein network by all (combinations of) proteins upon application of the heating temperatures. Throughout the rest of phase 1, G' continued to increase, at a lower rate however, until reaching a plateau towards the end of the isothermal heating step. This indicates further fortification of the protein network until reaching a more or less constant gel strength. During the cooling phase, additional structure formation occurred as seen by the increase in G' for all (combinations of) proteins.

In the case of 100ZM, 75ZM:25PS and 50ZM:50PS, the samples with the highest insect protein contents, this increase took place in 2 steps. A first, rather gradual, increase of G' was thereby followed by a much faster rise of G' , indicating an extra surge of structure build-up. Finally, during the second isothermal step (phase 3), G' remained relatively constant for all (combinations of) proteins, with the exception of 100ZM. These samples, containing only insect protein, showed an additional increase in G' at the beginning of phase 3, after which G' also became more or less constant. Therefore, it seemed that insect protein gel strength increased considerably during both cooling (phase 2) and keeping the samples at 20 °C (phase 3). By adding and increasing the meat protein content in the samples, this effect was diminished. [Yi et al. \(2013\)](#) and [Zhao et al. \(2016\)](#) also found an increase in G' during both heating and cooling of extracted proteins (15 % protein concentration, pH 7) under non-isothermal conditions. According to [Yi et al. \(2013\)](#), the formation of hydrogen bonds was responsible for the rise in G' during cooling. Similar results were also obtained by [Mishyna, Martinez, Chen, Davidovich-Pinhas, et al. \(2019\)](#) during non-isothermal heating and cooling of insect powder. However, none of these studies reported an additional surge of insect protein gelation during cooling, as was observed in the current study. This was also not reported in our previous study, where the same time-temperature profile as used in the current study was applied to model systems made from *Z. morio* larvae, containing whole insects rather than protein extracts ([Scholliers et al., 2020](#); [Scholliers et al., 2019](#)). Similar to the results for meat protein in the current study, [Glorieux et al. \(2017\)](#), [Liu and Foegeding \(1996\)](#) and [Xiong and Blanchard \(1994\)](#) found an increasing G' during isothermal heating of myofibrillar proteins, while gel firming during cooling was also reported by [Westphalen et al. \(2006\)](#).

The G' values at the end of phase 1 ($G'_{\text{end heating}}$) and at the end of phase 3 ($G'_{\text{end temperature sweep}}$) (Fig. 2) were statistically analyzed to obtain more insight into the effect of heating temperature and insect:meat ratio on the viscoelastic properties. The results showed significant interactions ($p < 0.05$) between the effects of temperature and insect:meat ratio for both $G'_{\text{end heating}}$ and $G'_{\text{end temperature sweep}}$. In general, a trend was observed towards higher $G'_{\text{end heating}}$ and $G'_{\text{end temperature sweep}}$ values with increasing heating temperature, indicating that higher gel strength was obtained when the samples were heated at higher

temperatures. This effect was most pronounced and statistically significant for 100PS, containing only meat protein. This is in accordance with [Glorieux et al. \(2017\)](#) who also found higher G' values for meat proteins heated at higher temperatures. However, in case of pure insect proteins, the effect of heating temperature on gel strength was small and not statistically significant. [Mishyna, Martinez, Chen, Davidovich-Pinhas, et al. \(2019\)](#) also found that, at pH 5 and 7, increasing the temperature from 70 to 85 °C did not result in a considerably higher percentage of coagulated *Apis mellifera* proteins. [Lee et al. \(2019\)](#), however, found that *Tenebrio molitor* protein aggregation did increase with increasing temperature (75 to 95 °C). As for the samples containing both insect and meat proteins, the effect of temperature was also rather small. Compared to the results shown in Fig. 2, a considerably more outspoken effect of temperature on the G' values was found in previous research for model systems containing whole *Z. morio* larvae and/or pork shoulder ([Scholliers et al., 2020](#)).

Contrary to the effect of temperature, the effect of insect:meat ratio was different for $G'_{\text{end heating}}$ compared to $G'_{\text{end temperature sweep}}$. As seen in Fig. 2A, samples containing 50 to 100 % insect nitrogen (50ZM:50PS, 75ZM:25PS and 100 ZM) all showed similar $G'_{\text{end heating}}$ values, which were rather low. Only upon increasing the percentage of meat nitrogen above 50 %, G' rose, with samples containing only meat proteins (100PS) exhibiting the highest G' values. Meat proteins therefore seemed to contribute more to gel strength during heating compared to insect proteins. For $G'_{\text{end temperature sweep}}$, however, a different effect occurred (Fig. 2B), as samples containing only insect proteins (100ZM) showed highest G' values at 70 °C and second to highest G' values at 80 °C. While, during heating, meat proteins showed the best gelling properties, cooling and keeping the samples at 20 °C appeared to be of significant importance for the insect protein gelation. As hydrophobic interactions increase with increasing temperature (from 25 to 75 °C) ([Liu & Foegeding, 1996](#)) and hydrogen bonds are formed during cooling ([Yi et al., 2013](#)), this may indicate that hydrophobic interactions and hydrogen bonds played a crucial role during gelation of meat proteins and insect proteins, respectively.

However, the samples containing both insect and meat proteins (75ZM:25PS, 50ZM:50PS and 25ZM:75PS) showed lower $G'_{\text{end temperature sweep}}$ values compared to the pure insect and meat protein samples. The lowest $G'_{\text{end temperature sweep}}$ values were found for the samples containing equivalent

amounts of insect and meat nitrogen (50ZM:50PS). To the best of our knowledge, there are currently no other studies on the gelation of a combination of extracted insect and meat proteins. However, the effect of the addition of other non-meat proteins to myofibrillar protein gels has been reported. [Brewer, Peterson, Carr, McCusker, and Novakofski \(2005\)](#) found an antagonistic interaction between gelatin and myofibrillar proteins, as the combination of both resulted in weaker gels than the pure gelatin and myofibrillar protein gels. They ascribed this to different and incompatible gelling mechanisms of both proteins. It must be noted that gelatin is a cold gelling protein, while meat proteins (as well as insect proteins) are warm gelling proteins. Upon addition of non-modified soy protein isolate to myofibrillar proteins, [Jiang and Xiong \(2013\)](#), [Han, Zu, Xu, and Zhou \(2015\)](#), [J. Sun, Yin, Chen, and Li \(2019\)](#), [Feng and Xiong \(2002\)](#) and [Ramírez-Suárez and Xiong \(2003\)](#) reported lower G' values compared to pure myofibrillar protein samples after non-isothermal heating. Similar results were obtained for mixtures of myofibrillar and pea proteins by [X. D. Sun and Arntfield \(2012\)](#) and for a combination of myosin and native β -lactoglobulin by [Vittayanont, Steffe, Flegler, and Smith \(2003\)](#). Furthermore, through compression rheology, [McCord, Smyth, and O'Neill \(1998\)](#) found diminished gel strengths when non-meat proteins (whey and soy protein) were added to salt-soluble meat proteins. It has to be noted, however, that soy proteins ([Feng & Xiong, 2002](#); [Jiang & Xiong, 2013](#)), pea proteins ([X. D. Sun & Arntfield, 2012](#)) and β -lactoglobulin ([Vittayanont, Vega-Warner, Steffe, & Smith, 2001](#)) have higher denaturation temperatures compared to meat proteins. The heating temperatures applied in the above mentioned studies were often insufficient to fully denature the non-meat proteins. As a result, even pure solutions of these non-meat proteins exhibited poor gelling properties under the conditions studied ([Feng & Xiong, 2002](#); [Ramírez-Suárez & Xiong, 2003](#); [X. D. Sun & Arntfield, 2012](#)). This is in contrast to our study, in which both insect and meat proteins are known to denature and aggregate below 70-80 °C (see also section 3.3 on protein aggregation). Furthermore, the results in Fig. 2B conflict with results from previous research, which showed considerably less structure formation in insect model systems compared to meat model systems ([Scholliers et al., 2020](#); [Scholliers et al., 2019](#)) and intermediate structure formation in hybrid (containing both insect and meat) model systems ([Scholliers et al., 2020](#)). Apparently, the insect proteins in the model systems, containing whole insects rather than extracted proteins, were not able to

gel as well as the extracted proteins. This indicates that other constituents of the insect biomass, e.g. pieces of exoskeleton, may interfere with insect protein gelation in the model systems.

3.2.2 Frequency sweep

Immediately following the temperature sweep, a frequency sweep was performed (Fig. 3). As, for all insect:meat ratios at both heating temperatures, G' was predominant over G'' and both G' and G'' showed a slight frequency-dependence, it can be concluded that weak gels with more solid-like characteristics were formed ([Rao, 2007](#); [Tabilo-Munizaga & Barbosa-Cánovas, 2005](#)).

Increasing the heating temperature from 70 to 80 °C resulted in (slightly) higher G' and G'' values throughout the frequency sweeps. This effect of temperature was most pronounced for 100PS, containing only meat proteins. Furthermore, similar to the G' end temperature sweep, the pure insect and meat protein samples (100PS and 100ZM) showed highest G' and G'' values. These results are in accordance with the results from the temperature sweep (see section 3.2.1).

3.2.3 Stress sweep

Subsequent to the frequency sweep, the samples were subjected to a stress sweep to determine the length of the LVR (Fig. 4). This was used as a measure for gel stability, as a longer LVR implies that more stress is needed to break down the gel structure, which in turn indicates higher gel stability ([Steen et al., 2014](#)). Statistical analysis showed a significant interaction ($p < 0.05$) between the effect of temperature and insect:meat ratio on the length of LVR.

Increasing the heating temperature from 70 to 80 °C, resulted in longer LVR's and, thus, higher gel stability for all insect:meat ratios. However, this effect was only significant for the samples containing both insect and meat proteins, not for the pure samples. Furthermore, for both heating temperatures, the gel stability increased with increasing meat protein content. Even though the samples containing the most insect protein (100ZM and 75ZM:25PS) showed relatively high G' end temperature sweep values (see section 3.2.1), this did not result in high gel stability. This suggests that the protein interactions formed in these samples may have been rather weak and broke under relatively low amounts of stress. These results are in accordance with previous research, where the same effect of temperature was found on

insect, meat ([Scholliers et al., 2019](#)) and hybrid model systems ([Scholliers et al., 2020](#)). Furthermore, similar to current results, the latter also found lowest and highest structure stability in insect and meat model systems, respectively, with intermediate stability in hybrid model systems.

3.3 Protein aggregation at different heating temperatures

SDS-PAGE was performed on the non-aggregated proteins from protein solutions that were isothermally heated at 20 to 80 °C (Fig. 5). Through this SDS-PAGE, insight into the molecular weight distribution of the proteins in the extracts and into the aggregation of the proteins at the different heating temperatures was obtained.

Comparing the patterns of the unheated meat proteins (100PS, 20 °C) and the unheated insect proteins (100ZM, 20 °C) clearly showed a distinctly different molecular weight distribution of the proteins in both extracts. In the meat protein extract, multiple high molecular weight proteins (>100 kDa) were present, presumably including myosin heavy chain (MHC), with a molecular weight of approximately 200 kDa ([Xiong, Brekke, & Leung, 1987](#)). As gel strength is related to the molecular weight of the proteins involved during gelation ([Wang & Damodaran, 1990](#)), the presence of multiple high molecular weight proteins in the meat protein extract may contribute to the excellent gelling properties of the meat proteins (see section 3.2). The most abundant band in the meat protein extract most likely represented actin, which has been reported to have a molecular weight of approximately 45 kDa ([Xiong et al., 1987](#)). The molecular weight distribution of the proteins in the meat protein extract showed many similarities to those found by [Glorieux et al. \(2017\)](#), [Han et al. \(2015\)](#) and [Feng and Xiong \(2002\)](#). Contrary to the meat protein extract, the insect protein extract showed only 1 diffuse band (at approximately 230 kDa) with a molecular weight above 100 kDa, which might represent MHC. The insect protein extract also showed 2 abundant bands at approximately 50 and 65 kDa. [Yi, Van Boekel, Boeren, and Lakemond \(2016\)](#) identified MHC (molecular weight of 262 kDa) in *T. molitor* protein extract. Furthermore, [Yi et al. \(2013\)](#) also performed SDS-PAGE on a water-soluble protein extract of *Z. morio* larvae, showing only proteins with molecular weights < 95 kDa and no band at 230 kDa. The unheated mixtures of insect and meat proteins (75ZM:25PS, 50ZM:50PS and 25ZM:75PS) showed intermediate protein band patterns.

The aggregation of the proteins at different temperatures can also be studied through the SDS-PAGE shown in Fig. 5. When certain protein bands diminish or disappear at a certain temperature, it indicates that the respective proteins (partially) aggregated at that temperature. After heating the meat proteins (100PS) at 40 °C, the bands presumably representing MHC and actin (as identified through their molecular weight) already started to diminish. When heated at 50 °C, all high molecular weight proteins were aggregated and heating temperatures of 60 °C or higher were sufficient for aggregation of all meat proteins. A similar effect of heating temperature on the aggregation of myofibrillar proteins was observed by [Glorieux et al. \(2017\)](#). Compared to the meat proteins, higher heating temperatures were needed to induce insect protein aggregation (100ZM). As seen in Fig. 5, the first protein bands only started to diminish after heating at 60 °C and only heating at 70 and 80 °C was sufficient to aggregate all insect proteins. SDS-PAGE has also been performed to study *A. mellifera* protein coagulation at different temperatures and at different pH values by [Mishyna, Martinez, Chen, Davidovich-Pinhas, et al. \(2019\)](#), who observed protein aggregation even at temperatures < 55 °C. Furthermore, similar to the results presented in Fig. 5, proteins with a molecular weight of 50-75 kDa were primarily responsible for coagulation at temperatures of 55-70 °C and 70-85 °C (at pH 5 and 7). Most insect and meat proteins in the mixtures of both (75ZM:25PS, 50ZM:50PS and 25ZM:75PS) followed the same trends as in the pure insect and meat protein solutions. What stands out, however, is that certain proteins, that aggregated in the pure insect or meat protein samples, did not aggregate in the mixtures. The diffuse high molecular band at approximately 230 kDa, which represents an insect protein as it was present in the insect protein extract (100ZM), did not disappear in 75ZM:25PS and 50ZM:50PS, indicating that, even at 80 °C, these proteins did not aggregate. The same goes for the insect and/or meat protein bands at approximately 16 kDa, which remained present at all heating temperatures in all mixtures (75ZM:25PS, 50ZM:50PS and 25ZM:75PS). This indicates that, due to the mixing of the insect and meat proteins, certain proteins were unable to aggregate. This may explain the lower gel strength observed in the temperature sweep experiments of protein mixtures (see section 3.2.1).

3.4 Surface hydrophobicity

In section 3.2.1 it was hypothesized that hydrophobic interactions and hydrogen bonds played a crucial role during gelation of meat proteins and insect proteins, respectively. Therefore, the surface hydrophobicity of the insect and meat proteins (in the different insect:meat ratios) was determined at different heating temperatures, to obtain more insight into the amount of hydrophobic interactions that can be formed. The surface hydrophobicity of pure insect (100ZM) and meat proteins (100PS) after heating at 20 to 80 °C is shown in Fig. 6. The results for the mixtures of insect and meat proteins (75ZM:25PS, 50ZM:50PS and 25ZM:75PS) were intermediate between the pure insect and meat proteins and are therefore not shown. Statistical analysis of the effect of temperature and type of protein (insect or meat protein) on the surface hydrophobicity index (S_0) showed a significant interaction ($p < 0.05$).

Upon heating the meat proteins at 40 °C, a significant increase in S_0 occurred, indicating (the onset of) protein denaturation at this temperature. Between 40 and 60 °C, S_0 continued to increase in function of the applied heating temperature, although further increasing the heating temperature to 70 or 80 °C did not result in higher surface hydrophobicity. These results are in accordance with the results from the SDS-PAGE (see section 3.3), as those showed that meat protein aggregation (which is a consequence of protein denaturation) already began at 40 °C and that at 60 °C all proteins had aggregated. Similar results were obtained for red and white chicken myofibrillar proteins by [Glorieux et al. \(2017\)](#), though hydrophobicity already increased at 30 °C and the maximum hydrophobicity was reached at 50 and 60 °C for red and white chicken myofibrillar proteins, respectively. As for the insect proteins, a gradual increase of S_0 was observed, starting from a heating temperature of 40 °C and reaching a maximum at a temperature of 70 °C. This too is in accordance with the results from the SDS-PAGE, which showed that all insect proteins had aggregated when heated at 70 or 80 °C. Furthermore, these results are in accordance with [Lee et al. \(2019\)](#), who observed an increase in surface hydrophobicity of *T. molitor* proteins in function of increasing heating temperature (55-95 °C). [Mishyna, Martinez, Chen, Davidovich-Pinhas, et al. \(2019\)](#) also found an increased *A. mellifera* protein surface hydrophobicity after heating at 85 °C (at pH 5 and 7) compared to unheated proteins.

When comparing the surface hydrophobicity of the meat and insect proteins, it was observed that, even unheated (20 °C), the meat proteins showed significantly higher surface hydrophobicity compared to the insect proteins. Upon heating, the difference became even more pronounced, resulting in approximately 3-fold higher S_0 values for meat proteins compared to insect proteins at 60-80 °C. Higher surface hydrophobicity indicates a higher potential for the formation of hydrophobic interactions, resulting in higher gel strengths. This also confirms the hypothesis that hydrophobic interactions played a crucial role during meat protein gelation (see section 3.2.1).

4 Conclusions

In this study, the gelation of a combination of insect and meat proteins and the effect of heating temperature and insect:meat ratio thereon was investigated.

Results showed that, during heating, meat protein gelation was most prominent. As heating resulted in high surface hydrophobicity in meat proteins, especially compared to the insect proteins, it is highly likely that hydrophobic interactions contributed significantly to meat protein gelation.

For insect proteins, on the other hand, a considerable amount of additional structure was formed during cooling and keeping the samples at 20 °C, most likely due to the formation of hydrogen bonds. This resulted in relatively high final gel strengths, comparable to those of meat proteins. However, the gel stability was considerably lower and generally decreased with increasing insect protein content.

Even though pure insect and meat proteins both showed comparable final gel strengths, this parameter decreased upon combining both protein sources, with lowest $G'_{\text{end temperature sweep}}$ values found at equivalent amounts of insect and meat nitrogen. This antagonistic effect between insect and meat protein gelation may result from the fact that some proteins (with molecular weights of 230 and 16 kDa), which aggregated in the pure insect and meat protein samples, did not aggregate in the mixtures. Therefore, these proteins probably did not contribute to gelation in the protein mixtures, explaining the lower gel strengths. However, further research is needed to better understand why these proteins did not aggregate and to what extent the insect and meat proteins interact with each other during gelation.

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446   **Conflict of interest statement**

447   We wish to confirm that there are no known conflicts of interest associated with this publication and
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Figure 1: Changes in G' and δ of insect and meat proteins in different insect:meat ratios during isothermal heating at 70 °C and 80 °C (1 h), cooling to 20 °C (2 °C/min) and holding at 20 °C (1 h) (G' , the solid black lines; $G' \pm SE$, the dashed black lines; δ , the solid grey lines; $\delta \pm SE$ the dashed grey lines; Temperature, the dotted black lines) ($n \geq 3$). ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.

Figure 2: Effect of heating at 70 or 80 °C on (A) $G'_{\text{end heating}}$ and (B) $G'_{\text{end temperature sweep}}$ of insect and meat proteins in different insect:meat ratios. Different letters indicate significant differences ($p < .05$). Mean values and standard errors are presented ($n \geq 3$). ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.

Figure 3: Frequency sweeps of insect and meat proteins in different insect:meat ratio after being heated at 70 or 80 °C, cooled to 20 °C and held at 20 °C, with (A) 100 ZM, (B) 75 ZM:25 PS, (C) 50 ZM:50 PS, (D) 25 ZM:75 PS and (E) 100 PS. Confidence intervals are not shown to preserve readability of the graph ($n \geq 3$). ZM: ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.

Figure 4: Effect of heating at 70 or 80 °C on the length of LVR of gels formed by insect and meat proteins in different insect:meat ratios. Different letters indicate significant differences ($p < .05$). Mean values and standard errors are presented ($n \geq 3$). ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.

Figure 5: SDS-PAGE of the non-aggregated proteins in isothermally heated (1 h at 20-80 °C) protein solutions with different insect:meat ratios. The outer lanes represent the molecular weight markers. ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.

Figure 6: Effect of different heating temperatures (20-80 °C) on the surface hydrophobicity (S_0) of insect and meat proteins. Different letters indicate significant differences ($p < .05$). Mean values and standard errors are presented ($n \geq 3$). ZM: proteins extracted from *Z. morio* larvae; PS: proteins extracted from pork shoulder.