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Influence of proteins on mechanical properties of a natural chitin-protein composite

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# 1 **Influence of Proteins on Mechanical Properties of a Natural Chitin-Protein Composite**

2

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4

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10

## 11 **ABSTRACT**

12 In many biogenic materials, chitin chains are assembled in fibrils that are wrapped by a protein fold.  
13 In them, the mechanical properties are supposed to relate to intra- and inter- interactions among chitin  
14 and proteins. This hypothesis has been poorly investigated. Here, this research theme is studied using  
15 the pen of *Loligo vulgaris* as a model material of chitin/protein composites. Chemical treatments were  
16 used to change the interactions involving only the proteic phase, through unfolding and/or  
17 degradation processes. Successively, structural and mechanical parameters were examined using  
18 spectroscopy, microscopy, X-ray diffractometry, and tensile tests. The data analysis showed that  
19 chemical treatments did not modify the structure of the chitin matrix. This allowed to derive from the  
20 mechanical test analysis the following conclusions: (i) the maximum stress ( $\sigma_{\max}$ ) relies on the  
21 presence of the disulfide bonds; (ii) the Young’s modulus (E) relies on the overall correct folding of  
22 the proteins; (iii) the whole removal of proteins induces a decrease of E (> 90 %) and  $\sigma_{\max}$  (> 80 %),  
23 and an increase in the maximum elongation. These observations indicate that in the chitin matrix the  
24 proteins act as a strengthener, which efficacy is controlled by the presence of disulfide bridges. This  
25 reinforcement links the chitin fibrils avoiding them to slide one on the other and maximizing their  
26 resistance and stiffness. In conclusion, this knowledge can explain the physio-chemical properties of  
27 other biogenic polymeric composites and inspire the design of new materials.

28

29 **Keywords:** chitin; proteins; composites; disulfide bridges; mechanical properties.

## 1 1. INTRODUCTION

2 Composite materials are extremely common in nature[1] and are mostly a combination of organic-  
3 inorganic constituents, like shells,[2] teeth or bones,[3] or organic macromolecules, usually including  
4 a polysaccharide, as in woods or arthropod's exoskeletons.[4–7]

5 Among natural polysaccharide, chitin, the  $\beta(1,4)$ -linked N-acetyl-D-glucosamine polymer, has a  
6 primary biological importance. According to the International Institute for Species Exploration, chitin  
7 is the most diffuse biopolymer on Earth, being present in over 70 % of the known species (most of  
8 them in the animal kingdom).[8,9] In these species, chitin appears in a huge variety of functional  
9 materials, generally as composites with proteins, other biomolecules, or minerals. Those composite  
10 materials have frequently structural or protective purposes[10–12] but, having different morphologies  
11 and tunable properties, also play a fundamental role in photonic systems[13,14] and support  
12 structures.[15–18]

13 In living organisms, chitin is bio-synthesized in three polymorphs ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) that form crystalline  
14 filaments, called nano-fibrils. These fibrils, having a diameter from 2.5 to 2.8 nm, are typically  
15 wrapped in a protein matrix.[19] These composite fibrils are the building block for semi-crystalline  
16 micro-fibers, which are the major constituent of the final hierarchically organized matrix.

17 Biochemical studies on proteins associated with chitin from a wide variety of arthropod cuticles have  
18 identified a conserved chitin-binding sequence, called R&R consensus sequence.[20] Homology  
19 modeling investigations suggested that the preferred secondary structure of this chitin-binding  
20 domain (CBD) is an antiparallel  $\beta$ -sheet structured in a half  $\beta$ -barrel structure.[21] In this  
21 configuration, the aromatic residues face the internal  $\beta$ -barrel surface and interact with the chitin  
22 crystalline fibril. In many other of those chitin-interacting proteins, as in chitinases and chitin  
23 synthases, a different CBD is observed. This CBD is kept folded by essential disulfide bonds, usually  
24 three for insects[22] and four for plants[23]. Indeed, a drastic drop in the binding capability is  
25 observed after their reduction.[24] Both these typology of CBDs are highly selective for chitin, even

1 at the level of polymorph selection.[25] Moreover, it is common for structural proteins to present  
2 many CBDs on the same protein. As an example, Shi & al. 2009 reported a protein with 19 CBDs.[26]  
3 To date, only a few studies have addressed how those proteins govern chitin fibrils self-assembly and  
4 ultrastructural organization, and are distributed in different chitin matrices.[27,28] In the squid pen,  
5 the chitin molecules form nano-crystallites of monoclinic lattice symmetry, resulting in  $\beta$ -chitin nano-  
6 fibrils. These crystalline fibrils are wrapped in a protein layer forming composite nano-fibrils. About  
7 100 of these composite nano-fibrils are needed to form a 0.2  $\mu\text{m}$  thick micro-fiber. The latter arrange  
8 into bigger fibers that align to form layers, which generate the squid pen. The molecular structure is  
9 highly anisotropic with 90% of chitin crystallites oriented along the main axis of the pen.[10,11] This  
10 feature provides a strong correlation between the macroscale structure and the molecular orientation.  
11 Structural information on the squid pen proteins was reported for *Sepioteuthis lessoniana* and  
12 *Uroteuthis chinensis*. [11] This research reported the presence of proteins in an  $\alpha$ -helical coil  
13 conformation oriented along the pen main axis. Moreover, in the squid pen from *Todarodes pacifica*  
14 the protein amino acid composition was that typical of globular proteins, with increased content of  
15 tyrosine and histidine residues.[29]

16 In this research, we aim to characterize the influence of structural proteins on the mechanical  
17 properties of a chitin-based composite material, the pen of the squid *Loligo vulgaris* (Figure 1). This  
18 composite material is an internal vestigial not calcified shell that acts as a support structure for the  
19 animal and thus has an important mechanical role.[30] The native squid pen was subject to a series  
20 of chemical and enzymatic treatments to study the influence of proteins in the matrix mechanical  
21 properties. Since the squid pen once was mineralized,[31] a way to modify the protein structure is to  
22 remove metal ions by chelating agents, as ethylenediaminetetraacetic acid (EDTA).[32,33] moreover,  
23 changes in protein folding and interactions are obtained by denaturing agents (e.g. urea) or species  
24 able to reduce the disulfide bonds (e.g. mercaptoethanol).[34] A mixture of the above chemicals is  
25 generally used to extract proteins from matrices avoiding their primary structure degradation. On the

1 other hand, enzymatic[35] and alkaline treatments[36] are efficient degrading methods to remove  
2 proteins from chitin samples by degradation.

3 The hypothesis tested in this research is that the folding of the proteins controls the mechanical  
4 properties of natural chitin-protein composite. This investigation is of interest for the understanding  
5 of the biosynthesis and structure-function relationship of chitin-based composites, and for the design  
6 of new materials.

7

## 8 **2. RESULTS**

### 9 **2.1 Characterization of the proteins extracted from the squid pen**

10 To extract the proteins from the pen a denaturing aqueous solution (Mix. 24h) containing 8 M urea,  
11 4 vol.% mercaptoethanol, and 1 mM EDTA was used at room temperature for 24 h. The extracted  
12 proteins were stable in the denaturing solution, but largely precipitated upon dialysis against a 50 mM  
13 tris-(hydroxymethyl)aminomethane (TRIS) buffer at pH 7.4. The amino acid composition (Table S1)  
14 of this extract shows a relevant amount of oxidized cysteine dipeptide (1.1 mol.%) and is also rich in  
15 apolar amino acids, glycine (13.7 mol.%), alanine (13.5 mol.%), proline (11.5 mol.%) and valine (8.5  
16 mol.%), and of aromatic residues, histidine (9.0 mol.%) and tyrosine (6.7 mol.%). No reduced  
17 cysteine and methionine were detected.

18 The sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) analyses revealed the  
19 same protein bands from the precipitated proteins and the protein remained in solution (Figure S1).  
20 Six strong bands between 30 and 50 kDa plus three weak bands at about 97 kDa, 25 kDa, and 20 kDa  
21 were stained (Figure 2 and Figure S1).

22 The protein precipitate was also analyzed using Fourier-transform infrared spectroscopy (FTIR)  
23 spectroscopy (Figure 2). All the typical absorption bands of proteins were observed: amide A ( $3290$   
24  $\text{cm}^{-1}$ ), amide I ( $1655 \text{ cm}^{-1}$ ), amide II ( $1521 \text{ cm}^{-1}$ ), and amide III ( $1235 \text{ cm}^{-1}$ ). Other weak absorption  
25 bands related to side-chain amino acid vibration modes were also observed, no absorption bands that  
26 could be related to phosphorylation or glycosylation were detected.[37]

1 When the extraction of the proteins was carried out using the single components of the denaturing  
2 solution the SDS-PAGE showed no bands for 1 mM EDTA and 4 vol.% mercaptoethanol, and only  
3 a weak band at about 45 kDa for 8 M urea. When the denaturing solution was used again on the  
4 matrices treated using the Mix. 24h (Mix. 48 h) a slightly different pattern of bands was observed  
5 with respect to that observed using the Mix. 24 h (Figure 2). Increasing the amount of solution loaded  
6 in the gel other less intense bands appeared (Figure S2). Those bands were efficiently extracted using  
7 the mixture for 24 h, but a less intense signal of those bands was also detected after 48 h. Moreover,  
8 the urea 8 M treatment extracted selectively those bands.

## 9 **2.2 Structural characterization of treated squid pen**

10 The quantification of squid pen degree of de-proteination, which was carried out by gravimetric  
11 measurement (Table 1), indicated that no molecules were removed from the squid pen after treatment  
12 with a 1 mM EDTA (EDTA) or 4 vol.% mercaptoethanol (Merc) solution. The sample treated with  
13 the 8 M urea (Urea) solution lost about 6 wt.%, while those treated in Mix. 24h or Mix. 48h lost about  
14 40 wt.% and 50 wt.%, respectively. The treatment of the squid pen with a proteolytic mixture of  
15 enzymes (Enzymes) or with a 1 M NaOH (NaOH) solution led to a weight loss of about 40 wt.% and  
16 60 wt.%, respectively.

17 The NaOH and Enzymes samples, being protein-free, were analyzed by solid state nuclear magnetic  
18 resonance spectroscopy to determine the degree of acetylation that was  $89 \pm 1$  % and above 93 %,  
19 respectively.

20 The UV-Vis spectra (Figure 3) showed that the intensity of the aromatic residues absorption band  
21 (280 nm)[35] did not change for the samples EDTA, Merc, and Urea with respect to that of the native  
22 sample. This band intensity decreased similarly in the samples treated with Mix. 24h and Mix. 48h.  
23 In the Enzymes and NaOH samples this signal was absent. The spectra were acquired using samples  
24 of comparable thickness, which gave a comparable scattering.

25 The analysis of the FTIR spectra (Figure S3) showed that the typical protein absorption bands at 3290  
26  $\text{cm}^{-1}$  (amide A), 1650  $\text{cm}^{-1}$  (amide I), 1520  $\text{cm}^{-1}$  (amide II), and 1235  $\text{cm}^{-1}$  (amide III) were present

1 only in EDTA, Merc, Urea, and Mix. 24h sample. In addition to these absorption bands, other two  
2 bands at  $2963\text{ cm}^{-1}$  and  $1446\text{ cm}^{-1}$  were present only in the protein containing samples. Typical  
3 absorption bands of chitin were present in all samples (Table S2). In samples Mix. 48h, Enzymes,  
4 and NaOH only chitin bands were observed along with a change in shape and a shift of the band at  
5  $1520\text{ cm}^{-1}$  to  $1555\text{ cm}^{-1}$ , the typical wavenumber for chitin.

6 The squid pen sample showed a swelling of about 200 wt.%. A similar value was obtained for the  
7 samples treated in EDTA, Merc, and Urea. The swelling increased to about 800 wt.% for the samples  
8 Mix. 24h, Mix. 48h, and Enzymes while it was around 450 wt.% for NaOH samples. The statistical  
9 analysis on the swelling values of the matrices was reported in Table S3. The samples were studied  
10 hydrated after de-proteination treatments to avoid interactions induced by the drying. As control  
11 experiment the Mix. 24h, Mix. 48h, Enzymes, and NaOH samples were completely dehydrated. These  
12 samples showed a reduced common swelling around 300 wt.% (Table 1 and Table S4). The NaOH  
13 dehydrated and then rehydrated sample, named NaOH-dried, was used to study the influence of  
14 interaction among the chitin fibrils, being completely protein free.

15 Scanning electron microscopy (SEM) images of the cross-sections of lyophilized hydrated samples  
16 (Figure 4) showed that the overall ultrastructural organization of the wet state was conserved in each  
17 sample. However, while in the samples containing the higher content of proteins (EDTA, Merc, Urea,  
18 and Mix. 24h) the material appeared more compact, in the ones protein free (Enzymes, and NaOH),  
19 or almost (Mix.48h), the layered chitin matrix is well visible. As expected from the decrease in the  
20 swelling observed, a clustering of the layers was visible in the sample NaOH-dried. Coherently,  
21 decreasing the protein content the sample's surface was observed to lose its marked fibrillary  
22 morphology, leaving a smoother surface due to the thinner fibrils of the chitin matrix (Figure S4).

23 The synchrotron X-ray diffraction images of the samples are reported in Figure 5. All the samples  
24 showed the typical periodicities of di-hydrated  $\beta$ -chitin at about  $10.7\text{ \AA}$  and  $4.4\text{ \AA}$  along the equator  
25 direction, and  $5.1\text{ \AA}$ ,  $3.3\text{ \AA}$  and  $2.55\text{ \AA}$  along the meridian direction (Figure S5, Table S5).[37]  
26 Additional diffraction peaks, typical of di-hydrated  $\beta$ -chitin were also observed in the samples having



1 high diffraction intensity. Weak diffraction intensities were observed for Merc or Enzymes samples.  
2 No evident diffraction peaks that could be associated with proteins were detected. The broadening of  
3 the (002) reflection along the azimuthal angle was measured (Table S5). The data showed a small  
4 increase of the full width half maximum of this reflection, from about 28° to about 32° only in the  
5 samples NaOH and NaOH-dried.

### 6 **2.3 Mechanical characterization of treated squid pen**

7 The mechanical properties were analyzed by uniaxial deformation tests along the main axis of the  
8 pen. The Young's modulus ( $E$ ), maximum stress ( $\sigma_{\max}$ ), and maximum elongation ( $\epsilon_{\max}$ ) were  
9 calculated by the analysis of the stress–strain plots, summarized in Table 1, and plotted in Figure 6.  
10 These data showed that the sample treated with EDTA behaved as the native squid pen. The sample  
11 Merc showed a reduction of  $E$  and  $\sigma_{\max}$ , but did not change the  $\epsilon_{\max}$  with respect to the squid pen. The  
12 treatment with Urea did not affect the  $\epsilon_{\max}$  and the  $\sigma_{\max}$ , but reduced the  $E$  value. The use of the Mix.  
13 24h, Mix. 48h, Enzymes, or NaOH provoked a strong reduction of  $E$  and  $\sigma_{\max}$  to similar values, and  
14 an increase of  $\epsilon_{\max}$ , similar in the samples treated with NaOH, Mix. 48h, or Enzymes. This sample  
15 NaOH-dried showed an increase of the  $\epsilon_{\max}$  and  $\sigma_{\max}$  with respect to the un-dried one, but did not  
16 change the  $E$ . The clustering of the results was checked using a T-test ( $p = 0.05$ ,  $v \geq 8$ ), as reported  
17 in Table S3.

18

## 19 **3. DISCUSSION**

20 The goal of this research was to investigate the role of proteins in affecting the mechanical properties  
21 of natural chitin/protein composites. To our knowledge, no study has addressed this theme. This  
22 despite the number of organisms that use chitin/protein composites and the key role that they have in  
23 protecting them. Squid pen was chosen as model system because: (i) it is an example of stiff bio-  
24 polymeric fibrous material that has a  $E$  on the order of few GPa and is not very extensible ( $\epsilon_{\max} <$   
25 20 %);[38] (ii) it is an almost pure binary chitin/protein composite;[30] (iii) the proteins can be  
26 removed without affecting the ultra-structure of the chitin matrix;[36] (iv) the structure of the chitin

1 matrix is relatively simple and has been deeply investigated;[10,37] (v) it is formed by aligned  
2 fibrils;[11] (vi) it is widely distributed and commercially available.[39] Diverse chemical treatments  
3 on the Squid pens were treated chemically to obtain samples having the protein structure altered in  
4 composition and structure, without affecting the chitin matrix structure. Successively, the mechanical  
5 parameters of the samples were measured and compared with those of the native squid pen.

6 The analysis of the protein extract showed that the matrix is composed at least of nine structural  
7 proteins. These proteins were extracted with a different efficiency in the two sequential treatments,  
8 Mix. 24h and Mix. 48h. Two proteins having a molecular weight of about 97 and 20 kDa stained the  
9 gel only after the Mix. 48h treatment, suggesting that they were linked more strongly to the matrix or  
10 that the other proteins shielded them from extraction. This possibility agrees with the reported  
11 complexity of the protein wrap reported from *Sepioteuthis lessoniana* and *Uroteuthis chinensis*. [11]

12 The amino acid composition of the extract from Mix. 24h showed mostly apolar residues, which  
13 explained the low solubility of these proteins in water and buffers. Moreover, a high amount of  
14 aromatic residues (His and Tyr) was observed. These residues have been reported to have a crucial  
15 implication in binding chitin in CBDs, suggesting a significant presence of these domains. [20,40]

16 The amino acid analysis also highlighted the presence of about 1 mol.% of oxidized cysteine dipeptide  
17 (meaning a double molar amount of cysteine is present) and no reduced cysteine. This indicated high  
18 level of cross-linking in the matrix and/or being diagnostic for a high amount of cysteine-based  
19 CBDs. [20,40] As a performance comparison, in the vulcanization reaction of rubber a 1 to 3 wt.%  
20 amount of sulfur was used [41–43] and the tensile strength of rubber changed from 0.1-1 MPa [44–46]  
21 to 5-20 MPa. [47–49]. This shows how even a 1 mol.% of disulfide bridges in the squid pen protein  
22 can have a massive effect on its mechanical properties.

23 The results of the protein extraction experiments indicated that urea was efficient (around 40 wt.%)  
24 only when disulfide bridges (or other chemical bonds reduced by mercaptoethanol) were reduced.  
25 This indicated that the urea denaturation alone was not sufficient to remove with efficiency the  
26 proteins from the chitin matrix and that the disulfide bonds were crucial in preserving the protein

1 wrapping and/or the interaction with chitin. The about 6 wt.% of proteins extracted in urea could be  
2 associated with no structural and/or other tissues proteins present in the squid pen.

3 The data from UV-Vis spectra, FTIR spectra and gravimetric analysis indicated that proteins were  
4 massively removed from the squid pen only by degradation, either enzymatic (41 wt.% lost) or  
5 alkaline (60 wt.% lost). This different efficiency could be due to UV-Vis transparent chitin bonded  
6 peptides not degraded in the Enzymes samples, but hydrolyzed and solubilized in NaOH. On the other  
7 hand, no elimination of UV-Vis absorbing protein was observed in EDTA, Merc, and Urea samples.  
8 The Mix. 24h and Mix. 48h, instead, showed a comparable UV-Vis signal from proteins, despite  
9 presenting a different weight loss and relative intensity in the FTIR spectra. A hypothesis might be  
10 that some proteins were covalently bonded to the chitin matrix and un-extracted upon denaturation  
11 and reduction. This linkage could be an amide moiety on the amino groups of chitin, as reported for  
12 other composites.[38,50] Coherently, these residual proteins were cleaved when an alkaline or  
13 enzymatic treatment was used. Anyway, the chitin covalently linked proteins, if present, were a minor  
14 component in the squid pen composition (Table 1). The low solubility of those structural proteins  
15 might decrease the efficiency of the cleaning steps of the matrices. As consequence, an incomplete  
16 protein removal from the matrices treated using the Mix, especially in Mix. 48h, or Urea treatment  
17 (not Merc and EDTA since they did not show bands in the SDS-PAGE) could be present.

18 The results from the analyses of FTIR spectra and X-ray fiber diffraction patterns on dry samples  
19 showed that the  $\beta$ -chitin structure matrix was preserved.[36,51] Indeed, no differences were observed  
20 in the position of the X-ray diffraction peaks and FTIR absorption bands. Moreover, the spread of the  
21 (002) diffraction peak along the azimuthal angle was similar for all the samples (Table S5), suggesting  
22 that also the chitin fiber alignment was not affected by the chemical or enzymatic treatment. This is  
23 a piece of crucial information, which validates the initial research hypothesis. Indeed, once it was  
24 ascertained that the structure of the chitin matrix was preserved from the nano- to the macro-scale,  
25 all the further data could be related to the protein structure and composition.

1 All samples were further analyzed in their hydrated state, to preserve as much as possible their  
2 ultrastructure, unless the NaOH-dried sample. The latter was used to evaluate the mechanical  
3 properties of the pure chitin matrix, since the fibrils interacted after solvent elimination.

4 The inter-fibril interactions were estimated from the swelling data and the SEM observations. The  
5 swelling did not change significantly (T-test,  $p = 0.05$ , Table S3) in EDTA or Urea samples compared  
6 to the native squid pen (200-220 %). While, the reductive breakage of the chemical bonds, Merc  
7 samples, induced a slight increase of the swelling (~ 260 %). These data indicated that the chitin inter-  
8 fibril interactions due to proteins were almost unaffected by hydration, also when the disulfide bridges  
9 were broken. On the other hand, the presence of low amounts of proteins in the matrix (Mix. and  
10 Enzymes) drastically decreased the chitin inter-fibril interactions producing a high swelled state (~  
11 800 %). As reported, the chemical conditions controlled the assembly of chitin fibrils.[35] The  
12 complete removal of proteins (NaOH) favored the interaction among the chitin fibrils, as indicated  
13 by the lower swelling (~ 450 %) respect to the sample containing a low amount of proteins (Mix. and  
14 Enzymes). All these observations were supported by the SEM observations on the sample's cross  
15 sections, which coherently showed a loosen packing of the chitin layers increasing the deproteination.

16 The measure of the swelling after dehydration of these matrices gave values similar to that of NaOH-  
17 dry. This result indicated that the high swelled state of these samples was actually a consequence of  
18 the loss of fibril interaction after the protein removal. Coherently, after the dehydration a clustering  
19 of the chitin lamellas was observed in the SEM images, implying an increase of chitin fibril  
20 interactions.

21 The mechanical properties of the squid pen samples were measured through uniaxial deformation  
22 along the pen main axis, since the material is highly anisotropic.[10,11] Native squid pen, EDTA,  
23 Merc, and Urea samples showed a statistically similar low value (around 1.5%) of  $\epsilon_{\max}$  indicating that  
24 in the presence of proteins a minimum sliding of the fibrils occurred under the uniaxial deformation  
25 before the rupture of the samples. This agreed with the presence of strong inter-fibril interactions.

1 Interestingly the pen, despite the vestigial role of shell and the presence of calcium ions,[31] did not  
2 contain structural salt bridges, also according to the mechanical data.

3 The rupture of the disulfide bonds caused a decrease of E (from 4 GPa to 0.9 GPa, about 60 %) and  
4  $\sigma_{\max}$  (from 40 MPa to 17 MPa, about 65 %), as expected by the weaker inter-fibril interaction  
5 according to the swelling data. This decrease in stiffness could have two main contributors: i) the  
6 breaking of inter-protein disulfide bridges; ii) the breaking of structural disulfide bonds in the CBD,  
7 which consequently decreases the protein-chitin interaction. In the CBD the disulfide bridges were  
8 reported as necessary for the correct folding. This information was obtained by experiments with  
9 proteins in solution.[24,52] In a solid and compact state, as in the matrix, the folding might be only  
10 partially affected by the rupture of the disulfide bonds. The decrease of  $\sigma_{\max}$  and E without an increase  
11 in the  $\epsilon_{\max}$  in the Merc samples support the CBD unfolding hypothesis. In this scenario, the lack of  
12 interaction would lead to a collapse of the structure when the force applied overpass the weakened  
13 binding force between chitin fibril and CBDs. Moreover, a missing increment in the  $\epsilon_{\max}$  is not  
14 coherent with a disulfide based cross-linked matrix, reinforcing the implication of CBDs.

15 The Urea samples showed a decrease in the E (from 4 GPa to 2.4 GPa, about 40 %) compared to the  
16 native pen. This treatment induced a loss of stiffness due to a potential denaturation of the structure  
17 at the inter-protein interface, forming points of discontinuity. Despite that, the protein unfolding did  
18 not come along an increment of the swelling. This suggested that these discontinuities were not due  
19 to a higher amount of solvent in the structure, but most likely to protein unfolding. Considering that  
20 it is common for structural chitin-binding proteins to possess more than one CDB, a single protein  
21 could bind more than one fibril.[22,23] If that so, the denaturation would decrease the stiffness of the  
22 material due to an unfolding of the protein domain linking the CBDs.

23 A different value of  $\sigma_{\max}$  in sample Mix. 24h compared to Mix. 48h (11 and 6 MPa respectively) was  
24 observed. This result suggested that in Mix. 24h the higher amount of remaining proteins interacted  
25 with the chitin fibrils. Accordingly, this sample had a significantly higher value of  $\sigma_{\max}$  with respect  
26 to the other protein free samples, but not of E and  $\epsilon_{\max}$  (T-test,  $p = 0.05$ ,  $v \geq 10$ ).

1 The Mix. 48h, Enzymes, and NaOH treated samples did not show significant differences in their  
2 mechanical parameters, which could be due to the almost complete removal of the proteins. This  
3 suggests a convergent lack of interactions controlling the mechanical properties among chitin fibrils,  
4 even at different degrees of swelling. The values of  $\epsilon_{\max}$  were significantly higher in all the protein-  
5 poor samples than that of the squid pen (about four times), indicating a higher sliding among fibrils  
6 as consequence of protein removal. On the other hand, the values of E and  $\sigma_{\max}$  were significantly  
7 lower (over 90 % and 80 % respectively), showing the importance of the proteins in determining the  
8 stiffness and resistance of the material.

9 A significant increment in both  $\sigma_{\max}$  and  $\epsilon_{\max}$  was observed in the NaOH-dried samples, with respect  
10 to the native pen. In this sample the dehydration, before rehydration, allowed the chitin fibrils to stack  
11 better generating stronger interactions. This proved that in this sample, which showed a lower  
12 swelling compared to the other protein free samples, chitin fibrils interacted.

13 The missed increment in E showed how in protein-poor samples the stiffness was not strictly related  
14 to chitin inter-fibrils interactions. On the other hand, the  $\epsilon_{\max}$  values in the sample of re-hydrated  
15 chitin reached the 11% value. Here, it can be supposed that, since the chitin fibrils have a better stack,  
16 during the sliding non-specific interaction points were broken and regenerated allowing the matrix to  
17 reach higher values of  $\sigma_{\max}$  too.

18

#### 19 **4. CONCLUSION**

20 In conclusion, combining the experimental data, it was demonstrated that the correct folding of the  
21 proteins had a strong influence on the stiffness of the material. In particular, the chitin-protein  
22 interactions appeared strongly related to the presence of disulfide bridges in the CBDs and drastically  
23 influenced E and  $\sigma_{\max}$  of the matrix. On the other hand, the inter-protein interactions appeared to be  
24 mostly governed by a correct folding of the proteins, which could be altered by denaturing agents, as  
25 urea. The latter did not appear to influence the maximum resistance of the matrix, which mostly relied  
26 on the correct folding of the CBDs. Contrary, the maximum elongation appeared to be mostly related

1 to the presence of the proteins, even if partially unfolded. In fact, the partial or complete removal of  
2 the proteins led to a convergent result in the maximum elongation due to not or weakly interacting  
3 chitin fibrils. Finally, this study highlighted few crucial features on how the proteins in the squid pen  
4 acted as a stiff matrix that linked crystalline chitin fibrils avoiding their sliding, as would happen in  
5 a pure chitin material.

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## 7 **5. EXPERIMENTAL SECTION**

8 *Materials:* All reagents and solvents were purchased from Sigma Aldrich and utilized without any  
9 further purification. Squid pens from *L. vulgaris* were collected from a local market. Once hydrated,  
10 the lateral blades were isolated, cleaned with abundant distilled water (carefully eliminating eventual  
11 residual tissues), ethanol 70 vol.%, distilled water to remove the ethanol, and then stored dry in a  
12 desiccator.

13 *Sample preparation:* All samples were prepared using the squid pens previously washed as described.  
14 The squid pens were set in a vial with a solution to get a squid pen/solution ratio of  $10 \text{ mg} \cdot \text{mL}^{-1}$ . The  
15 vial was then set on a rocking table for 24 hours. The aqueous solvent tested were: 1) 1 mM  
16 ethylenediaminetetraacetic acid (EDTA); 2) 4 vol.% mercaptoethanol (Merc); 3) 8 M urea (Urea); 4)  
17 a solution with all the previous reported conditions, named Mix. All the solutions were shaken for 24  
18 h except the Mix. that was also tested for 48 h, changing the solution after the first 24 h of treatment.  
19 After the different treatments, the samples were washed with water (using PremilliQ water for the  
20 samples treated with EDTA or Mix), left in water, and set on the rocking table for 15 minutes gently  
21 shaking the vials. After that, the solution was changed and the process repeated for a total of 6 times.  
22 The alkaline treated samples,[36] instead, were obtained putting about 25 g of squid pen in 1 L of  
23 boiling 1 M NaOH solution, and stirring for 1 hour. Then, that solution was replaced with a fresh one  
24 and stirred under reflux for one hour more (counting the time from when the reflux restart). The  
25 obtained  $\beta$ -chitin was washed two times with a warm 1 M NaOH solution and then with distilled  
26 water until the washing water was neutral.

1 The enzyme treated samples were obtained as reported in Montroni et al..[36] Briefly, the squid pens  
2 (1 g) were soaked in 100 mL of a pH 2 HCl solution containing 20 mg of pepsin (an aspartic protease).  
3 The mixture was placed on a rocking table for 24 hours at 37 °C. After this first de-proteination, the  
4 squid pens were collected and washed carefully with distilled water. Then, the wet squid pens were  
5 soaked in 100 mL of a 100 mM phosphate buffer solution at pH 7.6 containing 20 mg of trypsin (a  
6 serine protease). As in the previous step, the mixture was placed on a rocking table at 37 °C for 24  
7 hours. Finally, the samples were washed as reported for the first conditions.

8 All the samples were stored in water at 4 °C, without any intermediate drying step except when  
9 specified.

10 *De-proteination weight lost and swelling measurements:* The measure of the chitin sample swelling  
11 in water was carried out first weighting a wet sample after the de-proteination, prior blotting it on a  
12 paper towel, and secondly weighting it after drying overnight in a desiccator under vacuum. The  
13 ‘NaOH dried’ sample’s swelling was measured weighting the dry sample before and after immersion  
14 for 24 hours in water. The weight loss due to de-proteination was determined by weighting the dry  
15 starting squid pens before and after the de-proteination processes. Mass measurements were  
16 performed using a Sartorius CP225D ( $\pm 0.01$  mg) on at least 100 mg of initial dry sample. Both  
17 swelling and de-proteination weight lost measurements were performed at least on three independent  
18 samples.

19 *Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE):* Polyacrylamide gel  
20 electrophoresis of SDS-denatured proteins was performed according to the description of  
21 Laemmli.[55] Protein samples were loaded on 12.5 % polyacrylamide gel and separated at constant  
22 power of 120 V for about 90 min. The detection was performed using a staining solution with  
23 Coomassie Brilliant Blue R-250 (methanol 50 vol.%, acetic acid 10 vol.%, and Coomassie Brilliant  
24 Blue R-250 0.25 wt.%). The excess of Coomassie was eliminated by washing with a de-staining  
25 solution (ethanol 25 vol.%, and acetic acid 8 vol.% in water). Proteins samples were prepared mixing  
26 different volumes of sample with a denaturing loading buffer (60 mM



1 tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 6.8, SDS 2 wt.%, glycerol 10 vol.%,  
2 bromophenol blue 0.025 wt.%, and  $\beta$ -mercaptoethanol 2.5 vol.%). Samples were boiled 5 min before  
3 loading.

4 *Amino acid analysis:* Amino Acid Analysis was performed in Xell (Germany) using a high-  
5 performance liquid chromatography (HPLC) System with an absorbance detector. A 6 M HCl  
6 solution was given to the dry sample and used for hydrolysis at 100 °C for 24 h. Then, sample was  
7 dried in vacuo over NaOH. The sample was resolved in 1 mL water, filtered and used for amino acid  
8 measurements. The depicted results were obtained by UHPLC-DAD on an Agilent 1290 system after  
9 the derivatization of amino groups. Two different System Suitability Tests (SSTs) were measured  
10 along with the samples to verify the calibration. Samples were measured in different dilutions. HCl  
11 hydrolysis converts of glutamine to glutamate and asparagine to aspartate.

12 *Spectroscopic analyses:* UV-Vis spectra were collected between 240 and 450 nm with a 1 nm  
13 resolution, and an average time of 0.1 s using a Varian Cary 300 Bio spectrophotometer. Fourier-  
14 transform infrared spectroscopy (FTIR) spectra were collected using a Nicolet IS10  
15 spectrophotometer. The samples were prepared as KBr disks and the sample concentration was about  
16 2 wt.%. The spectra were obtained with 4  $\text{cm}^{-1}$  resolution and 64 scans. Omnic software (Thermo  
17 Electron Corp., Woburn, MA) was used for data processing and baseline correction.

18 *Scanning electron microscopy (SEM):* Scanning electron microscopy images were acquired with a  
19 Philips SEM 515 using 15 eV. The wet samples were lyophilized, eventually cut with a scalpel to  
20 expose the section, glued on a carbon tape, dried overnight in a desiccator, and coated with 20 nm of  
21 gold prior image them.

22 *Synchrotron X-ray diffraction analysis:* X-ray fiber diffraction patterns were collected at ID23  
23 beamline, European Synchrotron Radiation Facility, Grenoble, France. Each frame was collected at  
24 the peak wavelength (0.87313 Å) using an exposure of 60 s. The X-ray diffraction patterns were  
25 analyzed using Fit2D software.

1 *Uniaxial tensile test:* Monotonic uniaxial tensile tests were performed using a universal testing  
2 machine (Mod. 4465 with Series IX software, Instron) and dedicated grips. The tests were performed  
3 with an actuator speed of  $5 \text{ mm}\cdot\text{min}^{-1}$  (resulting in a strain rate of about  $0.3 \text{ \%}\cdot\text{sec}^{-1}$ ) at room  
4 temperature. Each wet sample was cut in a proper dimension using scissors before the de-proteination,  
5 maintained hydrated after the synthesis, and tested hydrated. No difference in the sample geometry  
6 was observed after any of the treatments, except in the thickness due to an increase in the swelling of  
7 samples. The actual width and thickness of each hydrated sample were measured before testing them  
8 using a SM-LUX POL microscope collecting images with a 5.0 MP digital camera (Motic Moticam  
9 5+). The images were analyzed using ImageJ. Each sample was about 40 mm long, 5 mm wide, and  
10 0.15-0.50 mm thick (the thickness varied between samples, because of the intrinsic variability of the  
11 initial biological samples and the different swelling of the matrices). The samples were connected at  
12 the instrument, leaving a free length of about 30 mm between the clamps. As the curves were rather  
13 linear until failure started, the following parameters could be calculated, taking into account the actual  
14 dimensions of each specimen:

15 - The Young modulus (E) of elasticity: defined as the slope of the linear part of the stress-strain curve  
16 (usually between 30-80 % of the maximum strain) and calculated using a linear interpolation. The  
17 portion of this region considered was reduced in case of change in the linearity, considering only the  
18 initial linear region;

19 - The maximum stress ( $\sigma_{\text{max}}$ ) and strain ( $\varepsilon_{\text{max}}$ ).

20 The samples failed in mostly two ways: either abrupt fracture, perpendicular to the sample's axis,  
21 common for the protein rich samples, or progressive unraveling of the fibers, more common for the  
22 samples poor in proteins. At least five specimens were tested for each group. Slipping, early breakage,  
23 breakage at the grips, or inhomogeneity in data were not observed in the samples examined.

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## 25 **CONFLICTS OF INTEREST**

26 There are no conflicts to declare.

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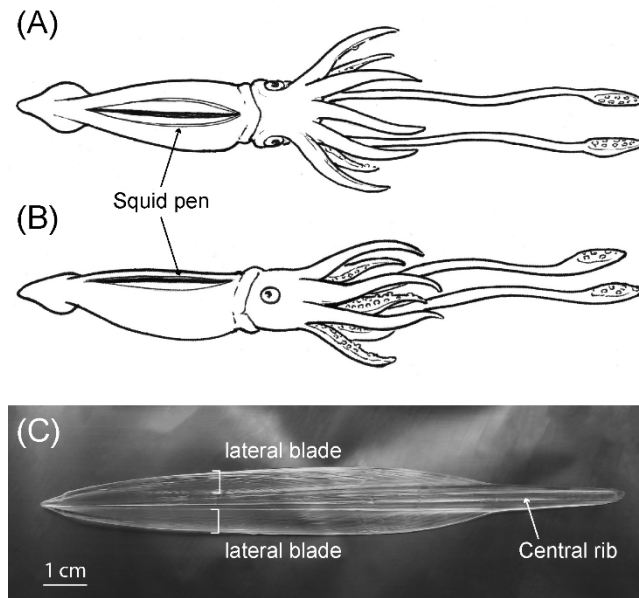


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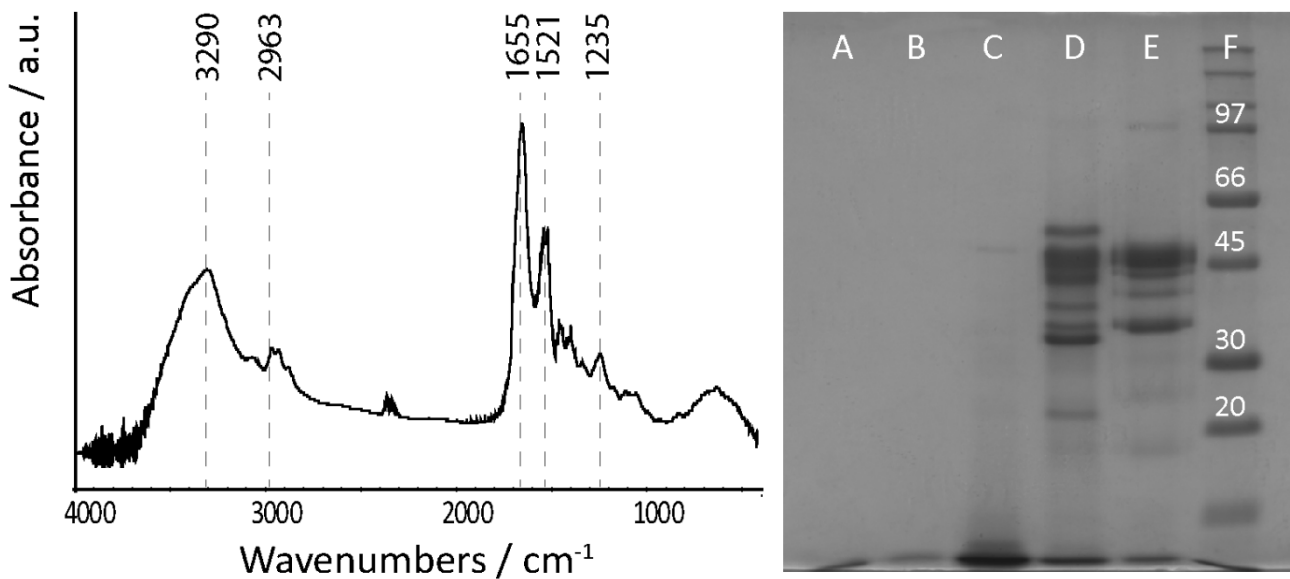
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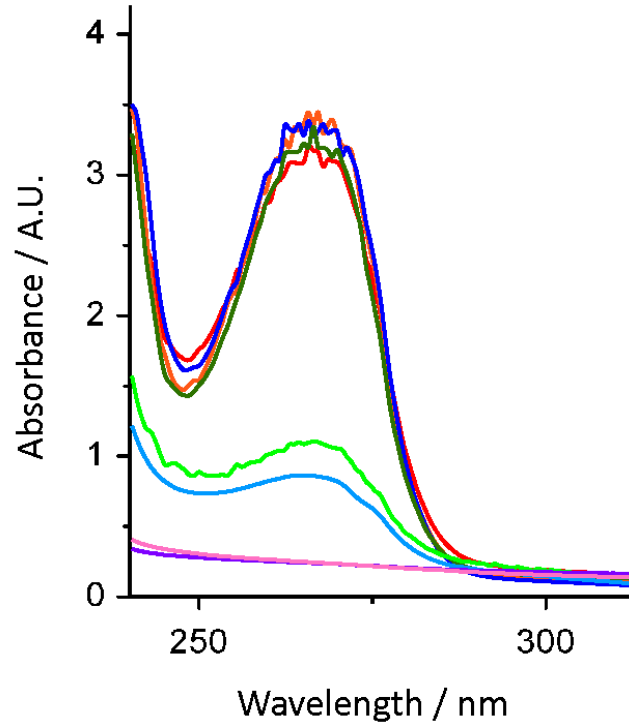
**Figure 1.** A schematic representation of a squid where the squid pen is illustrated even if, being an internal structure, is not visible from the outside of the animal. In (A) the squid is observed frontally while in (B) laterally. In (C) a camera picture of a squid pen.



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**Figure 2.** (left) FTIR spectrum of the proteins extracted from the squid pen using the Mix. 24h. The main absorption bands of proteins are labelled. (right) SDS-PAGE from the extracting solution: (A) 1 mM EDTA; (B) 4 vol.% mercaptoethanol; (C) 8 M urea; (D), Mix. 24h, and (E) Mix. 48h. (F) References for the molecular weight reported in kDa. 25  $\mu$ L of each sample were loaded in the SDS-PAGE, except for Mix. 24h were 2  $\mu$ L were loaded.

1

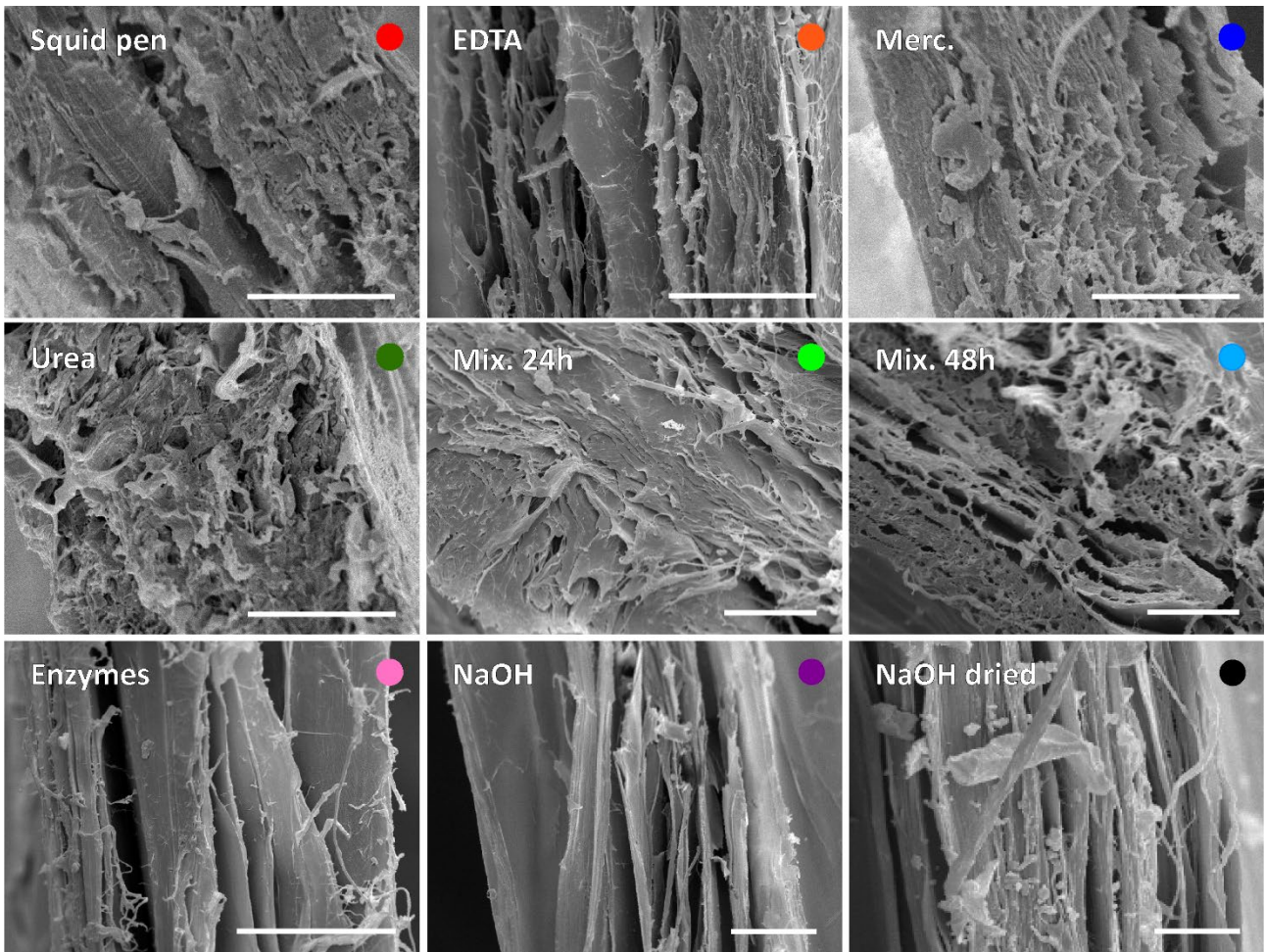


2

3 **Figure 3.** UV-Vis spectra of the different samples after chemical or enzymatic treatments. The  
4 samples reported, from higher to lower intensity of the aromatic residues signal at 280 nm, are the  
5 squid pen (red), EDTA (orange), Merc (blue), Urea (dark green), Mix. 24h (light green), Mix. 48h  
6 (light blue), Enzymes (pink), and NaOH (violet).

7

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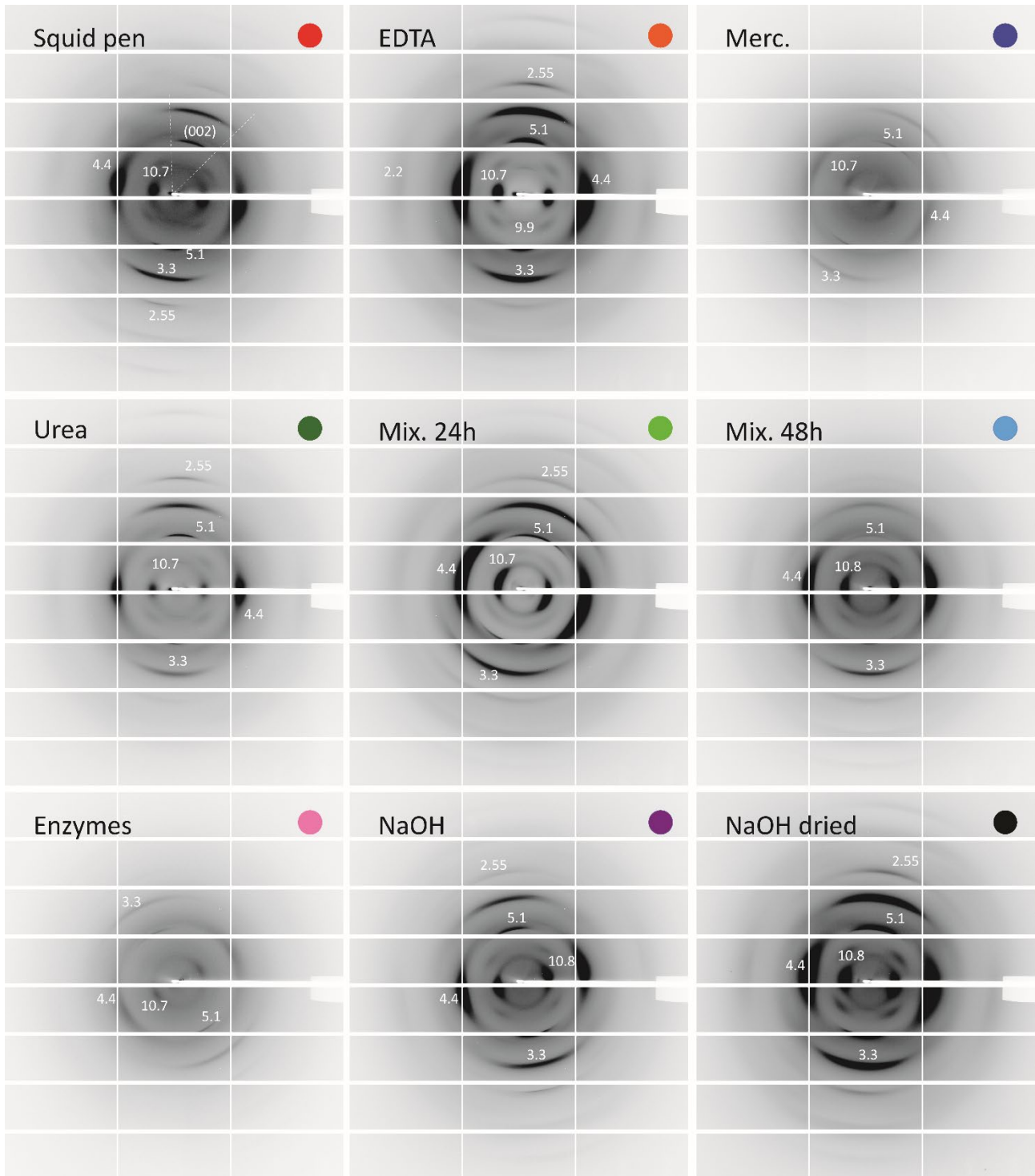


2

3 **Figure 4.** SEM images of the cross section of **the hydrated samples after freeze-drying**. The cross  
4 sections were obtained by mechanical rupture of liquid nitrogen frozen samples. When necessary the  
5 sample was tilted for the optimal observation of the cross section. Scale bar: 20  $\mu\text{m}$ .

6

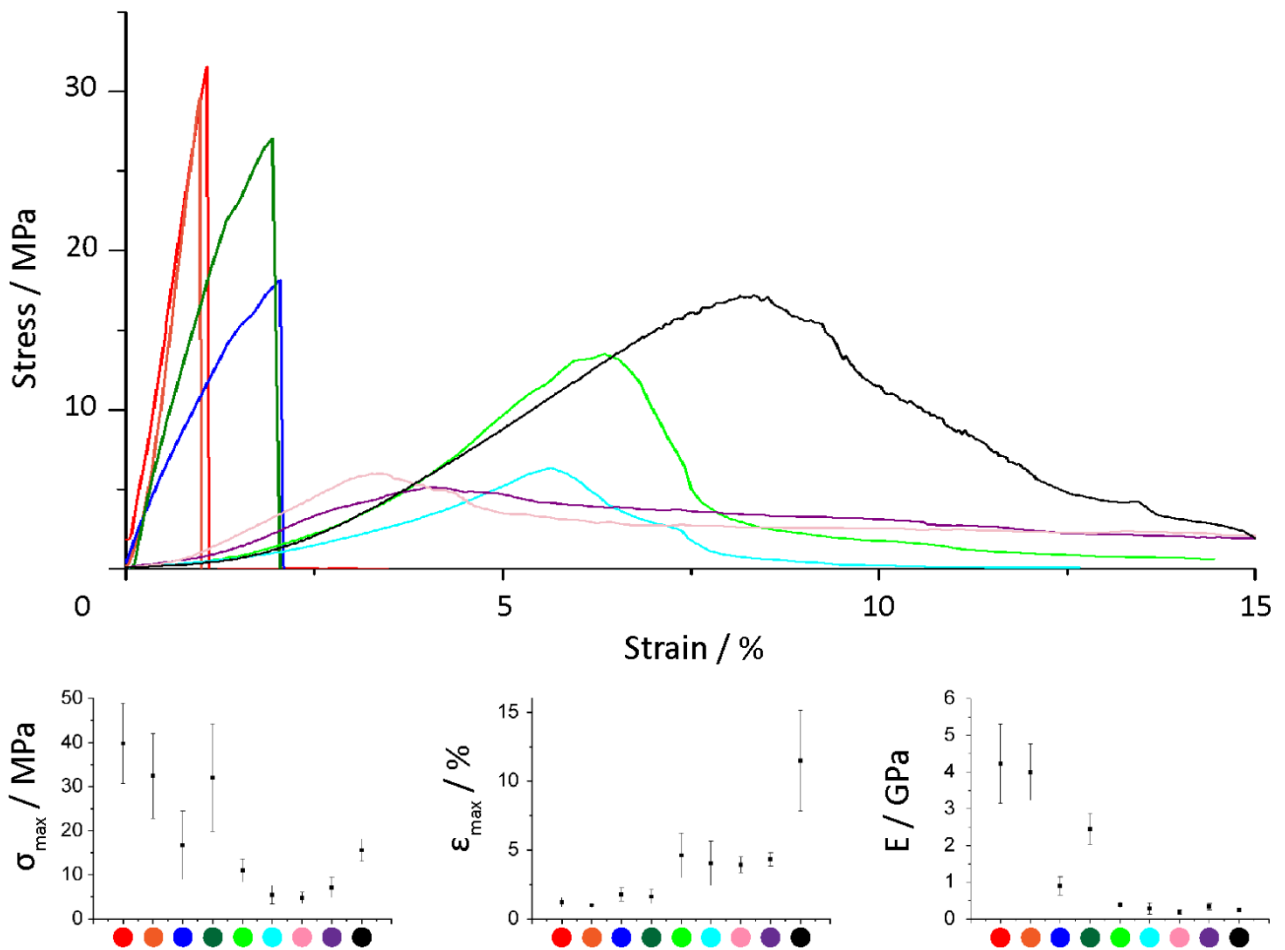
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3 **Figure 5.** Synchrotron X-ray diffraction images of the samples. The periodicities of di-hydrated  $\beta$ -  
4 chitin reflections are reported according to Sawada et al.[51]. The dotted line in the image squid pen  
5 indicate the angular spread of the (002) reflection.

6



1

2 **Figure 6.** (above) Uniaxial traction profiles of representative samples and (below) the mechanical  
 3 parameters of uniaxial traction, maximum stress ( $\sigma_{max}$ ), maximum strain ( $\epsilon_{max}$ ) and Young's modulus  
 4 (E). The data reported, generally from stiffer to softer, are : squid pen (red) samples, EDTA (orange),  
 5 Merc (blue), Urea (dark green), Mix. 24h (light green), Mix. 48h (light blue), Enzymes (pink), NaOH  
 6 (violet), and NaOH-dried (black).

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8

1 **Table 1.** Summary of the compositional, swelling, and mechanical properties of squid pen treated  
 2 samples. Weight loss (wt.%), swelling (wt.%), maximum stress ( $\sigma_{\max}$ ), maximum strain ( $\varepsilon_{\max}$ ), and  
 3 Young modulus (E) are reported.

4

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<b>Squid pen</b>	●	0*	199 ± 2	40 ± 9	1.2 ± 0.4	4000 ± 1000
<b>EDTA</b>	●	0 ± 3	212 ± 21	30 ± 10	0.99 ± 0.06	4000 ± 1000
<b>Merc</b>		0 ± 2	260 ± 20	17 ± 8	1.8 ± 0.5	900 ± 300
<b>Urea</b>		6.3 ± 0.4	220 ± 20	30 ± 10	1.6 ± 0.6	2400 ± 400
<b>Mix. 24h</b>	●	40 ± 9	800 ± 100	11 ± 3	5 ± 2	380 ± 80
<b>Mix. 48h</b>	●	52 ± 3	800 ± 100	6 ± 2	4 ± 2	300 ± 100
<b>Enzymes</b>	●	41 ± 9	760 ± 50	5 ± 1	3.9 ± 0.6	190 ± 50
<b>NaOH</b>	●	60.1 ± 0.9	450 ± 20	7 ± 2	4.3 ± 0.5	330 ± 90
<b>NaOH-dried</b>	●	60.1 ± 0.9	340 ± 10	16 ± 2	11 ± 4	250 ± 40

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5 \* Squid pen was used as reference weight.

6