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Extraction and characterization of protein fractions from five insect species

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ABSTRACT

Tenebrio molitor, *Zophobas morio*, *Alphitobius diaperinus*, *Acheta domesticus* and *Blaptica dubia* were evaluated for their potential as a future protein source. Crude protein content ranged from 19 - 22 % (Dumas analysis). Essential amino acid levels in all insect species were comparable with soybean proteins, but lower than for casein. After aqueous extraction, next to a fat fraction, a supernatant, pellet, and residue were obtained, containing 17 – 23 %,

33 – 39 %, 31 – 47 % of total protein, respectively. At 3 % (w/v), supernatant fractions did not form stable foams and gels at pH 3, 5, 7, and 10, except for gelation for *A. domesticus* at pH 7. At 30 % w/v, gels at pH 7 and pH 10 were formed, but not at pH 3 and pH 5. In conclusion, the insect species studied have potential to be used in foods due to: 1) absolute protein levels; 2) protein quality; 3) ability to form gels.

Keywords

Insect protein; Protein extraction; Protein characterization; Foaming; Gelation.

1. Introduction

1.1. Insects as a source of food

In most developed countries, human consumption of insects is infrequent, or even culturally inappropriate, although its nutritional value is comparable to conventional meat (van Huis, 2013). In many regions and countries of the world, insects form part of the human diet and it is a misconception to believe that this is prompted by starvation (van Huis, 2013). About 1900 insect species are consumed globally as human food in the world (http://www.ent.wur.nl/UK/Edible+insects/Worldwide+species+list/).

With an increase in the world population, increased consumer demand for protein, and the amount of available agricultural land being constrained, the sustainable production of meat will represent a serious challenge for the future. Insects can be considered as an alternative protein source with less environmental impact (van Huis, 2013). Insects can be consumed as a whole. However, they can also be processed in less recognizable forms, which may increase consumer acceptability. Insects are already used as natural food ingredients, *e.g.* the red colorant carmine (E120) used in yogurt is an extract of the female cochineal insect.

1.2. Edible insects

Insects are consumed in different life stages like eggs, larvae, pupae or adults. The main species consumed are, in order of importance: beetles (Coleoptera); caterpillars (Lepidoptera); ants, bees and wasps (Hymenoptera); grasshoppers and locusts (Orthoptera); true bugs, aphids and leafhoppers (Hemiptera); termites (Isoptera) and flies (Diptera) and some others. Lepidoptera, Coleoptera, and Diptera (including flies) are commonly consumed in the larval stage; while the Orthoptera, Hymenoptera, Hemiptera and Isoptera are mainly consumed in the adult stage.

Cultivating edible insects for food consumption has several advantages: 1) Insects have a high feed conversion efficiency compared with conventional livestock. For example, the feed conversion ratio of house cricket (*Acheta domesticus*) can be calculated twice as efficient as chickens, almost 4 times more efficient than pigs and over 12 times more than cattle (van Huis, 2013); 2) Cultivating insects for protein has less environmental impact than cattle ranching, due to the lower production of greenhouse gas and NH_3 emissions (van Huis, 2013); 3) Besides the higher production yield and less environmental impact, insect feeds can be obtained from a wider range of plants than that of conventional livestock, such as cattle or swine (Durst & Shono, 2010). Overall, insect farming can be introduced in terms of a sustainable form of agriculture.

1.3. Proteins of edible insects

As a food source, insects are potentially nutritious, rich in protein and fat, and providing a certain amount of minerals and vitamins. Studies on protein quality, nutritional value, protein content, and the amino acid composition of various insects are available (Ladrón de Guevara, Padilla, García, Pino, & Ramos-Elorduy, 1995); (Renault, Bouchereau, Delettre, Hervant, & Vernon, 2006); (Barker, Fitzpatrick, & Dierenfeld, 1998). The protein content of common

edible insects was around 9 – 25 % (Finke & Winn, 2004), and the Yellow mealworm beetle larvae (24 %) (Ghaly & Alkoaik, 2009), *Zophobas morio* larvae (19 %) (Finke, 2002), and *Acheta domesticus* adult (19 %) (Finke & Winn, 2004), conventional meat protein sources contain about 15 to 22 % protein (Ghaly & Alkoaik, 2009). In addition, some insects have not only protein content comparable to meat, but also to plant protein (up to 36.5 %).

People may consume insect food more easily when unrecognizable insect protein (extract) is incorporated in food in comparison to consuming whole insects. (Del Valle, Mena, & Bourges, 1982) also indicated that the extraction of proteins from insects for further use in food products is particularly relevant for countries that do not have the habit of consuming insects, such as Europe and North America.

In this study, there are five insect species selected based on their availability (species reared by companies in the Netherlands): three species of Coleoptera considered edible, including the Yellow mealworm (*Tenebrio molitor*), the Superworm (*Zophobas morio*), the Lesser mealworm (*Alphitobius diaperinus*) and one species of Orthoptera; the House cricket (*Acheta domesticus*) considered edible and one of the Blattodea; the Dubia cockroach (*Blaptica dubia*) not edible, but can be reared in large numbers and used for animal feed.

1.4. Objective

Although researchers from entomological and zoo-biology science have studied intact edible insects, still very little information from a food science point of view is available on characteristics and functionality of extracted insect proteins.

The aim of this study was to investigate if insects could be used as a future protein source in food. Therefore, insect protein characteristics and functionality were determined and evaluated for each of the five insect species. The specific objectives of this study were to: (a) extract proteins and characterize obtained fractions; (b) evaluate protein purity and yield of

the obtained fractions; (c) establish some functional properties of the protein fractions focused on foaming and gelation; (d) study protein quality by analysis of protein content and amino acid composition.

2. Materials and methods

2.1. Insects used

Tenebrio molitor, Z. morio, A. diaperinus, A. domesticus and B. dubia were purchased from the commercial supplier Kreca V.O.F, Ermelo, the Netherlands. *Tenebrio molitor, Z. morio, A. diaperinus* species were supplied in the larvae stage, *A. domesticus and B. dubia* in the adult stage. The feed for *T. molitor*, and *Z. morio* mainly consisted of wheat, wheat bran, oats, soy, rye, corn, carrot and beer yeast. The feed for *A. diaperinus, A. domesticus* and *B. dubia* mainly consisted of carrot and chicken mash obtained from Kreca V.O.F. All insects were sieved to get rid of feed and stored alive at 4 ºC for about one day before processing.

2.2. Analysis of water content, protein, and fat content

All fresh insects were frozen using liquid nitrogen and subsequently grinded using a blender (Braun Multiquick 5 (600 Watt), Kronberg, Germany). Frozen grinded insects were freeze-dried (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands) to determine moisture and dry matter content. The freeze-drying process was stopped at a stable sample weight. Next, the freeze-dried insects were used for protein content analysis. Crude protein content was determined by Dumas (Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience, Breda, the Netherlands) using a protein-to-nitrogen conversion factor of 6.25. D-methionine (Sigma, CAS nr. 348-67-4) was used as a standard. Furthermore, fat content was determined after hexane extraction (Biosolve, CAS nr. 110-54-3) in a Soxhlet apparatus for 6 hours. Afterwards, hexane was removed using a Rotary evaporator (R420,

Buchi, Switzerland). Defatted insect meal was stored at - 20 ºC. All experiments were performed in two duplications of the same sample.

2.3. Determination of amino acid composition and protein quality

Amino acid composition of freeze-dried insect powder was analysed using ion exchange chromatography, following the International standard ISO 13903:2005. Tryptophan was determined by reversed phase C_{18} HPLC using fluorescence detection at 280 nm, according to the procedure described by International standard ISO 13904:2005. The amino acid composition of the five insect species was compared to literature data of soybean protein and casein, representing high quality proteins among vegetable and animal proteins (Sosulski & Imafidon, 1990; Young & Pellett, 1994). Protein quality was evaluated by the essential amino acid index (EAAI), which is based on the content of all essential amino acids compared to a reference protein, being values for human requirements in this case (Smith & Nielsen, 2010). EAAI gives an estimate on the potential of using insects as a protein source for human consumption without correcting for protein digestibility (Eq.1).

$$\text{EAAI} = \sqrt[9]{\left(\frac{\text{mg of lysine in 1 g of test protein}}{\text{mg of lysine in 1 g reference protein}}\right) \times (\text{etc. for the other 8 essential amino acids})}$$

2.4. Protein extraction procedure

For protein extraction, 400 g of N_2-frozen insects was used. After adding 1200 ml demineralized water, that was mixed with 2 g ascorbic acid beforehand, blending for one minute took place (Braun Multiquick 5 (600 Watt), Kronberg, Germany). Then the obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 500 µm. The filtrates and residues were collected. After centrifugation at 15,000 g for 30 min at 4 ºC, three fractions were obtained from the filtrate: the supernatant, the pellet, and the fat fraction.

142 The residue, the pellet and the supernatant fractions were freeze dried for further analysis.
143 The freeze-dried supernatant and pellet fractions of all insect species studied were
144 characterized in terms of colour, protein content and molecular weight distribution using
145 SDS-PAGE. The extraction procedure was performed in duplicate starting twice with a new
146 insect batch.

147 *2.5. SDS-PAGE*

148 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to
149 determine the molecular weight distribution of the insect protein fractions. For the detection
150 of the supernatant, pellet and residue fractions, 12.5 % acrylamide Phastgels (15 kDa to 250
151 kDa) and 20 % acrylamide Phastgels (2 kDa to 150 kDa) (GE Healthcare Bio-Sciences AB,
152 Uppsala, Sweden) were used. The applied markers were ordered from SigmaMarker (S8445,
153 wide range, molecular weight 6.5 - 200 kDa SigmaMarker). The samples were dissolved in
154 20 mM Tris/HCl, 2 mM EDTA pH 8.0 buffers with protein concentration of 7 mg/ml and
155 placed in an ultrasonic bath for 10 min. The protein concentration of the samples was
156 calculated based on protein content (Dumas) and amount of dry matter. Next, protein
157 solutions were diluted with ratio 1:1 in a sample buffer, containing 20 mM Tris/HCl, 2 mM
158 EDTA pH 8.0 (Across Organics, Cas nr. 6381-92-6), 5 % (w/v) SDS (Sigma, Cas nr. 152-21-
159 3), 0.016 % (w/v) DTT (DL- Dithiothreitol, Sigma, Cas nr. 3483-12-4), 0.02 % Bromophenol
160 Blue (Merck, Cas nr. 115-39-9). Afterwards, the samples were heated at 100 ºC for 5 min and
161 centrifuged for 2 min at 10,000 rpm before applying to the gel.

162 *2.6. Foamability and foam stability*

163 The stability of foam stabilized by insect supernatant protein was determined using foam
164 tubes with a diameter of 2.0 cm, and a glass grid at the bottom (Deak, Murphy & Johnson,
165 2007). The tubes were filled with 20 ml supernatant solution with a concentration of 3 % w/v,

at pH 3, 5, 7, and 10. The solutions were aerated from below with nitrogen gas, at a flow rate of 10.0 ml/min. Some of the samples had insufficient foamability to form stable foam at these concentrations. For those samples with sufficient foamability, the samples were aerated until the foam level reached 30 cm. After stopping the flow of gas, the height of the foam was determined as a function of time. From these curves, the half-time of the foam (the time in which foam height is reduced by 50 %) was determined. All tests were performed in duplicate.

2.7. Gel formation

2.7.1. Visual observation of gelation

Insect supernatant solutions were heated in a water bath (86 ± 1 ºC) for 10, 20 and 30 min. The supernatant fractions were dissolved at concentrations of 3 % w/v and 30 % w/v at pH 3, 5, 7 and 10. Depending on the initial pH, the final pH was adjusted by slowly adding 1 and 5 M HCl/ NaOH solutions. Gel formation was determined through visual observation. If the liquid was not moving upon turning the tube, it was considered a gel. This method was previously used by (Beveridge, Jones, & Tung, 1984) for albumin gel formation. Experiments were performed in duplicate.

2.7.2. Strain sweeps

Freeze-dried supernatant fractions from five insect species were used for this experiment. Protein solutions were prepared as followed: freeze-dried supernatant fractions were dissolved in demineralised water at a concentration of 15 % w/v, stirred for 30 minutes at room temperature and adjusted to pH 7 using 1 M NaOH.

To determine the rheological properties of the supernatant protein solutions and gels made from them, oscillatory strain tests were performed on a stress-controlled rheometer (Physica

MCR 501, Anton Paar, Graz, Austria) with stainless steel and titanium CC-10 concentric cylinder geometry (diameter inner cylinder: 9.997 mm; diameter cup: 10.845 mm). After filling the geometry with supernatant solution, all samples were covered with a thin layer of silicone oil to prevent sample evaporation. Samples were first heated from 20 to 90 ^{o}C at a heating rate of 1 ^{o}C/min (phase 1), kept at 90 ^{o}C for 5 min (phase 2), and cooled to 20 ^{o}C at a rate of 3 ^{o}C/min (phase 3). During the temperature ramp, the storage modulus G' and loss modulus G" were determined by applying oscillatory deformations with a strain amplitude of 0.005 and a frequency of 0.1 Hz. The point at which G' started to increase and became greater than the background noise, was designated as the gelation temperature (Renkema, Knabben, & van Vliet, 2001).

After formation of the gel, an oscillatory strain sweep was performed on the samples, with strains ranging from 10^{-4} to 10, and a frequency of 0.1 Hz. Strain sweeps were also performed to confirm whether this strain was in the linear response regime. All samples were tested at a supernatant fraction concentration of 15 % (protein content of around 8 % for five types of insects) w/v. *Tenerio molitor* was also tested at concentrations of 7 % (protein content of 4.1 %), and 30 % (protein content of 16.6 %) w/v. Values for G' for this fraction from the linear response regime were plotted against protein concentration C , and the exponent n, in the relation $G' \sim C^{n}$, was determined using linear regression to obtain information on the structure of the gels. For all fractions the maximum linear strain, where G' starts to decrease as a function of increasing strain, was also determined. This was done by separately fitting the data points in the linear region and the fully nonlinear region, and extrapolating both curves to their point of intersection (see Figure 2C). This method of determining the maximum linear strain is only approximate, but since we are not interested in the absolute value of this strain, but rather in the differences in this strain for the various protein samples,

this approximation was considered sufficiently accurate. All tests were performed in duplicate.

3. Results and discussion

3.1. Chemical composition of five insect species

The proximate composition of five insect species with regard to moisture, fat, protein was determined on live weight basis (Table 1). The moisture content of the five insect species ranged from 60 % to 71 %, fat content ranged from 3.6 % to 16 %, and crude protein from 19 % to 22 % (including chitin nitrogen). Other components, calculated by difference, ranged from 3.4 % to 7.5 %.

The proximate composition of *T. molitor* was comparable to the results of (Barker, Fitzpatrick, & Dierenfeld, 1998); (Finke, 2002); (Jones, Cooper, & Harding, 1972); (Ghaly & Alkoaik, 2009). In addition, the crude protein content measured for *A. domesticus* and *Z. morio*, 19.3 % and 20.6 % respectively, was comparable to the range described in literature, namely 17.3 % to 20.5 % (Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002). For *A. diaperinus* and *B. dubia*, no crude protein data are available in literature. The measured crude protein contents of the five insect species might be relatively higher than their actual protein content, since amounts of nitrogen are also bound in the exoskeletons as chitin. (Barker, Fitzpatrick, & Dierenfeld, 1998) reported that 5 - 6 % of total nitrogen was measured as chitin-bound nitrogen in *T. molitor*. This would lead to an overestimation in protein content of 1.1 – 1.3 % on a fresh weight basis. It is a reasonable estimate for true protein content in most insect species. However, no detailed study on this issue is available.

The measured protein content of the tested insect species (around 20 %) in this study is comparable with that of beef (18.4 %), chicken (22.0 %) and fish (18.3 %) (Ghaly, 2009b). Further, measured insect protein content was higher than that of lamb (15.4 %), pork (14.6 %)

(Ghaly, 2009), eggs (13 %), and milk (3.5 %), but lower in comparison to soy (36.5%) (Young & Pellett, 1994).

3.2. Amino acid composition and protein quality of five insect species

The insect protein quality of the insect species was estimated by the amino acid composition (Table 2). The larvae of *A. diaperinus, T. molitor and Z. morio* contained all the essential amino acids in quantities that are necessary for humans (FAO/WHO/UNU, 1985).

Also, the sum of the amount of total essential amino acids (EAA) for *A. diaperinus, T. molitor and Z. morio* was comparable to that of soybean protein, but slightly lower than that of casein, as reported by (Young & Pellett, 1991). Furthermore, the sum of EAA for *A. domesticus* and *B. dubia* was lower than in casein and soybean protein, but EAA were available in quantities that are necessary for human requirement (sum of 277 mg/g crude protein).The amino acid profiles found for *T. molitor* were similar to the profiles that were reported by (Ghaly, 2009b); (Finke, 2002) and (Jones, Cooper, & Harding, 1972). The amino acid profiles of *Z. morio* reported by (Finke, 2002) and those of *A. domesticus* reported by (DeFoliart & Benevenga, 1989) were similar to ours. To our knowledge, no literature is reported on the amino acid profiles for *A. diaperinus* and *B. dubia* before.

The sum of total amount of amino acids (TAA) per g crude protein of *A. diaperinus* (927 mg/g), *T. molitor* (910 mg/g) *and Z. morio* (931 mg/g) was higher than that in *A. domesticus* (864 mg/g) and *B. dubia* (776 mg/g). The fact that the sum of the total amount of amino acids did not add up to 1000 mg/g crude protein is mainly explained by the presence of non-protein nitrogen in the form of chitin. *Acheta domesticus* and *B. dubia* are used in adult form and are known to contain a higher level of chitin as compared to *T. molitor, A. diaperinus and Z. morio*.

The calculated essential amino acid index (EAAI) of *A. diaperinus, T. molitor* and *Z. morio* was somewhat higher than that of soybean, but lower than that of casein, also indicating that the quality of the insect protein for these three insect species was comparable to conventional food protein sources. The EAAI of *A. domesticus and B. dubia* was the lowest in comparison to other insects, and lower than the EAAI for casein and soybean. For a more detailed insight in insect protein quality, digestibility data need to be taken into account in future studies, since digestibility is not included as a factor in determining EAAI. (Ramos-Elorduy, Moreno, Prado, Perez, Otero, & De Guevara, 1997) found that protein digestibility, calculated from a vitro study, ranged from 76 to 98 % for seventy-eight species of edible insects, representing twenty-three insect families in Mexico. Their study indicated that insect proteins might have a high nutritional value.

3.3. Protein distribution in obtained fractions and colour of supernatant fractions

A mass balance was built up based on protein content in the residue, pellet and supernatant fractions (Fig. 1). The amount of protein in the fractions was calculated based on protein content determined by Dumas, in combination with weight of the fractions (dry matter based). The protein recoveries ranged from 86.5 % to 103 % (Fig. 1). The losses did occur during the extraction procedure, especially for *B. dubia*. The pellet contained 32.6 % to 39.4 % of total protein and the residue 31.4 % to 46.6 % of total protein (Fig. 1). The obtained pellet and residue fractions were higher in protein content than that in the supernatant (17 % to 23.1 %) for all five types of insects. The amount of proteins in the residue was higher than that in the pellet, except for *Z. morio* (31.4 %).

In addition, the protein content on dry matter basis of each fraction ranged from 50 % to 61 % in the supernatant, from 65 % to 75 % in the pellet, from 58 % to 69 % in the residue and around 0.1 % in the fat fraction. All chitin-bound nitrogen is expected to be present only

in the pellet and residue fractions, because chitin is insoluble in aqueous solvents (Goycoolea, Argüelles-Monal, Peniche, Higuera-Ciapara, Doxastakis, & Kiosseoglou, 2000). Except for the presence of chitin-bound nitrogen, there is also uncertainty in the protein-to-nitrogen conversion factor of 6.25 leading to inaccuracy in the absolute protein content reported.

After aqueous extraction, the *B. dubia* had the lightest (light yellow), and the *T. molitor* the darkest, colour (dark brown) among all insect supernatant solutions. The colour of *A. diaperinus, Z. morio and A. domesticus* supernatant solutions was comparable. This visual observation indicated that chemical reactions took place during processing. Preliminary experiments showed that colour formation was most likely due to enzymatic browning reactions. In addition, the colour of residue and pellet fractions was similar to that of the supernatant fractions.

3.4. SDS-PAGE

The reduced SDS-PAGE using 12.5 % acrylamide gels results show a range of protein bands of the supernatant fractions < 95 kDa, and that of the pellet fractions < 200 kDa for all five insect species (Fig. 2). Five major groups of protein bands could be distinguished in Fig. 2, namely bands ≤ 14 kDa, 14 - 32 kDa, 32 - 95 kDa and > 95 kDa. Due to insolubility in sample buffer, protein bands of the residue fractions were absent on the gels used in this experiment.

Based on intensity, the bands ≤ 14 kDa were abundant, especially for *T. molitor*. SDS-PAGE analysis using 20 % acrylamide gels showed that the band ≤ 14 kDa consisted of a range of protein bands from 6.5 kDa to 14 kDa for all insect species studied (results not shown). For *T. molitor,* the bands ≤ 14 kDa could possibly originate from anti-freeze type of proteins ranging from 8.5 - 13 kDa, including hemolymph proteins having a molecular weight ~12 kDa (Graham, Liou, Walker, & Davies, 1997); (Liou, Thibault, Walker, Davies, & Graham,

1999); (Graham, Tang, Baust, Liou, Reid, & Davies, 2001). For the other insect species studied, no literature is available for specific proteins, not for those ≤ 14 kDa but also not for those > 14 kDa.

Next, the bands observed ranging from 14 to 32 kDa could possibly originate from *T. molitor* cuticle proteins with molecular weights predominantly between 14 and 30 kDa (Andersen, Rafn, Krogh, Hojrup, & Roepstorff, 1995), *e.g.* chymotrypsin-like proteinase (24 kDa) (Elpidina, Tsybina, Dunaevsky, Belozersky, Zhuzhikov, & Oppert, 2005),.

The bands observed ranging from 32 to 95 kDa in the *T. molitor* supernatant fractions could possibly be linked to enzymes and other proteins, *e.g.* melanization-inhibiting protein (43 kDa), β-glycosidase (59 kDa), trypsin-like proteinases (59 kDa), and melanization-engaging types of protein (85 kDa) (Ferreira, Marana, Terra, & Ferreira, 2001); (Zhao, Soderhall, Park, Ma, Osaki, Ha, et al., 2005); (Prabhakar, Chen, Elpidina, Vinokurov, Smith, Marshall, et al., 2007); and (Cho, Choi, Moon, Kim, Kwon, Homma, et al., 1999).

Above 95 kDa, no bands were observed in the supernatant fractions of *T. molitor*. Compared to *T. molitor*, the pattern of protein bands from supernatant fractions in *A. diaperinus* and *A. domesticus* were similar, but not identical. For *Z. morio* and *B. dubia*, more bands were found in the range of 30 to 95 kDa.

The observed bands with molecular weight > 95 kDa in the pellet fractions of *T. molitor* possibly originate from vitellogenin-like protein with a molecular weight of 160 kDa (Lee, Lee, Choi, Cho, Kwon, Kawabata, et al., 2000). No subunit structures of the proteins mentioned were found using UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5), so that actual molecular weight reported in literature is similar to apparent molecular weight on gel.

Besides the proteins mentioned before, proteins incorporated in the exoskeleton and muscle proteins are present in the five types of insects and in the fractions obtained. For the adult stage of *A. domesticus* and *B. dubia* muscle proteins include insect flight and leg muscles, which mainly consist of large size proteins, *e.g.* M-line protein, (flight and leg muscle, 400 kDa); kettin (leg muscle isoform, 500 kDa); kettin (flight muscle isoform, 700 kDa) (Bullard & Leonard, 1996); (Lakey, Ferguson, Labeit, Reedy, Larkins, Butcher, et al., 1990). For the larval stage of *T. molitor, A. diaperinus* and *Z. morio* skeletal muscles, which likely consist of large size proteins, are present.

3.5. Protein functionality measurements

Due to the insolubility of the pellet and residue fractions, only the supernatant fraction of the protein was tested for its functionality with respect to foamability, foam stability, and gelation.

3.5.1. Foamability and foam stability

As a reference for the foam stability measurements, albumin from chicken egg white was used at a concentration of 1.5 % w/v. The reference sample is a good stabilizer for foam, and was capable of producing foam with a half-time of 17 minutes. *Zophobas morio* formed foam at pH 3, 7 and 10 with a half-time of 6 minutes, *A. domesticus* at pH 3 with a half-time of 4 minutes, and *B. dubia* produced foam at pH 5 with a half-time of 5 minutes. Foams with half-time of < 6 minutes are not considered to be stable foams. All other supernatant fractions had negligible foam ability at a concentration of 3 % w/v, at pH 3, 5, 7, and 10. This may be due to the protein concentration in the supernatant fraction solution (around 1.7 % w/v) being too low to generate stable foam. The stability of the foam can be influenced by protein structure, protein concentration, and ionic strength. In addition, the stability of the foam can be also influenced by presence of oil. As mentioned by (Lomakina & Mikova, 2006), the effect of oil

355 at levels above 0.5 % reduced the volume of egg white foam. In our case, the supernatant
356 fractions obtained from five insect species also contained some amount of oil in
357 concentration of around 0.1 %, which may also influence foamability of proteins in
358 supernatant fractions.

359 *3.5.2. Gelation*

360 *3.5.2.1 Visual observation of gelation*

361 The visual appearance was determined of gels of five supernatant fraction solutions, with
362 fraction concentrations of 3 and 30 % w/v, at pH 3, 5, 7, and 10, after heating for 10 minutes
363 in a water bath at 86 ± 1 ^{o}C (Table 3). A heating time of 20 and 30 minutes was also tested,
364 but no differences were seen in gel formation (not shown). Factors affecting the gel
365 properties in general are pH, protein concentration, and thermal treatment. The protein
366 concentrations selected for gelation are in the range from 0.5 to 25 % concentration that are
367 used in general to make gels. At a concentration of 3 % w/v, none of the protein fractions
368 showed gel formation, except for *A. domesticus* at pH 7. At pH 5 and pH 7, for all samples
369 (except *A. domesticus* at pH 7) heating induced the formation of visible large aggregates
370 rather than gel formation.

371 All 30 % w/v supernatant fractions formed a gel at pH 7 and 10, but not at pH 3. At pH 5,
372 very weak gels were formed, that yielded when turned upside down. In table 3, these samples
373 are designated as "V" (viscous fluid). All samples at pH 7 and 10 were turbid, indicating that
374 the characteristic size of the structures forming the gel was larger than the wavelength of
375 visible light. All gels were already formed after 10 minutes and longer heating times had no
376 influence on the appearance of the gel.

377 Some insect proteins have an isoelectric point of about 5. For instance, the pI of proteins from
378 silkworm (*Bombyx mori)* and spider (*Nephila edulis)* are 4.37 - 5.05, and 6.47, respectively

(Foo, Bini, Hensman, Knight, Lewis, & Kaplan, 2006). If our protein fractions also have a pI of around pH 5, this may explain why all fractions at this pH formed aggregates at a concentration of 3 % w/v, and very weak gels at concentrations of 30 % w/v. Close to the pI, the electrostatic interactions between the proteins are very weak, which, upon denaturation, tends to lead to the formation of dense aggregates. These dense aggregates have a much higher gelling concentration than aggregates formed at a pH above or below the isoelectric point. To form a firm gel at this pH, higher protein concentrations are needed.

Samples at pH 3 and 10 at 3 % w/v were more transparent than samples heated at pH 5 and 7. The increased charge on the protein at pH 3 may prevent the proteins from aggregating, since even at 30 % w/v these fractions did not form a gel or even a viscous fluid. The decrease in turbidity observed at pH 10 suggests that the aggregates formed at this pH were less dense and/or smaller than the ones formed at pH 5 and 7.

3.5.2.2. Rheological properties of gels

According to the visual observation of gelation, at a pH of 7 and a concentration of 3 % w/v a weak gel was formed, and at 30 % w/v a strong gel was formed. Therefore, for studying gel strength, fraction concentrations in between these two values (7.5 and 15 % w/v) were chosen. For all five fractions, we determined the evolution of the storage modulus G' and loss modulus G" during the temperature ramp at a concentration of 15 % w/v and a pH of 7. The storage modulus is a measure for the elastic energy stored reversibly in a gel during deformation, and characterizes its stiffness; the loss modulus is a measure for the energy dissipated during deformation as a result of viscous friction. As an example, the results for the mealworm supernatant fraction (the other fractions showed similar results) are provided (Fig. 3A). G' gradually increased during the heating phase of the ramp. During the second phase, when the temperature was kept constant at 90 °C, G' kept on increasing gradually. This observation showed that the gel structure did not yet reach an equilibrium state. During

the cooling phase, both G' and G" increased sharply. This is typical for gels in which hydrogen bonds are formed between structural elements (Ould Eleya, Ko, & Gunasekaran, 2004). The gelation temperature observed ranged from about 51 °C to 63 °C (*T. molitor* 61.7 ± 1.1°C , *A. diaperinus* 58.2 ± 2.1 °C, *Z. morio* 51.2 ± 1.5 °C, *A. domesticus* 56.2 ± 0.7 °C, *B. dubia* 63.2 ± 0 °C, from which the lowest and the highest temperature were from *Z. morio* and *B. dubia* supernatant fractions respectively (results not shown).

To obtain more information on the gel structure, the value of log G' of *T. molitor* supernatants was determined as a function of log C (concentration) with fraction concentrations of 7.5 % w/v, 15 % w/v and 30 % w/v (corresponding to actual protein concentrations of 4.1 %, 8.3 % and 16.6 %) at 90 °C and 20 °C (Figure 3B). Values for G' at 90 ^{o}C were taken from end of phase 2 from the ramp, and values at 20 °C were taken from the end of phase 3, which is similar to the procedure of (Ould Eleya, Ko, & Gunasekaran, 2004). The values of the power-law exponent n in the scaling relation $G' \propto C^{n}$, were used for evaluation of gel structure (Shih, Shih, Kim, Liu, & Aksay, 1990). The parameter n had a value equal to 3.0 ± 0.4 at the end of the isothermal stage at 90 ºC, and a value of at 2.8 ± 0.6 from the end of the cooling stage at 20 ºC. These two values are comparable, so there were no significant structural rearrangements in the gel network upon cooling of the samples. An exponent n of about 2.8 is typical for fractal protein gels and points to a fractal dimension d_f which is close to 2 (Ould Eleya, Ko, & Gunasekaran, 2004).

Fig. 3C shows G' at the end of phase 3 of the temperature ramp as a function of strain, for insect supernatant gels at 20 °C and a concentration of 15 % w/v. The value for G' in the linear response region of *A. domesticus* supernatant gels was around 2500 Pa, which was almost 1.5 times stronger than that of *B. dubia* (around 1600 Pa), 6 times stronger than that of *Z. morio* (around 390 Pa), and 25 times stronger than that of *T. molitor* (around 100 Pa) and *A. diaperinus* (around 140 Pa). In interpreting these results, we must be careful, since the

actual protein concentrations in the fractions was lower than 15 % w/v, and differed slightly from fraction to fraction. As seen before, the actual protein contents were for *T. molitor* 8.3 %; *A. diaperinus* 9.2 %; *Z. morio* 7.6 %; *A. domesticus* 9.2 % and *B. dubia* 7.4 %.

Several conclusions can be drawn from these results. Although the *B. dubia* supernatant sample had the lowest actual protein content, it formed the strongest gels among all other three insect species, except *A. domesticus*. Supernatants from *A. diaperinus* and *A. domesticus* had similar protein concentration, but they showed significant differences in gel strength. In addition, supernatants from *B. dubia* and *A. domesticus* that were in the adult stage formed relatively stronger gels than the other three insect species that were in the larvae stage. Apparently, the insect growth stage influences the body protein composition, and different species differ in protein type and structure (Wilson, 2010).

All insect gels had a comparable maximum linear strain at supernatant fraction concentration of 15 % w/v, with a value of around 50 %. An example is shown for *Z. Morio* (Fig. 3C). The maximum linear strain is, of course, dependent on heating rate and protein concentration, and it would therefore be interesting to investigate the concentration dependence of this property, since it can provide additional information on the fractal dimension of the gels.

These detailed rheological results show that insect proteins can form gels that have similar properties as those formed from conventional food proteins. It therefore shows that insect proteins have indeed functionalities that are desirable for food application.

4. Conclusions

Proteins were extracted from five insect species and protein purity and yield of the obtained fractions was evaluated: Around 20 % of total protein was found back in the supernatant, the rest of the protein was divided about equally over the residue and the pellet fraction for all five insect species after aqueous extraction. The extraction method is easy and feasible to

453 apply, but the yield of extracted supernatant fractions is relatively low. The purity of
454 measured protein content expressed as percentage of dry matter ranged from 50 % to 61 % of
455 supernatant fractions, from 65 % to 75 % of pellet fractions and from 58 % to 69 % of residue
456 fractions depending on the insect species.

457 We established some functional properties of the protein fractions, focusing on foaming and
458 gelation: The soluble protein fractions of all five types of insects had poor foaming capacity
459 at pH 3, 5, 7, and 10, but could form gels at a concentration of 30 % w/v. At a concentration
460 of 15 % w/v at pH 7 and 10, *A. domesticus* supernatant formed the strongest gels among all
461 insect species. The gelation temperature ranged from about 51 °C to 63 °C for all insect
462 species at pH 7. In addition, all insect gels had a comparable maximum linear strain at this
463 concentration, with a value of around 50 %.

464 We studied the protein quality of whole insects by analysis of protein content and amino acid
465 composition. The protein content of the five insect species was comparable to conventional
466 meat products in terms of protein quantification. The sum of EAA per g protein for all insect
467 species was comparable with the sum of EAA for soybean protein, lower than that for casein,
468 but higher than that for the daily protein requirement of an adult (FAO/WHO/UNU, 1985).
469 Differences in calculated EAAI were similar.

470 Although differences are observed in protein content, amino acid composition, protein
471 distribution of the fractions obtained, SDS-PAGE data, foaming and gelation properties, the
472 similarities between the insect species are more apparent than the differences. The fact that
473 gels could be formed for all five insect species, using the soluble fractions obtained by a
474 simple aqueous extraction procedure, is promising in terms of future food applications. More
475 research is needed for developing further extraction and purification procedures, and for more
476 detailed insight into functional properties.

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605

Table 1. Proximate composition of five insect species on live weight basis (mean ± S.D., n=2).

Table 2. Amino acid pattern of five insect species, casein, soybean protein, recommendation for adult and calculated essential amino acid index of five insect species and casein & soybean protein (FAO/WHO/UNU, 1985) and (Young & Pellett, 1991).

Table 3. Gel formation of supernatant fractions from five insect species (X: no gel formation; A: aggregation; V: viscous fluid; O: gel formation).

Table 1.

Insects	Moisture (%)	Fat (%)	Crude protein (%) (including chitin nitrogen)	Other components (%) (*e.g.* carbohydrates, minerals and vitamins)
T. molitor	63.5±1.8	9.9±1.0	19.1±1.3	7.5±2.2
A. diaperinus	64.5±1.0	8.5±0.2	20.6±0.1	6.4±1.0
Z. morio	59.9±5.4	16.0±0.7	20.7±0.3	3.4±5.5
A. domesticus	70.8±2.0	3.6±0.4	21.5±0.5	4.1±2.1
B. dubia	67.4±2.1	7.7±0.1	19.3±0.9	5.6±2.3

[1]

[1] Table 1. Proximate composition of five insect species on live weight basis (mean ± S.D., n=2).

Table 2.

unit (mg/g crude protein)	*A.diaperinus*	*T.molitor*	*Z.morio*	*A.domesticus*	*B. dubia*	Casein	Soybean	1985 FAO/WHO/UNU
Essential amino acid (EAA)								
Histidine	34	29	31	21	23	32	25	15
Isoleucine	43	43	46	36	31	54	47	30
Leucine	66	73	71	66	56	95	85	59
Lysine	61	54	54	53	43	85	63	45
Methionine +Cysteine	26	26	24	25	23	35	24	22
Phenyl-alanine + tyrosine	120	100	111	92	93	111	97	38
Threonine	39	39	40	35	32	42	38	23
Tryptophan	12	12	14	9	8	14	11	6
Valine	58	61	63	55	52	63	49	39
Sum of EAA	459	437	454	392	361	531	439	277
Non-essential amino acid								
Alanine	66	70	68	81	71			
Arginine	54	54	54	65	46			

Aspartic acid	83	80	82	73	67		
Glutamic acid	123	109	127	110	96		
Glycine	46	50	48	51	53		
Proline	56	66	56	54	48		
Serine	40	44	42	38	34		
Sum of total AA	927	910	931	864	776		
EAAI	1.65	1.60	1.66	1.39	1.28	1.93	1.56

[2]Table 2. Amino acid pattern of five insect species, casein, soybean protein, recommendation for adult and calculated essential amino acid index of five insect species and casein & soybean protein (FAO/WHO/UNU, 1985) and (Young & Pellett, 1991).

Table 3.

	pH 3	pH 5	pH 7	pH 10
3 %				
T.molitor supernatant	X	A	A	X
A.diaperinus supernatant	X	A	A	X
Z.morio supernatant	X	A	A	X
A.domesticus supernatant	X	A	O	X
B.dubia supernatant	X	A	A	X
30 %				
T.molitor supernatant	X	V	O	O
A.diaperinus supernatant	X	V	O	O
Z.morio supernatant	X	V	O	O
A.domesticus supernatant	X	V	O	O
B.dubia supernatant	X	V	O	O

[3]

[3] Table 3. Gel formation of supernatant fractions from five insect species (X: no gel formation; A: aggregation; V: viscous fluid; O: gel formation).

Figure Captions and Tables

Fig. 1. Protein content of supernatant, pellet and residue fractions expressed as percentage of total protein and total recovery (n=2).

Fig. 2. Molecular weight distribution of *T.molitor* protein fractions, determined by SDS-PAGE using 12.5% homogeneous phastgel and (Samples from left to right: supernatant, pellet and marker); marker is ranging from 6.5 kDa to 200 kDa. Mw is molecular weight.

Fig. 3. A: Dynamic moduli G' and G" of T. molitor supernatant solution as a function of time. Heating and cooling phases are plotted as a secondary axis. B: Plots of the storage modulus G' as a function of protein concentration of mealworm supernatant fractions on a logarithmic scale at pH 7 (heating period 90 °C and cooling period 20 °C). C: Storage modulus G' (Pa) as a function of strain γ % for insect supernatant gelation at 20 °C at a supernatant fraction concentration of 15 % w/v.

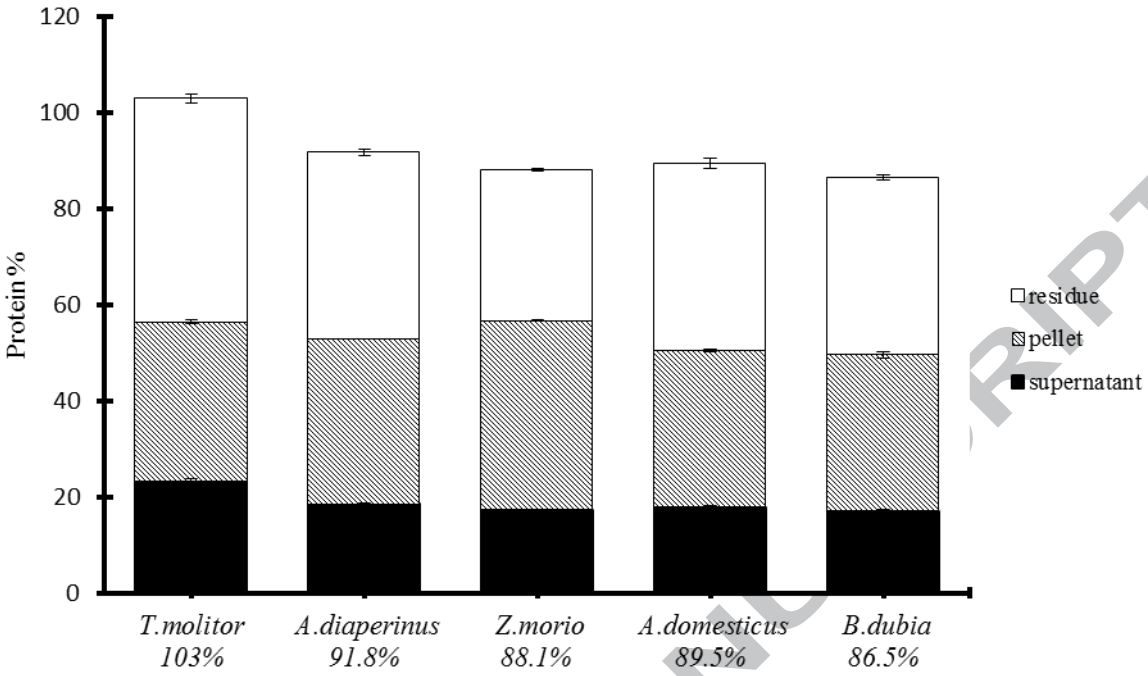


Fig. 1.

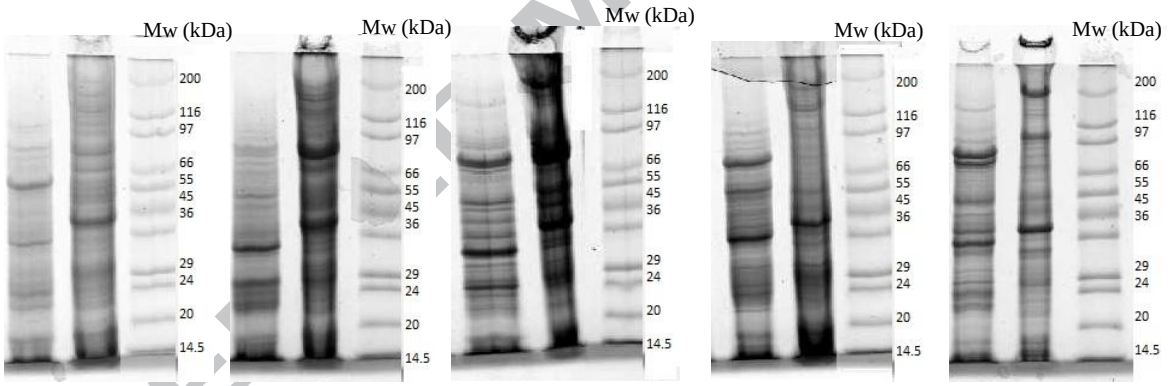
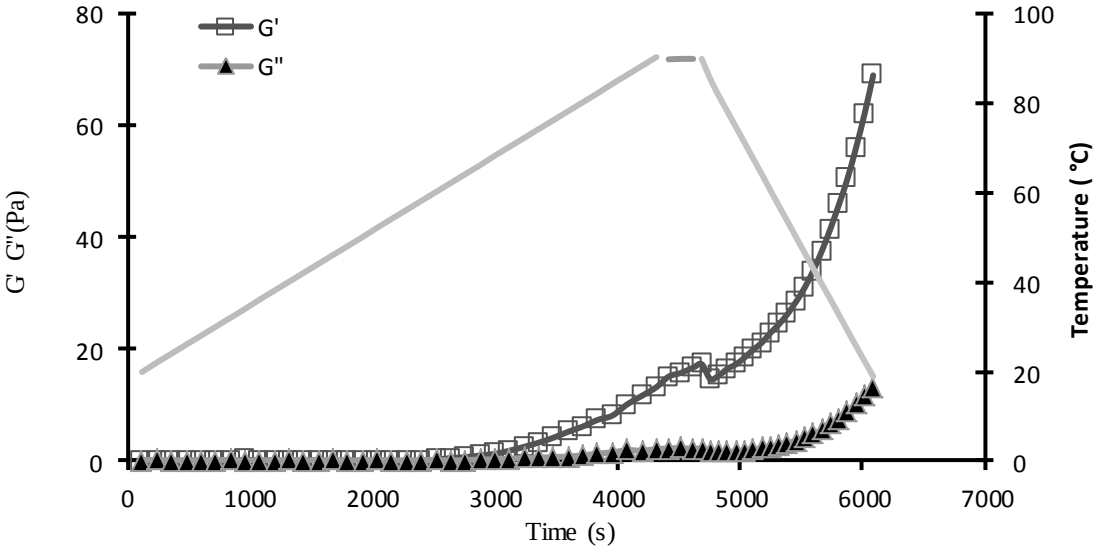
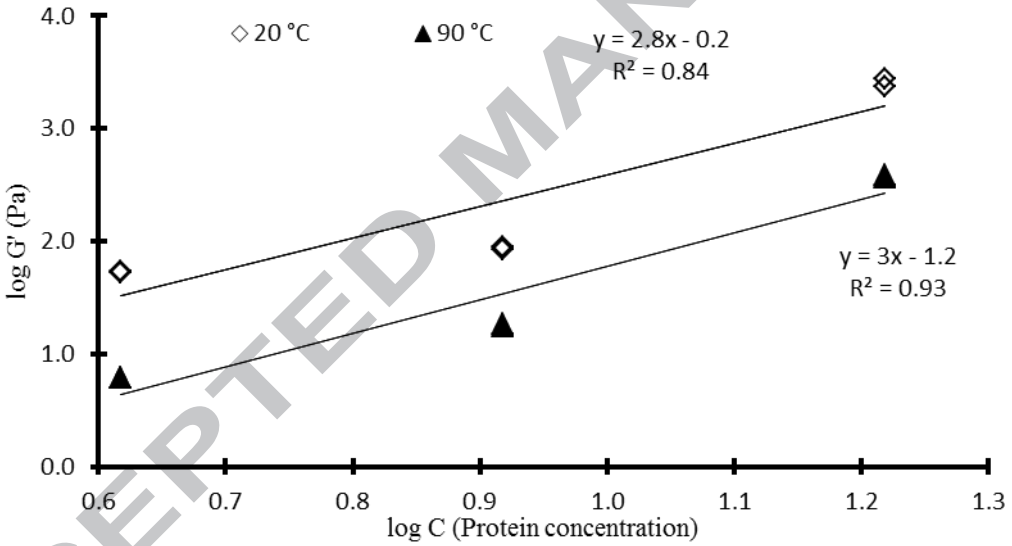


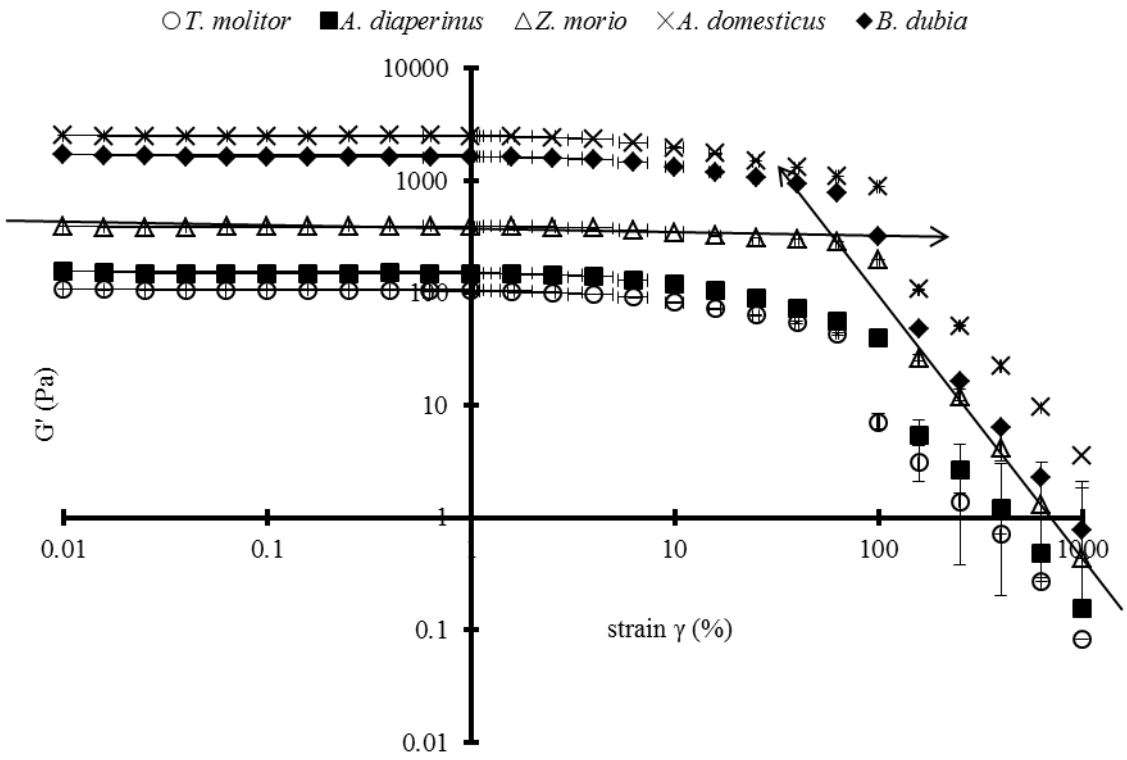
Fig. 2.



A



B



C

Fig. 3.

Highlights

- Crude protein content of insects was similar to conventional meat products.
- The amount of EAA of insects was higher than daily protein requirement of an adult.
- The supernatant, pellet, fat and residue fractions were obtained after an aqueous extraction.
- Protein bands were < 95 kDa for supernatant fractions and < 200 kDa for pellet fractions.
- Most supernatant fractions did not foam, but could form gels depending on protein concentration and the pH.