Microscopic Structure of Edible Insects Using LM and SEM

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Abstract

According to the valid legislation, insects are classified as novel foods, and they have already appeared on the market as a part of some products or individually. A number of methods are used to verify food authenticity, and microscopy is one such possibility. Microscopic methods work based on visual-image evaluation, so they can be a suitable method for identifying insects in food. The necessary prerequisite for proper identification is knowledge of the structures found in the insect body and the capability to compare them with other structures that are a common food component. The aim of this work was to describe the characteristic structure of the cuticle of the mealworm*(Tenebrio molitor).* The anatomical-morphological structure and the main features of this insect were studied using light microscopy (LM) and scanning electron microscopy (SEM).

microscopy, cuticle, new food trends, Tenebrio molitor

Introduction

Insects have become very popular as food in recent years for several reasons. In addition to being a food with high-value, environmental aspects are also gaining in importance. These aspects include lower emissions, lower feed and water consumption, and less demand for agricultural land (van Huis, 2013; Steinfeld, 2006). Insects and their derived products have huge dietetic potential. Protein content in their dry matter varies between 25% and 75% depending on the insect species (Finke and Oonincx, 2017), where its quality is determined by the representation of amino acids, including essential amino acids (Wu et al., 2020). Fats are the second significantly represented component, the content of which in dry matter ranges from 15% to 32% (Paul et al., 2017). Other nutritional benefits include the content of vitamins and minerals. According to some studies, insects are a good source of iron, copper and zinc. They also contain B vitamins, where differences between species are considerable. To illustrate, in the flour beetle, B12 is present at 1.08 g/100 g in dry matter, while for cockroaches, it is up to 13.2 µg/100 g in dry matter (Schmidt et al., 2019). Chitin is one of the key polysaccharides in the insect body, which, together with proteins, forms the cuticle. After consuming insects, it acts like fibre and is virtually indigestible to humans (Baiano, 2020).

From many points of view, both in terms of nutritional composition and ecological impact, it would be easy to classify insects as food and enjoy benefits at all levels; however, such consumption is still unacceptable to many consumers. The whole insect can be repulsive and unattractive. Maybe that is why many manufacturers are trying to incorporate this raw material into food in the form of various flours and fractions to make the product more attractive.

According to the applicable European legislation, Regulation (EC) 2283/2015 of the European Parliament and of the Council, it has been possible to produce insects as a novel food since January 2018, which is probably one of the reasons why many companies offering these products have emerged in the last few years. This regulation relates to the European Commission Implementing Regulation establishing a list of authorised foods. Insects are used whole or in the form of flour in different fractions. Flour is then used to produce confectionery or bakery products. Most insect products on the market come in the form of

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bars, biscuits, pasta, crackers, pastries, or loose mixtures. Some manufacturers use insects only partly, and add only protein extracts to their food. Under Regulation (EU) 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers, there is an obligation to indicate information about food composition. Besides, insects are potentially allergenic foods (De Castro, 2018). In order to prevent adulteration and ensure food safety, it is necessary to lay down verification procedures and methods for official controls.

Microscopic methods provide good results in the form of an image, and seem suitable for evaluating the food structure. The most commonly used method, i.e. light microscopy, together with targeted or clear staining, provides information on structure and morphology (Pospiech et al., 2011). Knowledge of cell organisation into typical tissues can be used to detect insects in food as well. As a characteristic feature, the insect body is divided into 3 parts: head, thorax, and abdomen. The thorax includes locomotor organs – legs and possibly wings. The body is oxygenated by means of cuticular spiracles located on the body surface. Tracheoles branch down to the smallest tubes and must provide a smooth exchange of gases with the body. This is a passive system where oxygen is received, and CO_2 is excreted simultaneously (Loudon, 1989). Gas diffusion occurs due to changes in internal pressure; in addition, it is also affected by movement. Movement is provided by the muscular system (Wigglesworth and Lee, 1982; Westneat, 2003). According to its function and location, the muscular system of insects is divided into two groups: visceral and skeletal. Skeletal muscles provide movement similarly to vertebrates and, among other things, have an important function in the period of so-called ecdysis – the period when the cuticle changes (Gillot, 2005). Insects only have striated muscles (Gullan and Cranston, 2014). The malpighian tubule is the most important organ of the insect excretory system. It provides osmotic and excretory function. With its blind ends, this tubular organ is connected to the posterior and middle intestine (Lipovšek et al., 2016). The digestive system of insects is divided into three parts:stomatodeum, mesenteron and proctodeum.. Chyme in the mesenteron intestine is separated by a peritrophic layer that passes into a lumen containing numerous folds and microvilli for the better absorption of nutrients. The peritrophic layer is partly composed of chitin molecules (Klowden, 2008). Chitin structures can also be found in the insect integument, i.e. the covering system. This can be divided into skin and cuticle. The cuticle contains several layers (Finke and Oonincx, 2017). The covering system plays an important role during ecdysis, where the old cuticle is replaced by a new one. This process takes place with the participation of hormones and the muscular system.

This paper aims to describe the microstructure of the mealworm(*Tenebrio molitor)* with light microscopy using histological staining of Mayer's haematoxylin and with scanning electron microscopy. The microstructure was described for larvae and flour prepared from the mealworms.

Material and methods

For analysis, a species of the larva of the mealworm beetle (*Tenebrio molitor*) was purchased. Live insects were killed by lyophilisation and placed in a fixative fluid. For light microscopy (LM) and scanning electron microscopy (SEM) examinations, a solution according to the methodology of Sánchez-Parda et al. (2008) and a 3% solution of glutaraldehyde were used, respectively. After 24 hours of fixation, the samples were dehydrated with an ascending series of alcohols. The sample for LM was placed from alcohol into xylene, and then saturated with paraffin. Paraffin blocks usable for slicing on a 2255 RM rotary microtome (Leica, Germany) were formed from the sample. Micro-sections were taken from the water surface on slides and stored in a thermostat before histochemical staining. Paraffin was removed from the samples by washing in xylene. Subsequently, the sections were stained by histochemical staining for mucin according to Mayer (Sigma Aldrich, USA). Washing in running water for 10 minutes, staining with haematoxylin for 10 minutes, staining with a 5% aqueous solution of methanyl yellow for 5 minutes, rinsing with distilled water, and immersion in an aqueous solution of 10% mucocarmine for 60 minutes. The sections were washed in distilled water before dehydration in alcohol and clarification in

xylene. After clarification, the samples were embedded in solacrylic. The chemicals used were from Merci (Czech Republic).

Before dehydrating, the samples for SEM were processed in the same way as those for LM. After dehydrating, the samples were dried to the CO2 critical point value of the Emitech K850 device (Quorum Technologies, United Kingdom). After drying, the samples were fixed with carbon tape to the SEM targets. The fixed samples were transferred to a Q150R ES sputter coater device (Quorum Technologies, United Kingdom) for sample plating. The sample was gold-plated at 10 nm and prepared for analysis under a MIRA3 microscope (Tescan, as., Czech Republic).

Results and Discussion

Samples of whole insects, as well as samples of flour, were examined by LM using histochemical methods. A larger number of structures were identified in samples of whole insects than in the monitored flour. The insect flour contained clear parts of the cuticle; muscle cells, fat, and part of a malpighian tubule were also identified; see Table 1.

Table 1: Insect structures detected by LM and SEM

	LM		SEM
Structures	Whole insect	Insect flour	Whole insect
Cuticle	+	+	+
Muscles	+	+	+*
Fat	+	+	+*
Spiracles	+	-	+
Tracheoles	+	-	+*
Malpighian tubules	+	+	-
Nervous ganglia	+	-	-

+ positive; - negative; *after body crack

For a microscopic description of the whole insect, staining according to Mayer was then used, employing the haematoxylin basic dye and staining the internal structures in shades of pink. Other parts are stained in dark pink – mucus, black for the cell nuclei, and yellows for other structures (Locquin and Langeron, 1983). After histochemical processing, the morphological and anatomical arrangement could be evaluated from the light microscope. The separation of the head from the body is well-marked. The insect surface is highly specific. The body is covered with a layer of cuticle, which separates from the skin in the same way as Merzendorfer (2003) states. At higher magnifications, individual layers of the integument are observable, see (Plate XX, Fig. 1). The epidermis consists of a single layer of cells with distinct nuclei, which stain brown to black due to the chosen staining. Towards the outer margin, the cuticle is divided into three parts: endocuticle, exocuticle, and epicuticle (Clark and Triblehorn, 2014; Moussian, 2010; Barbakadze, 2006; Wigglesworth, 1948). In some places, a wax layer appears that functionally contributes to protecting the body and prevents the larva from drying out (Pushkin et al., 2018). It appears to be of epicuticular origin and is composed predominantly of lipids. In (Plate XX, Fig. 1), it can be seen as a unified yellow line.

The body interior is separated from partially digested food by a lumen with a peritrophic membrane (Plate XX, Fig. 2, e); subsequently, it passes into the muscular part with areolar tissue (Pushkin et al., 2018). Figure 2 shows that, in addition to the developed muscular system, a relatively large amount of fat is present. In general, proteins and fats have the largest share in the composition of edible insects and insects, so it is no wonder they are found in such quantities.

Structures were identified for the whole mealworm by the SEM method, see Table 1. Some parts were possible to observe only after intentional body disruption. Without partial disruption, tracheoles (Plate XX, Fig. 3, E) and adjacent tissues would not have been possible to observe. The muscle surrounding the tracheoles has a typical structure; fat is present as well. In cross-section, it is possible to observe the cuticle separating from the skin (Plate XX, Fig. 3, D). Without intervention, the surface of the larva is firm, compact, and mainly fulfils a covering function. The cuticular surface, covered with a wax layer, is

porous – with numerous airholes located on the sides of the insect body. The opening and closing of the spiracles is controlled by muscles. The hole is raised along the edges (Plate XX, Fig. 3, F) and separated from the trachea it self by the atrium (Ras et al., 2018). The septum is covered with a large number of papillary protrusions of various sizes. Spiracles were located randomly, and their location does not appear to be standardised (Newton, 2020).

For SEM analysis, the insects were not observed in longitudinal section; therefore, the internal structures were not described. Using SEM, it was possible to detect the oral system, antennas, the thorax containing the prothorax with a pair of legs, and the abdomen (Silva Soares et al., 2018). The segmented body was noticeable.

Conclusion

Even though not all parts of edible insects have been described by microscopic methods, we are able to determine their presence in food. In particular, knowledge of the cuticle structure, which is highly specific for insects, provides proof. Histochemical staining used according to Mayer was confirmed as qualitative method suitable for specific proof of the insect cuticle, both in larval form and in the form of flour.. In addition, electron microscope images show surface integrity, shape, and provide an overview of the appearance and size of the mealworm larva. The results of both methods show that they can be used for further studies in the field of entomology and food adulteration.

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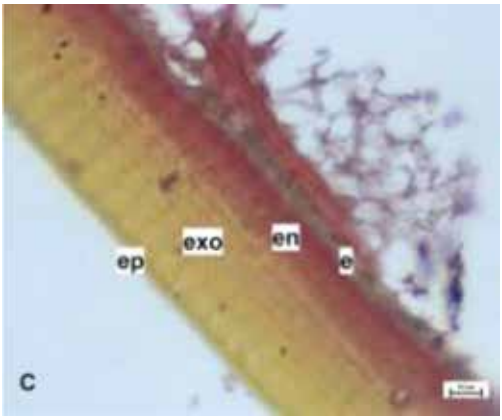

Fig. 1. Insect cuticle

Caption
Head part (A), mouthpart a, glands b, body part (B) cuticle c, fat d, peritrophic membrane e, midgut f, muscles g, cuticle (C), epicuticle ep, exocuticle exo, endocuticle en, epidermis e.

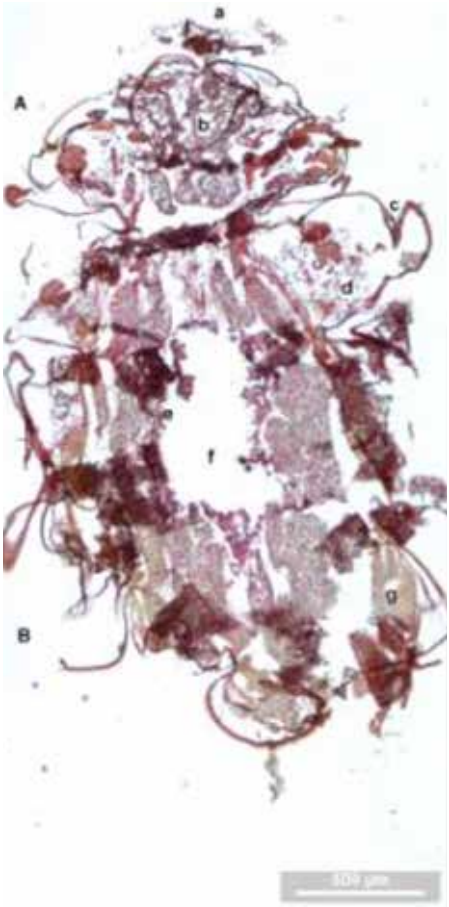

Fig. 2. Microstructure of larvae (Tenebrio molitor)

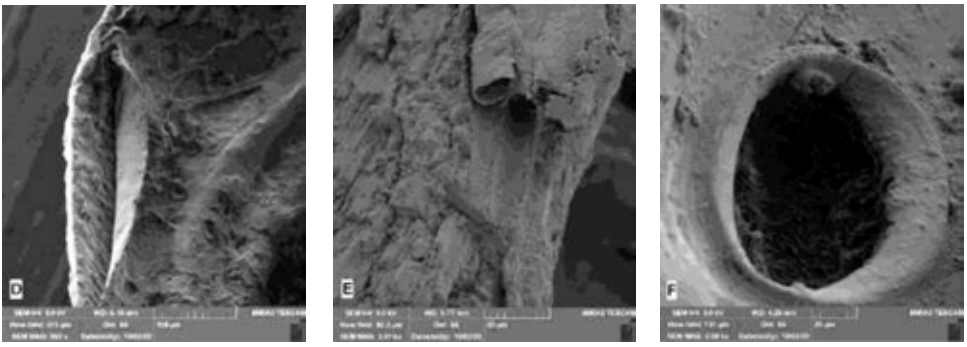

Fig. 3. Part of the insect body by SEM

Caption
Cuticle (D), tracheoles with muscle cover (E), spiracle (F)