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The exoskeleton of the lobster *Homarus americanus* as an example of a smart anisotropic biological material ☆

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Abstract

Many biological materials are composed of fibrils arranged according to well-ordered three-dimensional patterns. These materials often show a strong anisotropy in their properties. An essential characteristic of biological structures is their hierarchical organization from the nanometer to the millimeter scale. Lobster cuticle is a good example of this and a suitable model for studying these properties. In this study the structure of untreated as well as chemically and physically treated cuticle from the exoskeleton of the American lobster (*Homarus americanus*) was investigated using scanning electron microscopy. Fresh samples have been chemically decalcified and deproteinated and thermally treated to evaluate their resistance to degradation. Results showed that their structure is more complex than the commonly assumed model for arthropod cuticles. Stacked chitin–protein planes create the characteristic twisted plywood pattern found in arthropod cuticles. However, due to a well-developed pore canal system these planes are not simple arrays of parallel chitin–protein fibers. In lobster cuticle, interconnected fibers bend around the continuous lenticellate cavities of the pore canals to form a planar honeycomb-like structure. The chemically and thermally treated samples showed that the organic matrix retains its shape and structure despite the attack of chemical compounds or heat. It was also possible to study the distribution of the biominerals after the removal of the organic matrix. The observed residual structure gives a good impression of how the minerals (mainly calcite) are distributed inside the polymeric network.

Keywords: Lobster cuticle; Chitin; Thermal treatment; Chemical treatment

1. Introduction

Many skeletal tissues found in nature are composite materials with associated organic fibrils and mineral particles where the fibrous matrix is first deposited and then orientates the subsequent mineral nucleation and growth. In fact, skeletal tissues are fiber-reinforced composites where the whole is more important than the sum of its parts. Separately, the mineral or the fibrils show much weaker mechanical properties than the entire composite, as the mineral is made of brittle crystals and the polymer is supple. Nevertheless, as a composite structure they resist strong forces. The reason for this behavior is that crystal fractures stop where they meet fibrils, which in turn do not bend, because the distances between them are fixed in the mineral [1]. Good examples of this are the bones of vertebrates, the shells of molluscs and the cuticles of crustaceans. The cuticles of crustaceans and arthropods in general act as exoskeletons, functional units which supply mechanical support to the body of the animals, enable movement through the formation of joints and attachment sites for muscles, and provide protection against predators. In order to grow, these exoskeletons regularly have to be shed and replaced by a new, larger one. Our model organism, the American lobster *Homarus americanus*, is a

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decapod crustacean whose relatively large size and good availability makes it an excellent model for studying its exoskeleton.

The cuticle of the Crustacea comprises two main layers, the epicuticle and the procuticle. The epicuticle is a thin waxy layer which acts as a diffusion barrier to the environment. The procuticle is further divided into an exocuticle and an endocuticle which are chiefly designed to resist mechanical loads (Fig. 1b). The organic matrix of crustacean cuticles is secreted by a single-layered epithelium and is composed of chitin associated with proteins where eventually the calcite crystals will grow [2–4]. Chitin is a biopolymer whose ideal structure is a linear polysaccharide of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose, where all the residues are comprised entirely of *N*-acetyl-glucosamine, i.e. fully acetylated. However, in nature, the biopolymer exists as a co-polymer together with its deacetylated derivative, chitosan. When the number of acetamido groups is more than 50%, the biopolymer is termed chitin [5].

In many crustacean groups the hard parts of the exo- and endocuticle are mineralized, essentially by precipitation of crystalline calcium carbonate and, to a lesser extent, of amorphous calcium carbonate into the twisted lamellar structure of the chitin–protein cholesteric matrix [6–13]. The cuticle of the crustaceans is a complex structure in

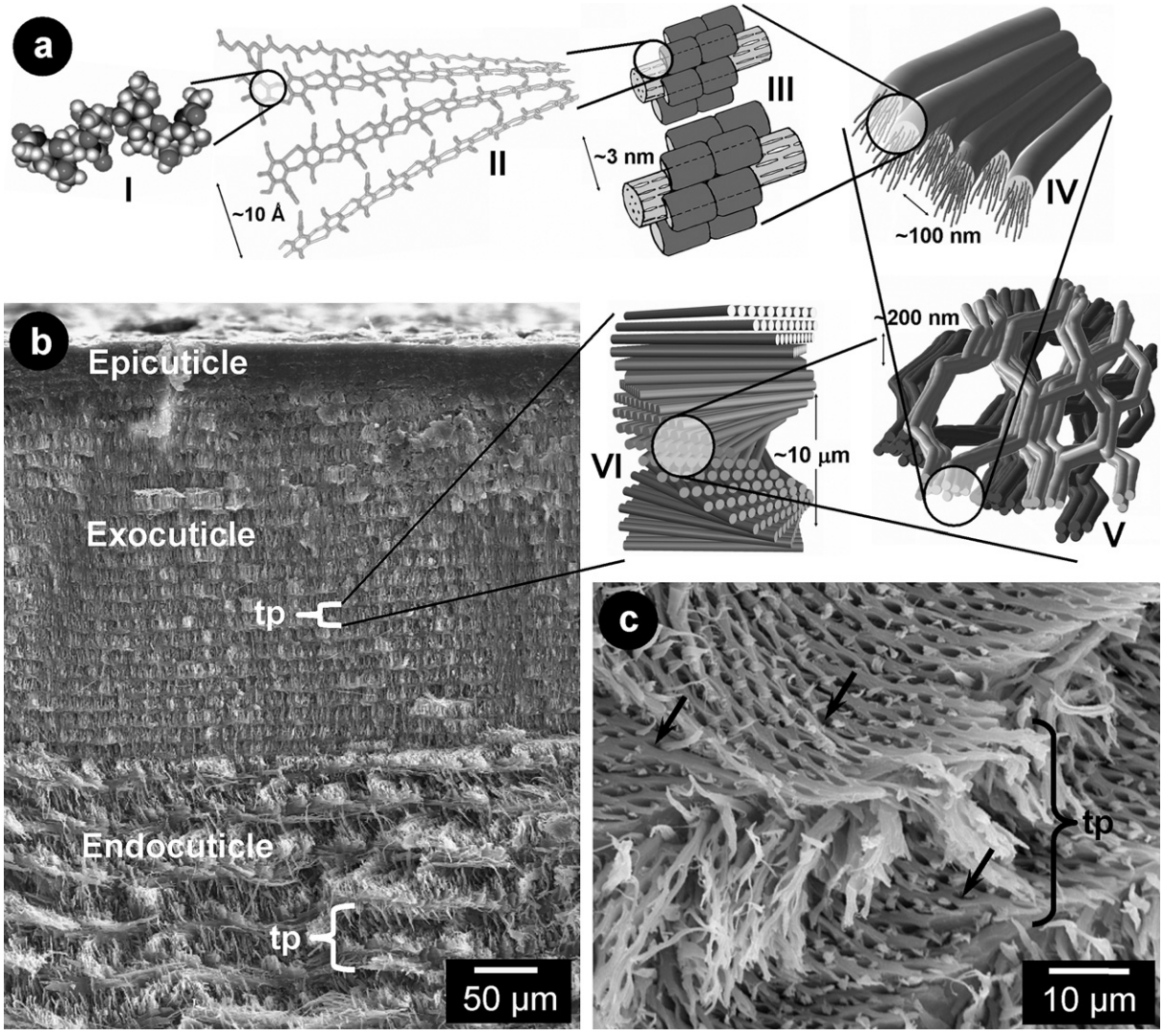

Fig. 1. Microstructure of lobster cuticle. (a) Schematic representation of the different hierarchical levels in the microstructure of lobster cuticle starting with the *N*-acetyl-glucosamine molecules (I) forming anti-parallel α-chitin chains (II). Between 18 and 25 of these molecules wrapped with proteins form nanofibrils (III), which cluster to form chitin protein fibers (IV) that are arranged in horizontal planes in which the long axes of the fibers are all oriented in the same direction. The fibers are arranged around the cavities originating from the extremely well-developed pore canal system which gives the structure a honeycomb-like appearance (V). These chitin protein planes are stacked with the orientation of the fibers in superimposed layers rotating gradually around the normal axis of the cuticle, thus creating a typical twisted plywood structure (VI). (b) SEM micrograph showing a cross-section through the three-layered cuticle. The different stacking density of the twisted plywood layers (tp) in the exo- and endocuticle can be clearly seen. (c) SEM micrograph of obliquely fractured endocuticle displaying two superimposed twisted plywood layers (tp) and showing their typical honeycomb-like structure. The arrows indicate the pore canals.

which initiation, growth and orientation of calcite domains are controlled by organic intra- and extracellular elements. This material provides a wide range of mechanical properties through local variations in composition and structure [4,14–19]. Characteristic of such composite materials is their strong microstructural hierarchy (see Fig. 1a). Hierarchical structures of the organic matrix are visible already in sections imaged by optical microscopy (in the range of 10–50 μm) [20].

The smallest subunits in the lobster cuticle are long chitin molecules. Between 18 and 25 chitin molecules then form nanofibrils with a length of approximately 300 nm and a diameter of about 2–5 nm which are wrapped by proteins. In the lobster the chitin chains are arranged in an antiparallel fashion, forming α-chitin, which is the most abundant of the three crystalline modifications of chitin occurring in nature. Crystallographic texture analysis of the chitin in lobster cuticle using X-ray wide-angle Bragg diffraction has shown that a large fraction of the α-chitin lattice cells is arranged with their longest axis parallel to the normal of the surface of the cuticle [21]. The protein-wrapped chitin nanofibrils cluster to form 50–250 nm thick, long chitin protein fibers that are arranged parallel to each other and form horizontal planes. These chitin protein planes are stacked and the longitudinal axis of the fibers in superimposed layers rotates around the normal axis of the cuticle, creating a twisted plywood or Bouligand structure. A stack of fiber layers that have completed a 180° rotation is referred to as one Bouligand or twisted plywood layer. Electron micrographs of the cross-section of the cuticle (Fig. 1b) show that the twisted plywood structure of the exocuticle is more densely packed than that of the endocuticle. The lobster cuticle additionally features an extremely well-developed pore canal system whose numerous canals penetrate it perpendicular to the surface (Fig. 1c). These canals contain long, soft and probably flexible tubes which play an important role in the transport of ions during the mineralization of the new exoskeleton after the animals molt [22]. The parallel fibers of the chitin protein planes meander around the lenticellate cavities of the pore canals, a formation which generates a structure that can be described as a twisted honeycomb (Fig. 1c) [23].

The combination of different types and quantities of materials and their interaction on different scales leads to a pronounced structural, topological and crystallographic directionality in the cuticle material relative to applied mechanical loads. The close connection between matter and construction is referred to as smart anisotropy and it seems to be a typical principle in biological materials [24]. However, these internal parameters of the biological materials are not the only ones to define their mechanical properties. They are also influenced by physiological parameters like the environmental conditions the organism lives in (i.e. temperature, pH, salinity, pollution) and its physiological state (i.e. molting stage, nutrition, diseases) together with artificial parameters like storage conditions of the samples, grade of hydration and state of decomposition. All these parameters influence each other and their combination finally defines the measurable mechanical properties of biological materials.

The aim of this study was to document the changes in the microstructure of lobster cuticle where selected components have been removed either chemically or by heat treatment using scanning electron microscopy.

2. Materials and methods

The exoskeleton of an American lobster (*Homarus americanus*) in intermolt stage acquired from a local seafood supplier was used. Molting stage was determined by the presence of the basal membrane on the inner surface of the cuticle. The preparation of the samples has been done as stated in the NIH Guide for Care and Use of Laboratory Animals. Both chemical and thermal treatments were conducted on specimens that were dissected from the chelipeds. Samples for chemical treatment were cleaved either perpendicular to the cuticle surface to expose the cross-section or parallel to the surface to expose the endocuticle. The cleavage was done prior to chemical attack to avoid the formation of structural artifacts. Chemical attacks were performed using NaOH (1 M, 1 week) to remove the protein structure, EDTA (0.15 M, 2 weeks) to remove the biominerals and a combination of EDTA (0.15 M, 2 weeks) followed by NaOH (1 M, 1 week) to obtain only the chitin network. All experiments have been carried out at room temperature under continuous mechanical stirring.

The end point of the decalcification was determined using Armin's method [25]. A solution of ammonium hydroxide (5%) and ammonium oxalate (5%) was added to 5 ml of the decalcifying medium at regular intervals. The formation of a cloudy solution caused by precipitation of calcium oxalate indicated that the specimen was not thoroughly decalcified. The decalcifying solution was replaced repeatedly until the cloudy solution was not longer formed. A transparent solution indicated that no more calcium was being removed from the specimen.

During deproteinization, covalent chemical bonds between chitin and proteins have to be destroyed. A 1 M NaOH solution is typically used for this with variations in treatment parameters like temperature and exposure time. The protein content of the whole cuticle is about 1% when the treatment is properly carried out. This method is traditionally used to purify chitin for commercial purposes [5].

Thermogravimetric analysis (TGA) has been carried out to determine the decomposition temperature of the different components of the native exoskeleton. TGA monitors the weight change in materials as a function of time and temperature. The measurements provide basic information about the thermal stability of a material and its composition. Samples for TGA were heated up to 1200 °C, at 5 °C min^{-1}, in an inert atmosphere using a Setaram SETSYS 16 thermobalance.

Thermal treatment was carried out in a conventional furnace under air. In order to reproduce the conditions of the TGA experiments, samples were heated in the furnace at a rate of 5 °C min^{-1} and held at the appropriate temperatures (220, 370 and 750 °C) for 10 min.

All obtained samples were air-dried, sputter-coated with 5 nm of gold and imaged using a high-resolution scanning electron microscope (Zeiss Gemini 1540 XB). The contrast and brightness of the digital images were adjusted where necessary using Adobe Photoshop CS2 (Adobe Inc.). Qual-

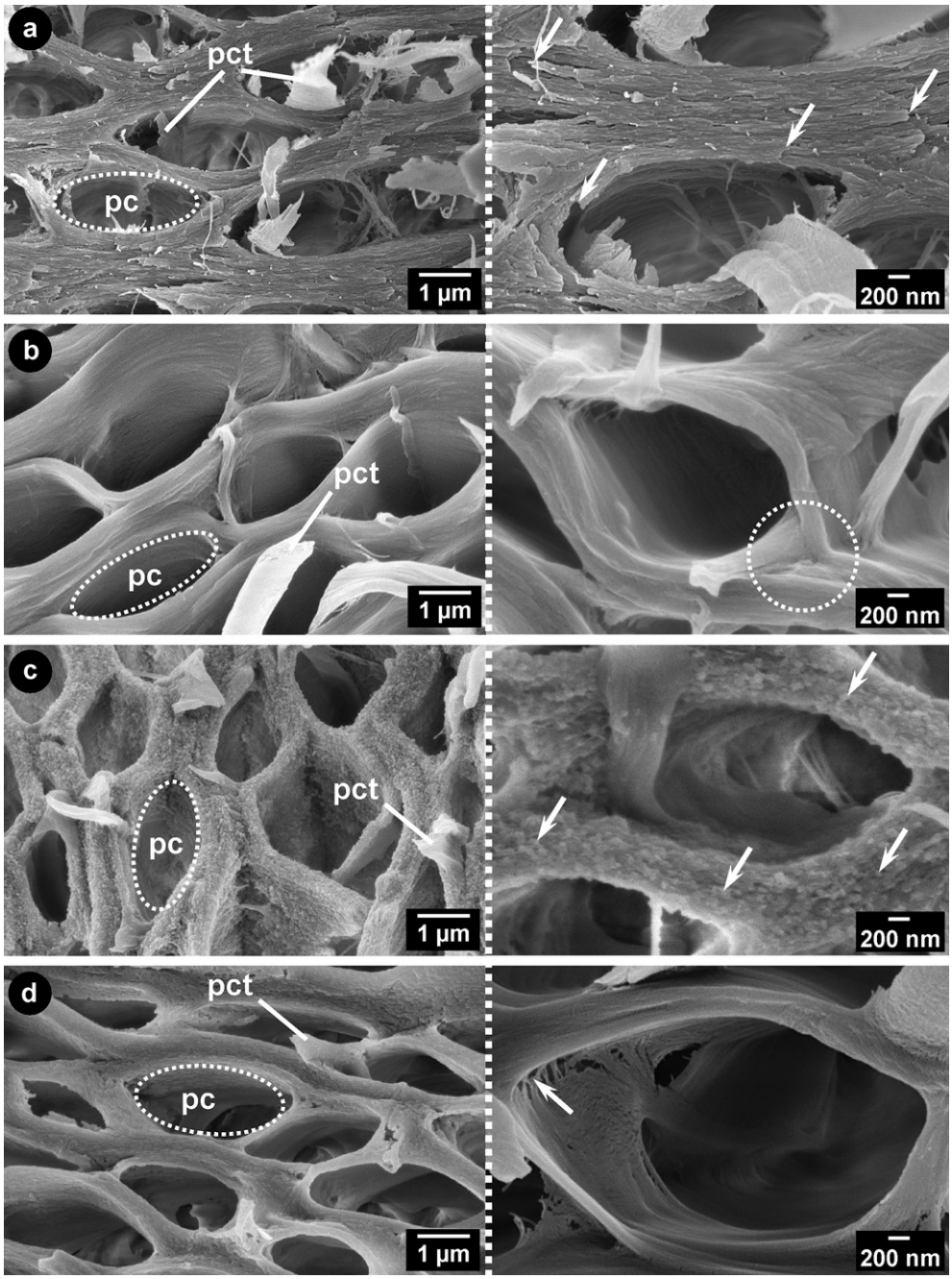

Fig. 2. Microstructure of lobster endocuticle fractured parallel to the surface. The SEM micrographs are overviews (left) and detail images (right) of untreated and chemically treated material. (a) Untreated cuticle, overview showing thick walls around the pore canals (pc) and residues of the tubes (pct) therein. The detail image shows the discontinuous, blocky fiber structure with numerous broken fiber ends (arrows). (b) Decalcified cuticle (EDTA, 0.15 M), overview with pore canals (pc) and pore canal tubes (pct). The high-resolution image shows the smoothness of the thin-walled structure and the way how separate fiber bundles contact each other to form the corners of the pore canals (encircled area). (c) Deproteinated cuticle (NaOH, 1 M), overview showing walls with a rough surface around the pore canals (pc) which contain pore canal tubes (pct). The detail image shows that the structure is covered with small spherical granules with diameters of 20–50 nm (arrows). (d) Decalcified and deproteined cuticle (EDTA, 0.15 M + NaOH, 1 M), the overview shows smooth walls, pore canals (pc) and residues of tubes (pct) therein. The high-resolution image shows a similar smooth structure to that of the decalcified samples but with occasional interruptions between the fibers (arrow).

itative energy dispersive X-ray (EDX) spectra of the decalcified and the deproteinated samples were recorded using an Edax (Leo 1540 XB) EDX detector mounted on the scanning electron microscope.

3. Results

3.1. Chemically treated material (decalcified, deproteinated and both)

High-resolution scanning electron micrographs of untreated lobster cuticle (Fig. 2a) show its characteristic microstructure formed by parallel oriented fibers with diameters between 25 and 50 nm and blocky appearance. The walls of the honeycomb-like structure formed by the stacked fiber layers appear homogeneous and solid. All over the investigated area broken fibers with square ends can be observed, which leads to a rough surface of the sample with hardly any continuity in the fibers visible.

In cuticle decalcified with EDTA (Fig. 2b) it is hard to discern individual fibers. The honeycomb-like structure around the pore canals is well defined but the walls formed by the stacked fiber bundles are very smooth. The walls of adjacent pore canals do not merge homogeneously but show separated fiber bundles contacting each other in certain areas and then separating again to form another pore canal. The only occasionally observed interruptions in the fibrous structure have rounded ends. The qualitative EDX analysis shows that calcium and magnesium have been completely removed (Fig. 3a).

Samples which were deproteinated using NaOH (Fig. 2c) display a well-defined honeycomb-like structure with visible shallow depressions dividing some of the honeycomb structure's walls along the longitudinal axis of the pore canals. These structures on the fracture surface give the impression that there exist contact areas between the walls of individual pore canals. Single fibers cannot be discerned. The whole structure is covered with small spherical structures with diameters of 20–50 nm. The qualitative EDX analysis of the samples shows strong signals for calcium, magnesium and phosphorous (Fig. 3b).

The structure of decalcified and deproteinated cuticle (Fig. 2d) closely resembles that of decalcified cuticle, but the walls of the pore canals are slightly thicker and appear less compact, giving it a more collapsed and loose, swollen look. The honeycomb-like structure appears also very smooth and slightly less well defined.

3.2. Thermally treated material

The diagram of the TGA (Fig. 4a) shows that at temperatures between 50 and 270 °C water and proteins are removed from the untreated cuticle. The degradation of chitin starts at about 270 °C. At temperatures between 500 and 600 °C the chitin is completely removed and above 650 °C the decarboxylation of the $CaCO_3$ starts.

The second TGA graph (Fig. 4b) additionally shows the mass change of the chemically treated samples during the thermal analysis. These data can be only qualitatively and not quantitatively related to one another since the initial mass of each sample was not the same. The curve of the deproteinated and demineralized sample presented a single drop at 230 °C, indicating the beginning of the degradation of the biopolymer. In the untreated cuticle this temperature is around 270 °C, i.e. slightly higher, probably due to some residual proteins still being present. As mentioned above, the chemical bonding between chitin and protein is very strong. Those results are in good agreement with García-Alonso et al. [26] who showed that protein decomposition in lobster shells takes place between 180 and 220 °C and chitin degradation begins at 280 °C.

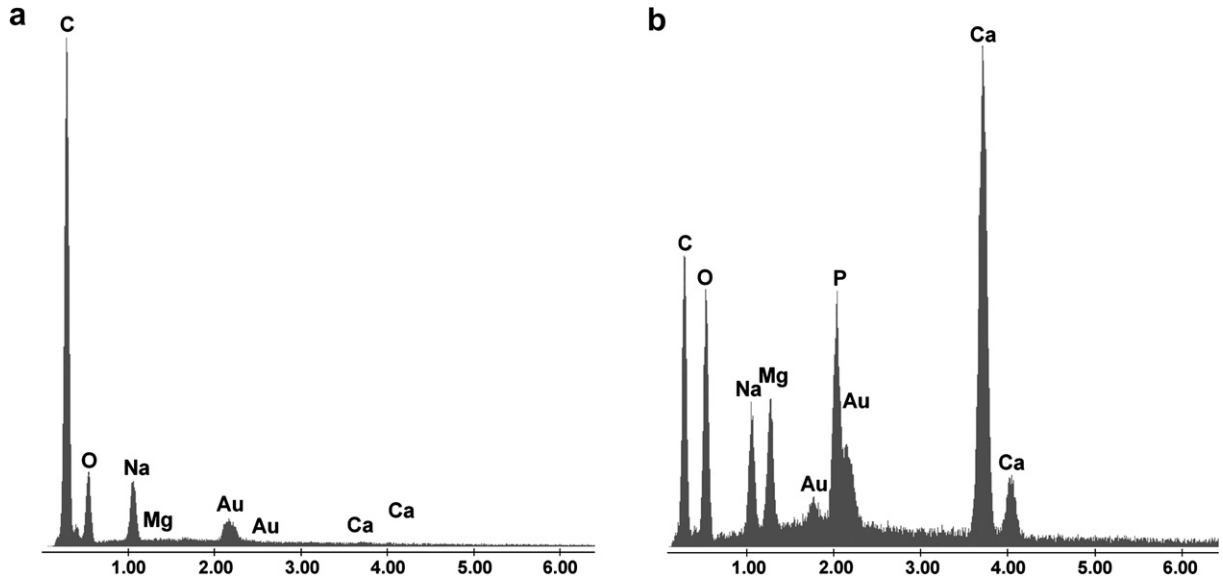

Fig. 3. Qualitative energy dispersive X-ray (EDX) analysis of decalcified (a) and deproteinated (b) lobster cuticle.

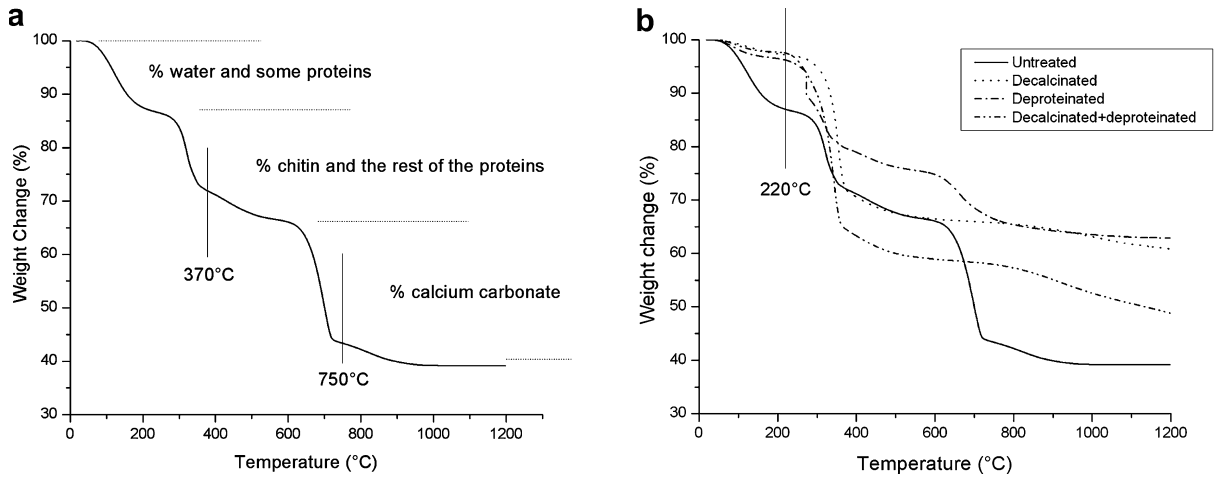

Fig. 4. Thermogravimetric analysis of lobster cuticle. (a) Untreated cuticle: the diagram shows that water and proteins are removed between 50 and 270 °C, the chitin degrades between 270 and about 600 °C and the decarboxylation of $CaCO_3$ starts above 650 °C. Samples heated up to 370 and 750 °C were investigated using scanning electron microscopy (SEM). (b) TGA diagrams for untreated and chemically treated cuticle. Samples which were both decalcinated and deproteinated were examined with SEM after heating them up to 220 °C.

In Fig. 4a (for the untreated sample) and Fig. 4b (for the deproteinated and demineralized sample) the temperatures at which the thermal treatments have been carried out are indicated. A temperature of 370 °C ensured the major decomposition of the biopolymer, while 750 °C ensured the decarboxylation of calcite. Regarding the deproteinated and demineralized sample, 220 °C kept the chitin network almost intact while the major protein–water removal had already taken place.

Untreated cuticle heated up to 370 °C displays a gradual change in structure preservation from the inside of the sample to the surface. In the centre of the sample, the honeycomb-like structure is well preserved (Fig. 5a). The individual fibers have a blocky appearance and are broken,

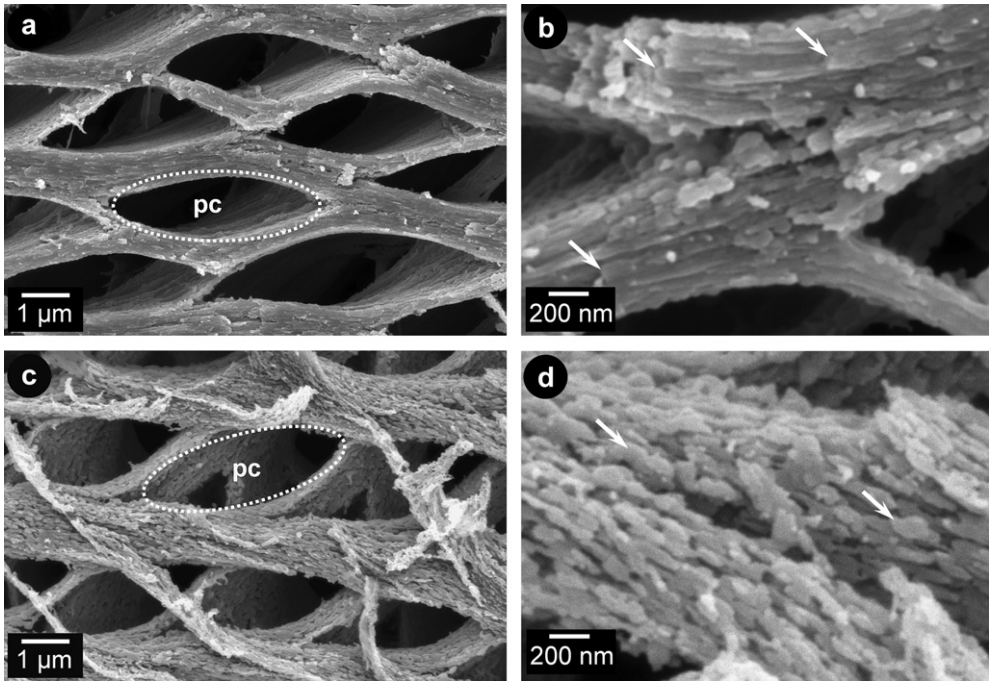

Fig. 5. Microstructure of native cuticle heat-treated at 370 °C. (a) Structure in the inner part of the sample; the fibers building up the well-preserved original structure closely resemble those of untreated cuticle. (b) Detail image showing the blocky appearance and sharp fractures (arrows) of the fibers. (c) Structure in the surface areas of the sample; the original geometry of the honeycomb like structure is preserved but the formerly continuous fibrilar structures have transformed to agglomerations of consecutive ellipsoidal particles. (d) Detail image of pore canal walls, showing particles which have a length of about 200 nm (arrows). pc, pore canals.

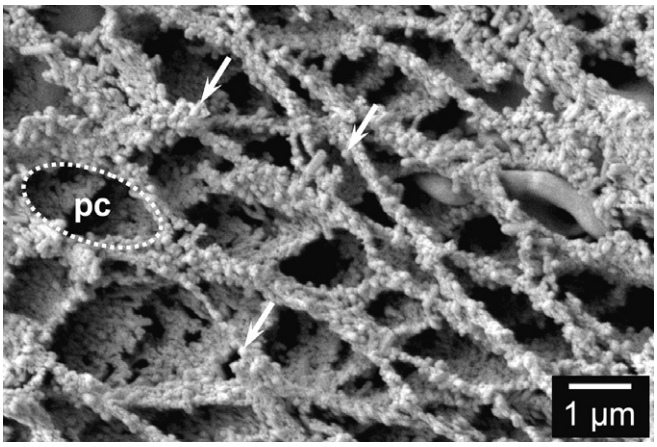

Fig. 6. Microstructure of native cuticle heat-treated at 750 °C. The original honeycomb-like structure can still be distinguished. The wall structures are very thin and covered with spherical particles with diameters between 120 and 150 nm (arrows). pc, pore canals.

displaying sharp, square fractures (Fig. 5b). They form solid-shaped honeycomb walls which appear slightly porous due to numerous small cavities between them. In areas near to the surface the microstructure displays still a very well-developed honeycomb structure but its walls are not compact (Fig. 5c). Their formerly fibrilar structure is no longer visible but is now an agglomeration of consecutive ellipsoidal particles, each one with a length of about 200 nm, which retain the original geometry (Fig. 5d). In the sample heated at 750 °C, the honeycomb structure is not nearly as well preserved, although the shape and the dimensions of the lenticellate pore canals can still be distinguished (Fig. 6). The wall structures are much thinner and consist of spherical particles with diameters between 120 and 150 nm.

Samples with minerals and proteins chemically removed before heat treatment and subsequently heated up to 220 °C show a pronounced fibrous structure. The holes of the pore canal system are narrower and the planar parallel fibers are very long and continuous, and showing no signs of fracture (Fig. 7a). At higher magnifications the parallel arrangement of individual fibers in superimposed layers becomes much more obvious than in untreated or only chemically treated material (Fig. 7b). The fibers have diameters of about 30 nm and adjacent fibers seem to be connected to each other through very small fibrilar structures, which can be seen in Fig. 7b (inset).

4. Discussion

Our results showed that both chemical and thermal treatments had a severe impact on the lobster cuticle microstructure. During decalcification with EDTA the $CaCO_3$ was gently removed and the remaining structure was formed by chitin–protein fibers, which themselves are composed of protein-wrapped nanofibrils. The missing calcium and magnesium peaks in the qualitative EDX analysis indicate that the structure is free of minerals after decalcification. The smoothness of the structure without $CaCO_3$ is in contrast to the blocky appearance of the untreated cuticle, indicating that the biominerals were indeed located between and around the chitin–protein fibers, as it is known from previous studies [3,27]. The absence of structural roughness caused by the removal of the minerals also shows that the chemically treated material loses the brittleness present in the natural state. For the deproteinated samples, the removal of the proteins seems to affect the structural integrity of the composite in a way that the fractured structure resembles that of the decalcified ones, although there are still minerals inside. The appearance of depressions resembling contact areas between the walls of individual pore canals is probably due to the dissolution of branched chitin fibrils caused by the removal of their protein hulls. As these depressions are only present in the peripheral areas of the fractured honeycomb structure, one can assume that some of the fibrils are lost in the deproteination solution before they can bind again to others, or that they bind to adjacent chitin fibrils without branching. The strong signals of calcium, magnesium and phosphorous obtained by qualitative EDX analysis confirms the presence of mineral in the structure. The small spherical particles appearing in the deproteinated sample are therefore most likely biomineral crystals which become

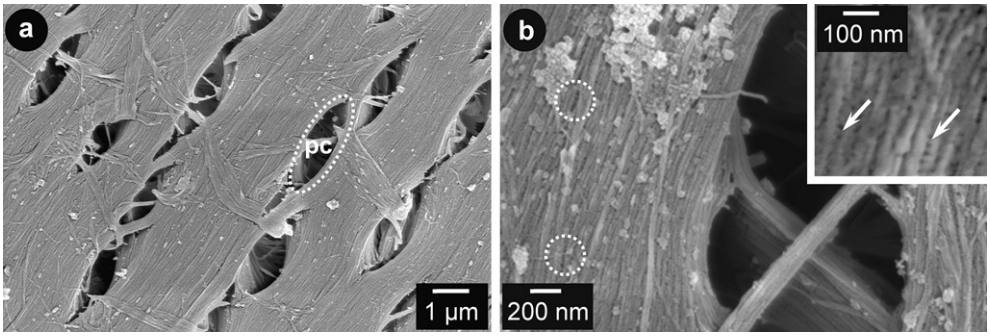

Fig. 7. Microstructure of purified chitin heat-treated at 220 °C. (a) The overview of the structure parallel to the cuticle surface shows narrow pore canals (pc) and very long and continuous parallel fibers. (b) Detail images of the fibers near a pore canal. Adjacent fibers seem to be connected by small fibrilar structures (encircled areas, arrows in inset).

visible after the removal of the proteins. This indicates that the proteins stabilize not only the structure but the crystalline state by binding the mineral together with the chitin polymer. At present it is not possible to determine whether the particles represent original crystallites formerly enclosed in the fibers or the products of a recrystallization process which has taken place due to the chemical attack. After removal of both proteins and minerals the residual chitin fibers retain their shape. Therefore, it can be assumed that even though the proteins are responsible for the fiber shape of the chitin polymer, the removal of them does not make the polymer loose its honeycomb-like structure. This is most likely due to the formation of new bondings between the now unwrapped chitin nanofibrils.

In the 370 °C heated sample the proteins and the major part of the chitin polymer are eliminated, as can be seen in Fig. 4b where a sample of chemically purified chitin presents major weight loss starting at 270 °C and finishing at around 400 °C. The treatment was carried out at a slow heating rate to avoid structural damage while removing the organic compounds. Nevertheless the samples investigated by scanning electron microscopy showed a strong gradient in the quality of structural preservation from the inside to the surface. Inside the material the structure still resembled the original untreated state with slightly higher porosity, most probably caused by the evaporation of organic components. This indicates that the structure still contains chitin that is not yet degraded. In the peripheral areas no more polymers can be distinguished and the residual mineral has taken the shape of ellipsoidal particles, probably due to a recrystallization process. This structure represents an intermediate state between the sample heated up to 370 °C and the one heated up to 750 °C. The heat treatment of the material obviously removed the chitin present in superficial areas but was not long enough to allow its complete removal.

At 750 °C the decarboxylation of the $CaCO_3$ has already started, leading to a loss of material. The reaction that takes place produces two types of residual products, solid CaO and gaseous carbon dioxide and water vapor. Due to the slow heating the organic compounds have most probably been gently removed and the solid product of the decarboxylation reaction, calcium oxide, has retained the architecture of the original biomineral. It is possible that due to time and temperature, coalescence of the mineral phase has occurred. This process would be equivalent to sintering in metallurgy and could explain the presence of the relatively large spherical particles.

The chemically purified chitin was heat treated at 220 °C, just before its thermal decomposition starts (see Fig. 4b). The structure preservation is remarkably good and the stacked rotating planes of parallel fibers, which represent the basic construction principle of the cuticle, become clear. The heat has probably helped the pore canals to constrict almost to the point of collapsing (Fig. 7a). Individual chitin fibers become easier to discern (Fig. 7b), probably due to the temperature eliminating all possible proteinaceous residues left by the chemical attack (Fig. 4b). Considering this fact, the small fibrilar structures connecting individual fibers are most probably pure, unwrapped chitin fibrils that are cross-linking the larger fibers.

Chemical treatment is already an established method for purifying chitin obtained from nature [5]. Attempts have been made to produce chitin networks with defined porosity. Chow et al. [28] have prepared porous matrices by loading $CaCO_3$ into a chitin solution, which led to the formation of a gel. The $CaCO_3$-loaded gels were subjected to a 1 M HCl solution, and gaseous carbon dioxide together with calcium chloride and water were produced. The evaporation of carbon dioxide gas created the desired porous morphology of the chitin gel. Pore dimensions and the porosity of chitin matrices can be controlled by varying the amount of $CaCO_3$ loaded into the chitin solution.

In our case, the well-developed pore canal system of the lobster cuticle provides continuous pores with a defined size of about 2 μm. Our investigation shows that it is possible to treat the cuticle to obtain a pure chitin matrix which retains the porosity of the original material. Materials with directional porosity and defined pore size, which can be produced through a simple and inexpensive process, could be made suitable for example for medical applications. Porous biodegradable materials are already in use for controlled drug release systems, enzyme immobilization support, molecular fractionation or transition-metal adsorption. These materials are also being explored widely in tissue engineering, for tissue repair and fabrication [29]. In particular, regeneration of damaged tissues is an objective because of insufficient organ and tissue donors for the high demands [30]. In the case of lobster cuticle the complete absence of proteins that were removed by heat treatment is an additional advantage because they are known to be a source of allergic reactions.

5. Conclusions

We have compared the microstructure of cuticle from the exoskeleton of the American lobster *Homarus americanus* in its natural state with cuticle that had been chemically decalcified and deproteinated as well as cuticle that had been heat treated. The results show that both types of treatment have little influence on the overall structure. However, on the scale of the individual fibers the structure is altered significantly through the selective removal of the different cuticle components. Through chemical etching and subsequent heat treatment of the native material, a defined porous matrix of highly purified chitin can be obtained. This product could be regarded as an example for the development of innovative medical applications.

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References

[1] Bouligand Y. The renewal of ideas about biomineralisations. C R Palevol 2004;3:617–28.
[2] Bouligand Y. Twisted fibrous arrangement in biological materials and cholesteric mesophases. Tissue Cell 1972;4(2):189–217.
[3] Guiraud-Guille MM. Plywood structures in nature. Curr Opin Solid State Mater Sci 1998;3:221–7.
[4] Vincent JFV. Structural biomaterials. Princeton (NJ): Princeton University Press; 1990.
[5] Khor E. Chitin: fulfilling a biomaterials promise. Amsterdam: Elsevier Science; 2001.
[6] Lowenstam HA. Minerals formed by organisms. Science 1981;211:1126–31.
[7] Mann S, Webb J, Williams RJP. On biomineralization. Weinheim: VCH Press; 1989.
[8] Lowenstam HA, Weiner S. On biomineralization. Oxford: Oxford University Press; 1989.
[9] Weiner S, Addadi L. Design strategies in mineralized biological materials. J Mater Chem 1997;7:689–702.
[10] Mann S. Biomineralization and biomimetic material, chemistry. J Mater Chem 1995;5:935–46.
[11] Manoli F, Koutsopoulos S, Dalas E. Crystallization of calcite in chitin. J Cryst Growth 1997;182:116–24.
[12] Falini G, Albech S, Weiner S, Addadi L. Control of aragonite or calcite polymorphism by mollusk shell macromolecules. Science 1996;271:67–9.
[13] Luquet G, Marin F. Biomineralisations in crustaceans: storage strategies. C R Palevol 2004;3:515–34.
[14] Travis DF. Structural features of mineralization from tissue to macromolecular levels of organization in the decapod crustacea. Ann NY Acad Sci 1963;109:177–245.
[15] Hadley F. The arthropod cuticle. Sci Am 1986;255:98–106.
[16] Vincent JFV. Arthropod cuticle: a natural composite shell system. Composites: Part A 2002;33:1311–5.
[17] Raabe D, Sachs C, Romano P. The crustacean exoskeleton as an example of a structurally and mechanically graded biological composite material. Acta Mater 2005;53:4281–92.
[18] Neville AC. Biology of fibrous composites. Cambridge: Cambridge University Press; 1993.
[19] Vincent JFV, Wegst UGK. Design and mechanical properties of insect cuticle. Arthropod Struct Dev 2004;33:187–99.
[20] Guiraud-Guille MM, Belamie E, Mosser G. Organic and mineral networks in carapaces bones and biomimetic materials. C R Palevol 2004;3:503–13.
[21] Raabe D, Romano P, Sachs C, Fabritius H, Al-Sawalmih A, Yi SB, et al. Microstructure and crystallographic texture of the chitin–protein network in the biological composite material of the exoskeleton of the lobster *Homarus americanus*. Mater Sci Eng A 2006;421:143–53.
[22] Cameron JN. Post-molt calcification in the blue-crab *Callinectes sapidus* – timing and mechanism. J Exp Biol 1989;143:285–304.
[23] Raabe D, Romano P, Sachs C, Al-Sawalmih A, Brokmeier HG, Yi SB, et al. Discovery of a honeycomb structure in the twisted plywood patterns of fibrous biological nanocomposite tissue. J Cryst Growth 2005;283:1–7.
[24] Raabe D, Al-Sawalmih A, Romano P, Sachs C, Brokmeier HG, Yi SB, et al. Structure and crystallographic texture of arthropod biocomposites. Mater Sci Forum 2005;495–497:1665–74.
[25] Luna LG. Histopathologic methods and color atlas of special stains and tissue artifacts. Gaithersburg (MD): American Histolabs; 1992.
[26] García Alonso I, OviedoVega D. Thermoanalytical studies on lobster shell. J Therm Anal 1990;36:1347–54.
[27] Falini G, Fermani S. Chitin mineralization – review. Tissue Eng 2004;10(1/2):1–6.
[28] Chow KS, Khor E. Novel fabrication of open-pore chitin matrixes. Biomacromolecules 2000;1:61–7.
[29] Patrick CW, Chauvin PB, Hobley J, Reece GP. Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng 1999;5(2):139–51.
[30] Kang HW, Tabata Y, Ikada Y. Fabrication of porous gelatin scaffolds for tissue engineering. Biomaterials 1999;20:1339–44.