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Short communication

The presence of α-chitin in Tardigrada with comments on chitin in the Ecdysozoa

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ABSTRACT

We used Fourier Transform Infrared Spectroscopy (FT-IR) to characterize for the first time chitin in the cuticle of a eutardigrade (*Macrobiotus* cf. *hufelandi*). Analysis of the isolated cuticles of single individuals and comparison with commercial α-chitin isolated from shrimp shell and β-chitin from squid pen revealed that the amide I band was split into two peaks characteristic for α-chitin. In the current literature cuticles containing α-chitin are considered as an apomorphic character of the Ecdysozoa (Cycloneuralia, Panarthropoda). This is a plausible assumption, although α-chitin has been unequivocally demonstrated only in the cuticle of the Panarthropoda, i.e. Onychophora, Tardigrada (this article) and Arthropoda, and in the Priapulida (Cycloneuralia), whereas chitin in the cuticle of the other cycloneuralian taxa either was not further specified or appears to be absent.

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1. Introduction

Tardigrada is a monophyletic group of microscopic metazoans living in a variety of permanent (e.g. deep sea, psammon of the marine and freshwater environments) or temporary (soil, lichens, mosses etc.) aquatic habitats. Following the current phylogenies, Tardigrada belong to the monophyletic clade Ecdysozoa, well supported by numerous molecular analyses, which includes all animals that periodically shed their cuticle as they grow (e.g. Aguinaldo et al., 1997; Mallatt et al., 2004; Telford et al., 2008; Dunn et al., 2008; Borner et al., 2014). Ecdysozoa are divided into two groups, the widely accepted Panarthropoda comprising Arthropoda, Onychophora, Tardigrada and the Cycloneuralia including Nematoda, Nematomorpha, Priapulida, Kinorhyncha. and Loricifera (see Nielsen, 2012; Westheide and Rieger, 2013). Monophyly of Ecdysozoa appears well supported (see literature cited above), whereas that of Cycloneuralia and Panarthropoda is less clear, for details, controversial discussions, and different clustering of certain groups see for example Aguinaldo et al. (1997), Dunn et al. (2008), Hejnol et al. (2009), Paps et al. (2009), Meusemann et al., 2010; Rota-Stabelli et al. (2010), Campbell et al. (2011), Borner et al. (2014), and Yamasaki et al. (2015).

The cuticle of Ecdysozoa is structurally extremely diverse depending on the taxon considered. Generally it consists of at least three layers often called epicuticle, exo- and endocuticle – summarized in Schmidt-Rhaesa et al. (1998) and modern textbooks of Zoology (e.g. Westheide and Rieger, 2013) –, and contains various proteins and in most taxa chitin (poly-β-(1-4)-*N*-acetyl-D-glucosamine). Chitin is a widespread biopolymer occurring in three different crystalline forms that are classified according to the different orientations of its polymeric chains, i.e. α—(antiparallel chains), β—(parallel chains), and γ—(combination of parallel and anti-papal chains)-chitin.

Although α-chitin appears to be the most common form in animals and in nature generally, also β-, and γ-chitin have been demonstrated in animals (Jeuniaux, 1975, 1982; Rudall and Kenchington, 1973; Neville 1975; Muzzarelli, 1977; Jang et al., 2004). Modern textbooks of Zoology (e.g. Westheide and Rieger, 2013) and summarizing articles (e.g. Schmidt-Rhaesa et al., 1998) suggest that the ecdysozoan cuticle typically contains α-chitin, most often in layers close to the epidermis, which character obviously is considered an autapomorphy. However, checking the literature it appears that in most Ecdysozoa the presence of this specific form of chitin is implicitly foreseen rather than explicitly demonstrated with adequate techniques. This holds also for the cuticle of Tardigrada, in which only the presence of chitin was

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demonstrated without specifying its form (see Baccetti and Rosati, 1971; Bussers and Jeuniaux, 1973a,b; Jeuniaux, 1975; Greven and Peters, 1986; Kristensen and Neuhaus, 1999).

In the present article we tried to demonstrate the type of chitin in the cuticle of a eutardigrade via Fourier Transform Infrared Spectroscopy (FTIR) and summarize briefly what is known about chitin in other Ecdysozoa.

2. Material and methods

2.1. Sample collection

Eutardigrades (*Macrobiotus* cf. *hufelandi*; see Fig. 1) were extracted from mosses from a stall roof in a low mountain range (Eifel, Germany). As eggs were not found in the samples a precise determination was not possible. Voucher specimens mounted in polyvinyl-lactophenol are deposited in the Zoological Museum, Arachnology, Center of Natural History, University of Hamburg (Acc. No. ZMH A1/16). Animals extracted from the moss were rinsed in distilled water and then transferred in hot ethanol (70–90%).

2.2. Isolation of chitin

Tardigrades were kept in 5 M HCl-solution at room temperature for 40 min, then transferred into distilled water and then treated with 4 M NaOH for 1 h. Then, individual specimens were rinsed again with distilled water, bleached by using 0.5% sodium hypochlorite (NaClO) for 30 s, and rinsed several times to remove the NaClO. After this procedure the remnant (a tiny white particle) was transferred on the plate of the FT-IR machine (see below). The preparation steps were carried out at room temperature. To minimize possible experimental error more than ten analyses were carried out in the same way using a single specimen per analysis. In each run, almost the same absorbance bands were observed in the FT-IR spectra.

Commercial α-chitin isolated from shrimp shell Commercial α-chitin isolated from shrimp shell (SIGMA-ALDRICH with Pcode: 1001416772) and β-chitin from squid pen, from our collection in Aksaray University Central Laboratory, generated in the same way as the chitin of the tardigrades, were used for comparison.

2.3. Fourier transform infrared spectroscopy (FT-IR)

The IR spectra of chitin from the tardigrades, commercial α-chitin and β-chitin from squid pen were conducted via an ATR FT-IR spectrometer (Perkin Elmer 100, Massachusetts, USA) over the frequency range of 4000–625 cm^{-1}. To demonstrate the presence of chitin in any material amide I, II, and III bands are crucial. These bands are responsible for “C=O secondary amide stretch”, “N—H bend, C—N stretch” and “CH_2 wagging” respectively (Jang et al., 2004; Kumirska et al., 2010).

3. Results

FT-IR spectra of commercial α-chitin, tardigrade chitin and squid pen β-chitin are shown in Fig. 2. A more detailed analysis revealed that the amide I band of the tardigrade chitin was divided into two peaks at 1651 and 1624 cm^{-1} proving that the tardigrade chitin was present in α-form like the commercial α-chitin. The amide I band of commercial α-chitin was divided as 1655 and 1619 cm^{-1} like tardigrade chitin (Fig. 2a and b). As is seen from Fig. 2c, amide I band of β-chitin from the squid pen was represented only a single peak at 1630 cm^{-1}. Amide II and III bands of the tardigrade chitin were recorded at 1544 and 1311 cm^{-1}. All the other bands for α-chitin isolated from the shrimp shell, and the tardigrade cuticle as well as for β-chitin from squid pen are given in Table 1. Tardigrade chitin and commercial α-chitin exhibited a clear “V” (sharp) shaped amide I band, but β-chitin from squid pen possessed a“U” (wide) shaped amide I band (see Fig. 3).

4. Discussion

FT-IR spectroscopy is a well established and reliable tool to characterize chitin in any material by using very small quantity of sample and to assign chitin as α, β and γ crystal forms. The spectra show a series of absorption bands, typical of crystalline polysaccharide samples, i.e. the amide I-, the amide II-, and the amide III-band. The divided amide I band into two peaks is characteristic to α-chitin, while a single amide I band is characteristic to β-chitin. The degree of crystallinity of α-chitin is higher than that of β-chitin. Further, due to its higher crystallinity the amide I band of α-chitin is sharper (V-shaped) than that of β-chitin (U-shaped) (e.g. Jang et al., 2004; Kumirska et al., 2010). Therefore, our results clearly prove that the chitin of *Macrobiotus* cf. *hufelandi* is highly crystalline like the commercial α-chitin.

Chitin in the cuticle of tardigrades has long played an important role in the discussion about relationships of tardigrades. Already in the 19th century Kaufmann (1851) took the resistance of the tardigrade cuticle to caustic potash (potassium hydroxide) as evidence for its chitinous nature and used this finding to stress the affinity of tardigrades to arthropods. Later, however, Marcus (1928) failed to demonstrate chitin in the tardigrade cuticle using zinc iodine chloride, a technique that was considered to be specific for the detection of chitin at that time. Since then the presence of chitin in tardigrades was a matter of debate. Meanwhile, however, its presence in the tardigrade cuticle was repeatedly documented using different techniques, e.g., extraction of ultrathin section with chitinase (Baccetti and Rosati, 1971), digestion of complete tardigrades with a highly purified chitinase (Bussers and Jeuniaux, 1973a,b; Jeuniaux, 1975), and labelling with wheat germ agglutinin, a lectin with binding sites specific for *N*-acetyl-glucosamine residues, coupled with colloidal gold (Greven and Peters, 1986; Kristensen and Neuhaus, 1999). With the latter technique chitin was also demonstrated in the pharyngeal cuticle of tardigrades (Kristensen and Neuhaus, 1999). More recently the composition of the chitinous parts of the buccopharyngeal apparatus of a eutardigade was studied using the autofluorescence of chitin (see Guidetti et al., 2015; Savic et al., 2016).

Despite the remarkable structural diversity and complexity of the tardigrade cuticle, especially when comparing the cuticles of species of the two ‘classes’ Eu- and Heterotardigrada (summarized in Greven, 1984; see also the discussion in Kristensen and Neuhaus, 1999 and the literature cited above), it was always the layer closest to the epidermis, i.e. the procuticle, that contained chitin. This again prompted the authors to stress the relations of tardigrades to arthropods.

As noted already in the introduction, chitin in all three forms is widespread among animals of different evolutionary lines (e.g. Rudall, 1955; Jeuniaux, 1982; Muzzarelli, 1977; Rudall and Kenchington, 1973). To name but a few, α-chitin has been found in such diverse organism as bryozoans (Kaya et al., 2015a) and sponges (Ehrlich et al., 2007a,b), β-chitin in the chaetae of annelids (e.g. Lotmar and Picken, 1950; Rudall, 1955; Rudall and Kenchington, 1973) and all three forms in the peritrophic membranes of insects (Rudall and Kenchington, 1973) and in different parts of the squid. These and other findings support the view, (i) that the production of chitin is an ancestral (plesiomorphic) metazoan trait, and (ii) that α-chitin may be the earliest (most ‘primitive’) form of chitin (see the discussion in Jeuniaux, 1982; Ehrlich et al., 2007a,b). This means also that the occurrence of chitin cannot read-

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