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Cellulose-protein moieties as bioingredient: Their origin, composition, structure and industrial application

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Abstract

Globally, around 2815 tunicate species have been recorded from shallow coastal waters to deep waters. These organisms have cellulose and protein in their structural and chemical composition. Extraction of tunicate cellulose nanofibers (T-complex) can be done using a wide variability of methods. Phylogenetic analyses of ascidian cellulose synthase suggested that the genes might have been acquired from a prokaryote by horizontal gene transfer prior Tunicate CesAs have only been identified in two ascidians, Ciona savignyi and C. intestinalis. To support the cytoskeleton, body structure and functions, cellulose is produced by multimeric cellulose synthase located in the Terminal Complexes (TCs) inserted in the plasma membrane. Considering this, the protein-cellulose moieties or complexes (PC-tun complex) have been researched in many studies without elucidation of their bio-arrangement in the tunicate cell. Chemically, the principal constituents of the tunics are proteins with amino groups and tryptophan, scleroproteins, collagen and elastic fibers as well as high quantities of acid mucopolysaccharides and neutral polysaccharides.

This family of proteins was first discovered and isolated in the study of general mechanisms of adhesion of E-cadherin. β-catenin is part of many human canonical and non-canonical process inter and extracellular. This molecule has been researched acting as regulator signal in many drug-delivery systems as metabolic routes, showing properties that influence diseases as cancer, metabolic disorders, cognitive disorders, osteoporosis. The function of these polysaccharides in the tunic of the ascidian is a matter of speculation. Since the tunic of ascidians is an external supportive and protective skeleton and these polysaccharides occur in concentrations which resemble the great quantities of the glycosaminoglycans that are characteristic of cartilages. Finally, bioingredients as T-complex can be obtained from marine and terrestrial sources and their bioactive compounds has been well recognized in connection with health promotion, disease and risk reduction in health care costs, food and packaging material applications. Because of its unique functional properties, these compounds have a widely used in food industries, pharmaceutical preparations, cosmetic and material industries.

Keywords: Tunicates; Health and Care; Complex diseases; Nanocellulose; Protein

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Introduction

The cosmetics and biomedicine industry has demanded sustainable bioingredients to replace fossil sources. Nanocellulose is an attractive option to replace synthetic solutions. Nanofibrillated Cellulose (CNF) mainly from wood fibers has been the most used raw material applied as rheological additive and structural matrix reinforcement in different sectors. The industry sector well knows the properties of the CNF from wood and this study will show a new source of raw material with different composition

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and structural properties to produce a new grade of CNF, called T-complex, a new bioingredient obtained from fibrils extracted from Tunicates. Tunicates are organisms that present cellulose and protein in their structural and chemical composition. Tunicates are a class of marine invertebrates present throughout the globe; they grow in coastal communities and present themselves as residues and invaders in the marine ecosystem. Extraction of tunicate cellulose nanofibers (T-complex) can be done using a wide variability of methods. This new category of nanocellulose increases the accessibility and exposure of the protein-cellulose complex, T-complex. The chemical composition of the T-complex leads the application areas as for cosmetics; barrier and coatings; biomedicine and pharmaceutical.

Literature Review and Product Design

Oceanographical distribution

The precise origin of *Styela plicata* is unknown. The type specimen was described from a ship in the Delaware River, Philadelphia, Pennsylvania in 1823 [1]. Tunicates species it was apparently well-established south of Cape Hatteras by the late 19th and early 20th centuries before regular collecting began [2]. Globally, around 2815 tunicate species have been recorded from shallow coastal waters to deep waters [3]. Ascidians, usually abundant on artificial substrates, often are one of the most frequent gender of species of the reported introduced sessile taxa [4-6]. It is impossible for them to disperse over great geographic distances. Transoceanic spreading is only possible by human-mediated transport [5]. The current presence of ascidian species was identified in 15 coastal regions. These regions can be classified as (WIPac) West Indo-Pacific; (NWPac) Northwest Pacific; (CIPac) Central Indo-Pacific; (Taus) Temperate Australasia; (NEPac) Northeast Pacific; (TEPac) Tropical East Pacific; (SEPac) Southeast Pacific; (NWAtl) Northwest Atlantic; (TWAtl) Tropical West Atlantic; (SWAtl) Southwest Atlantic; (NES) Northern European seas; (Lusit) Lusitanian Sea; (Med) Mediterranean Sea; (TEAtl) Tropical East Atlantic; (SEAtl) Southeast Atlantic [5]. The most important environmental variables associated with the natural distribution of ascidians are seawater temperature and salinity [7-9].

Figure 1 shows the global distribution of *S. plicata* in tropical and warm-temperate oceans, based on occurrences reported in the literature. [2,10]. Ascidians are sensitive to low salinity, which can fluctuate significantly on a daily basis in certain regions due to tidal influence. As a result, environmental suitability may have been overestimated in areas with highly variable freshwater input. Also, using species with a history of introduction tends to concentrate records in harbors, where eutrophication is common due to proximity to major urban centers [5].

Nineteen (19) species of Ascidian was identified [5], and one of them is *S. plicata*. From all the 15 coastal regions, *S. plicata* occur in 12 and don't appear in 3 specific areas, Tropical East Pacific (TEPac), Southeast Pacific (SEPac) and Northern European seas (NES) [5]. The major impacts currently reported for introduced ascidians are associated with the bivalve industry because aquaculture facilities create an ideal environment for ascidians, with abundant suspended food and unlimited locations for attachment (bivalve shells, ropes and buoys) [5].

Phylogenetic

Globally, around 2815 tunicates species have been recorded from shallow coastal waters to deep waters [3,11]. Although this marine organism is considered invasive, it presents numerous opportunities for use as a source of materials in various fields, due to its unique composition and structure. Tunicates are soft-bodied solitary or colonial (60%) sessile marine organisms belonging to the family Ascidiacea under the subphylum Urochordata, phylum Chordata [3,11,12]. These organisms are hermaphroditic, filter feeders, and appear in different body colors, with a life span ranging from two months to one year [3,11-15].

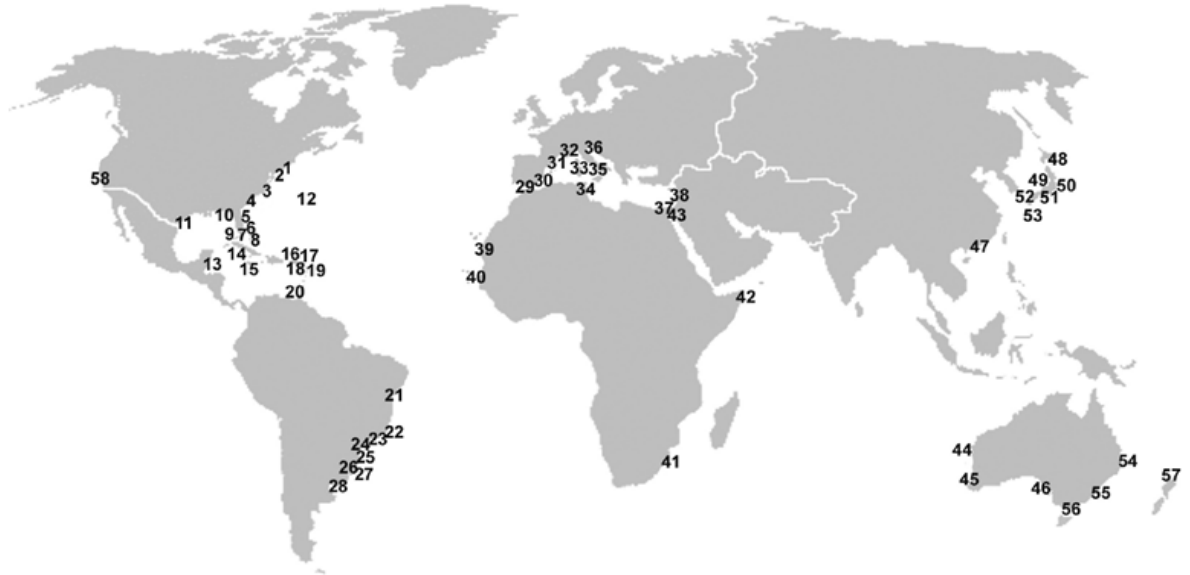

Figure 1 World distribution of *Styela plicata*. Numbers means the concurrency according to the register and location [2,10].

They are classified into 4 major clades such as (a) Appendicularia, (b) Thaliacea + Phlebobranchia + Aplousobranchia, (c) Molgulidae, and (d) Styelidae + Pyuridae, based on the phylogenomic transcriptomic approach [3,15]. **Figure 2** shows tunicates' structure in solitary and colonial form. The intestine is on the left side of the pharynx. The internal longitudinal branchial veins are flat. The pharynx surrounds the gonads from both sides Stolidobranchiata. Branchial tentacles are simple in structure, unbranched. The specie researched in this work have number of folds in both valves of the pharynx 4, there are considered Stigmatatas and are long from the Styelidae family, recognized as *S. plicata* [16].

Cytoskeleton

A major advantage of using ascidian embryos to study cell fate specification is that their tadpole larvae, which share the characteristic chordate body plan, contain a relatively small number of cells. This feature makes it possible to draw a comprehensive picture of cell fate specification in an embryo at the single-cell level. Because cell fates are mostly restricted in a single cell and determined by the 110-cell stage [17]. Cell development in Ascidians could be divided into four distinguished stages called: (1) Fertilization; (2) Embryogenesis; (3) Gastrulation and (4) Neurulation. After the last stage, some important major tissues differentiation starts. The major tissues and their constituents as protein-cellulose moieties that constitute the ascidian tadpole are the epidermis, nervous system, notochord, muscle, mesenchyme, Trunk Lateral Cells (TLCs) and Trunk Ventral Cells (TVCs), and endoderm **(Figures 3-5)** [17].

The TLCs and TVCs are small groups of cells located bilaterally dorsal and ventral, respectively, to the mass of mesenchyme cells. The TLCs become body wall (oral siphon and longitudinal mantle) muscle and blood cells, while the TVCs give rise to the body wall (atrial siphon and latitudinal mantle) muscle, heart, and pericardium [17]. Phylogenetic analyses of ascidian CesAs suggested that the genes might have been acquired from a prokaryote by horizontal gene transfer prior Tunicate cellulose synthase (CesAs) have only been identified in two ascidians, Ciona savignyi and C. intestinalis [18].

To support the cytoskeleton, body structure and functions, cellulose is produced by multimeric cellulose synthase terminal complexes (TCs) inserted in the plasma membrane where TCs are presented in a stationary, linear organization [18]. Post-metamorphic and pre-metamorphic are also surrounded by a tunic composed in part of cellulose **(Figure 4)** [18]. Post-metamorphic stages of the latter two groups of Ascidians incorporate cellulose into a tough integument, the tunic, which surrounds the animal and forms in part the filter-feeding apparatus [18-20]. Pre-metamorphic, non-feeding, larval ascidians are also surrounded by a tunic composed in part of cellulose, and in addition to its protective function, cellulose has a role in the control of Ciona metamorphosis.

Larvaceans do not live inside a rigid tunic, but instead repetitively secrete and discard a complex, gelatinous filter feeding house. The house comprises of cellulose [18,21] and of at least 30 proteins [18,22,23] and both components secreted by a polyploid oikoplastic epithelium [18,24]. Of eleven (11) characterized house proteins, none of them show significant similarity with proteins

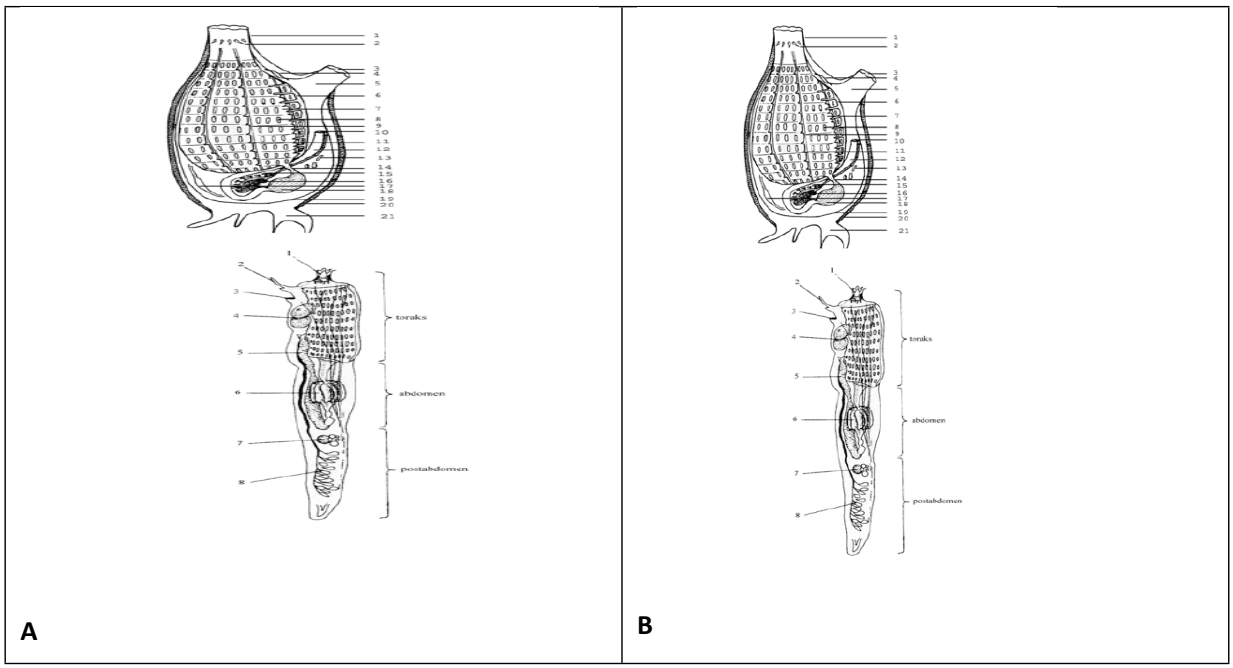

Figure 2 Structure of ascidians [1]. A) solitary adult ascidian 1: Branchial siphon, 2: Branchial tentacle, 3: Atrial siphon, 4: Neural complex, 5: Peribranchial space, 6: Dorsal lamina, 7: Pharynx, 8: Stigmatata, 9: Internal longitudinal branchial vessels, 10: Anus, 11: Papillary, 12: Intestine, 13: Renal vesicle, 14: Esophagus, 15: Testis, 16: Ovary, 17: Heart, 18: Stomach, 19: Mantle, 20: Tulum, 21: Stolon [15]. B) Zooid of colonial ascidians 1: Branchial siphon, 2: Atrial appendage, 3: Atrial aperture, 4: Embryo, 5: Intestine, 6: Stomach, 7: Ovary, 8: [18].

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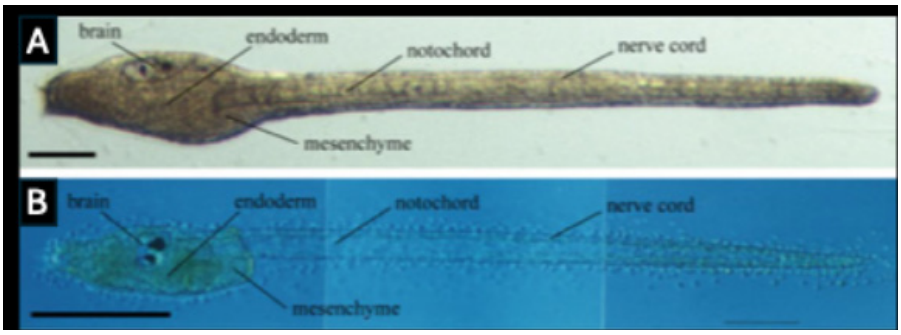

Figure 3 Major tissues that constitute the ascidian cytoskeleton. A and B: Tadpole larvae of A) *Halocynthia* and B) *Ciona intestinalis* [19].

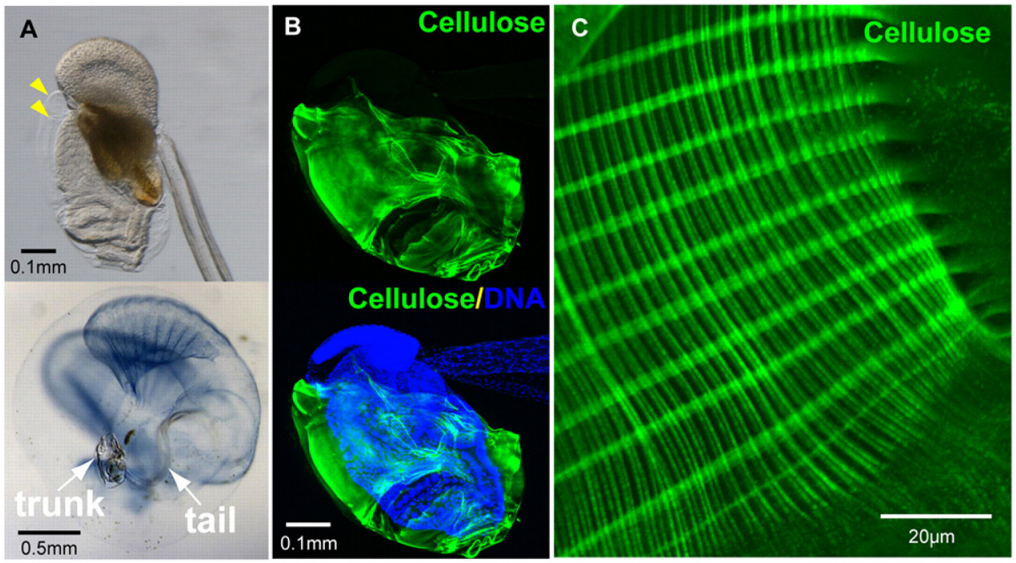

Figure 4 Cellulose structures in post-metamorphic *Oikopleura dioica*. A) Upper: day-5 animal with gonad at the top and mouth at the bottom has two uninflated pre-house rudiments (arrowheads) secreted around the trunk. Lower: day-3 animal inside an inflated house stained with India ink. The ribbed food-concentrating filter is visible at the top. B) Confocal image stack of cellulose (green) in the rudiment (upper) superimposed on stained nuclei (blue) of the oikoplastic epithelium responsible for secretion of house components (lower). C) Confocal image stacks of mesh formed by cellulose microfibrils in the maturing inlet filter [20].

in the sequenced ascidian genomes of C. intestinalis or C. savignyi, or in a broader sense, with any proteins in public databases, suggesting that these innovations are specific to the larvacean.

In ascidians, β-Catenin, is the protein that sits at the top of a hierarchy so far that leads to endoderm formation. β-Catenin, which is the protein uniformly present in the entire embryo, activates the expression of several genes. Despite its role in initiating endoderm formation, the ubiquitous expression of the protein is not consistent with that of what might be a maternal cytoplasmic determinant for endoderm formation. Results from earlier cytoplasmic transfer experiments suggested that it is localized to the vegetal pole of the fertilized egg and inherited by the cells of the vegetal hemisphere. Several other downstream genes of β-Catenin are, in fact, expressed in endoderm and mesoderm cells [17]. **Figure 5** illustrates the schematic cytoskeleton structure of ascidian after the last phase (4-neurulation) of the development in Ascidians with the differentiated tissues.

Protein-cellulose bio arrangement

The characterization tools applied in this study have been chosen to elucidate the lack of information in some specific areas of knowledge about tunicates, considered a new source of raw material for nanocellulose production and application. Nanocellulose from fiber materials are well known established in some areas as: (1) Production route is clear for many producers independently of the source, (2) Applications fields are well discovery for many materials that's not involve specific human contact, health and risk assessment regulations and the last major area, (3) Raw materials remain still a lack of information of some structures and challenge intrinsically components, their modifications and what they can impact in the application field.

Considering this, the protein-cellulose moieties or also called protein-cellulose complexes (PC-tun complex) have been researched in many studies without elucidation of their bio-arrangement in the tunicate cell. The ultra and suprastructure

of cellulose is established mainly for wood-derivated materials, as well the cellulose-lignin complexes in the wood. In the case of the tunicates the binding of protein in the cellulose fibrils is still non well documented, thus **(Figures 6-8)** bellow show are my proposed schematic model of cellulose-protein bio-arrangement and supraorganization, secondary metabolites and cellular structures in the tunic in the completely process of defibrillation for nanocellulose production (Before, during and after mechanical fibrillation) based on all literature background and initial results from this project.

Chemically, the principal constituents of the tunics are proteins with amino groups and tryptophan, scleroproteins, collagen and elastic fibers as well as high quantities of acid mucopolysaccharides and neutral polysaccharides [25,26]. Figure 6 shows the TS (Transversal section of natural tunic of tunicate); PSC (Proteins complexes and secondary components); PC-tun complex (Protein-Cellulose complex) and CF (Cellulose fibrils). Assuming that the nitrogen content originated from protein, 25.82–38.08 % of the dry weights of SP (*S. plicata*), CI (*Ciona intestinalis*) and HR (*Halocynthia roretzi*), while the lowest protein content (17.74 %) was found in AS (Ascidia sp) [25].

To enhance and hold the links involved in the PC-tun complex, some residual sugar was identified in different classes [27]. Three fractions of different molecular weights: one fraction has a molecular weight of 100,000 (C1: Class 1) or more and more two with approximately 20,000 (C2: Class 2) and 8,000 (C3: Class 3) Daltons. In each class some residual sugar could be identified as: C1 (Mainly Galactose, Gal); C2 (Mainly galactose and glucose, Gal and Glu); C3 (Mainly hexoses and sulfated esters, Gal, Glu, Manose-Man and Xylose-Xyl) [27-29]. These sugars are the linkage between cellulose and protein in the PC-tun complexes **(Figures 6-8)** and. It is important to mention that the binding interface of protein and cellulose is governed for specific linkages and the most frequent are O-linked glycans [25].

For C1, hexosamines are classified as N-glycans components (GalNH and GlcNH) linked β-1→4 0-glycan [27-28] and possible OH terminals in the cellulose for this binding are in the carbons C2, C4 and C6. For classes C2 and C3, sulfated esters, sulphated glycans and N-terminal units are classified as R-OSO_3- linked at carbons C3 and C4 [28,29]. Sulfated glycans are mainly classified as α-L→galactopyranose [29]. Finally, N-end-units are mainly classified as Gal and Xyl major monosaccharides at the reducing terminal of glycans [29]. One unique property of the PC-tun complex is that from these 3 classes of sugar and their linkage involved, C1 could be considered as neutral charged and C2 and C3 due to R-OSO_3- residues could be considered anionic [29]. PSC

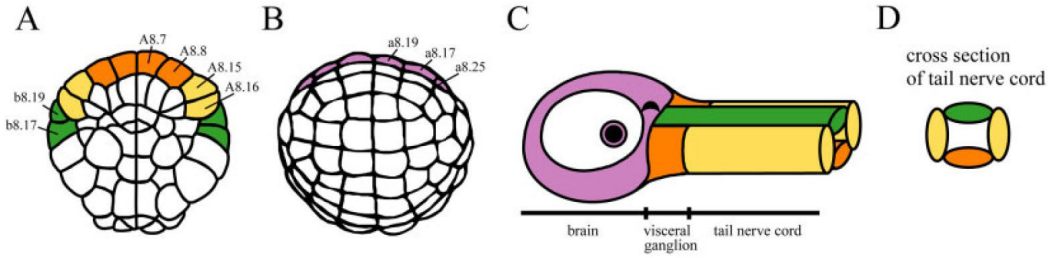

Figure 5 Schematic cytoskeleton structure of ascidian [19]. Fate map of the ascidian Central Nervous System (CNS). The origins in the 110-cell stage embryo of the brain (purple), and the dorsal (green), lateral (yellow), and ventral (orange) rows of the ependymal cells in the nerve cord are shown. A) A vegetal view of the 110-cell stage embryo. Anterior is up. B) An animal view of the 110-cell stage embryo. C) Schematic drawing of the larval CNS. Anterior is to the left with dorsal up. D) A diagram of a cross-section of the posterior nerve cord [19].

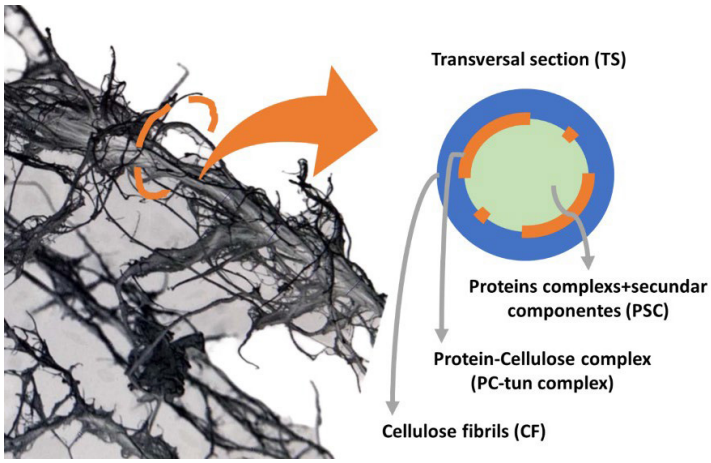

Figure 6 Schematic model of Protein-Cellulose bio-arrangement before mechanical fibrillation. TS (Transversal Section of natural tunic of tunicate); PSC (Proteins complexes and secondary components); PC-tun complex (Protein-Cellulose complex) and CF (Cellulose Fibrils).

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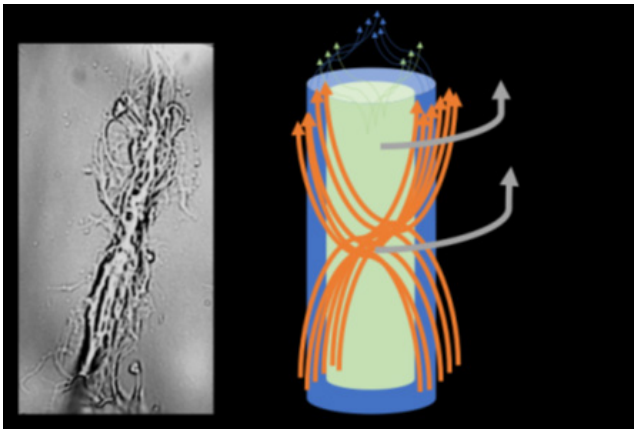

Figure 7 Schematic model of Protein-Cellulose bio-arrangement movement during and after mechanical fibrillation. Shear forces resulted from mechanical fibrillation are normally applied using grinders, homogenizers and refiners. LS (Longitudinal Section of natural tunic of tunicate and from the illustrated proposed model); PSC (Proteins complexes and secondary components); PC-tun complex (Protein-Cellulose complex) and CF (Cellulose Fibrils).

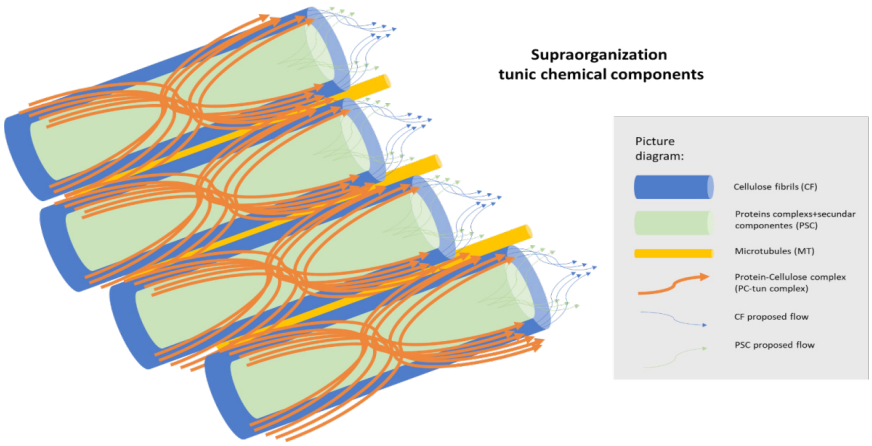

Figure 8 Bio-arrangement and supraorganization of cellulose, protein and secondary metabolites in the tunic during and after mechanical fibrillation.

complexes are also immerged in a matrix that contain secondary metabolites as showed in Figures 6-8. Between 0.28% and 4.25% of the tunic was composed of fatty acids, representing its lipid content. [25]. This fraction is composed of volatile and non-volatile materials also and is the first to be removed when pre-treatments are applied in the natural tunicate material [25].

Figure 7 shows the proposed schematic model of Protein-Cellulose bio-arrangement movement during and after mechanical fibrillation. The fibrillation of compacted cellulose involves the application of substantial shear forces normally applied mainly from grinders, homogenizers and refiners. Arrows in Figure 7 represent the proposed movement for the exposition of the chemical components in the tunicate matrix after the application of 2-step of energy that was converted in mechanical stress. This arrangement was inspired in the light microscope images obtained in this same study and in the literature for another wood fibers. The proposed movement promoted by the mechanical forces applied present enough shear forces to expose cellulose fibrils and their additional components linked to their structure.

Figure 8 shows the bio-arrangement and supraorganization of cellulose, protein and secondary metabolites in the tunic during and after mechanical fibrillation, and an important component, the MT (microtubules) that is also present in the tunicate cell and act as physical support for the cellular structures (cellulose, protein and secondary metabolites. In the tunic, cellulose and proteins are bound together, forming a compact structure known as PC-tun complexes, as previously described.. An important role for the PC-tun complexes is that in the larval phase of the tunicates, the tadpoles, present specific structures called collocytes. Collocytes are cellular components that differentiated papillary regions in the tunicate larval phase. Thus, collocytes localized in the papillae region secret green-glues called mucoadhesives composed for protein-cellulose linkages [30].

One of our hypotheses is that these cell-regional structures are responsible for secreting the cellulose–protein moieties involved in tunic formation, in coordination with cellulose synthesis by CESA during the tadpole larval stage. Post larval phase cellulose could be considered a major component in the tunic.

Regarding the type of protein involved in tunic composition, maternal β-catenin plays a key regulatory role at the top of the developmental hierarchy leading to endoderm formation. Therefore, identifying the structure and function of this protein presents a unique opportunity to advance potential applications of T-complexes containing PC-tun structures, which are expected to be associated with β-catenin activity.

β-catenin

β-catenin belongs to the catenin family of molecules. This family of proteins was first discovered and isolated in the study of general mechanisms of adhesion of E-cadherin [31-34]. β-catenin is part of many canonical and non-canonical process inter and extracellular. This molecule has been researched acting as regulator signal in many drug-delivery systems as metabolic routes, showing properties that affects [33] diseases as cancer, metabolic disorders, cognitive disorders, osteoporosis. This protein is a multitasking and evolutionary molecule present in many groups **(Figure 9)** [35] and in Ascidians. Conserved molecule that in metazoans exerts a crucial role in a multitude of developmental and homeostatic processes. More specifically, β-catenin is an integral structural component of cadherin-based adherent's junctions, and the key nuclear effector of canonical Wnt signaling in the nucleus. In adult organs, Wnt signaling continues to play indispensable roles in tissue homeostasis, cell renewal, and regeneration [35].

β-catenin presents a structure containing 781 amino acid residues in humans as a member of the Arm superfamily [31, 34]. Its primary structure **(Figure 10)** consists of the NH2-terminal pre-exchange (NDT), the central region, represented by 12 MER repeats (R1–12), and COOH terminal end (CTD) **(Figure 10)** [31,33,36]. Each Arm repeat includes approximately 42 amino acid residues forming 3 helices. All together, the MER repeats form a supercoil that forms a long positively charged groove [31].

The initial role of β-catenin in subdividing embryos **(Figure 11)** into ectoderm and (mes)endoderm has been reported in many phyla [37-45]. In Ciona, *C. elegans*, and P. dumerilii, nuclear β-catenin asymmetry is observed during the segregation of mesoderm and endoderm lineages with β-catenin localized to the nucleus of the endoderm precursor [37, 46-49]. In both Ciona and *C. elegans*, β-catenin asymmetry is required for this fate segregation. A shared mechanism for mesoderm and endoderm segregation between sea urchins and ascidians would be particularly remarkable given their very different modes of embryogenesis [37]. These observations probably represent only the tip of the iceberg and future studies may find that the signaling role of β-catenin is broader and more complex than we thought [21] when the primary canonical route was first described in cells and also their functions in Ascidians organisms.

Amino Acids (AA) from *styela plicata*

The sulfated glycans in the tunic of *S. plicata* differ from the glycosaminoglycans of animal tissues and from the sulfated polysaccharides isolated from marine algae. The ascidian glycans occur primarily as three fractions that differ markedly in molecular weight and chemical composition [50]. This glycosaminoglycans could be named as hexosamines iminoacids. The high molecular weight fraction encompasses a broad range of molecular weights but is chemically homogeneous and contains an unusual amount of galactose. The 20,000 molecular weight polysaccharide is rich in galactose and glucose, whereas the 8,000 molecular weight fraction is enriched in amino sugars and contains the neutral hexoses galactose, glucose, and mannose [50]. Polyacrylamide gel electrophoresis of the ascidian polysaccharides shows

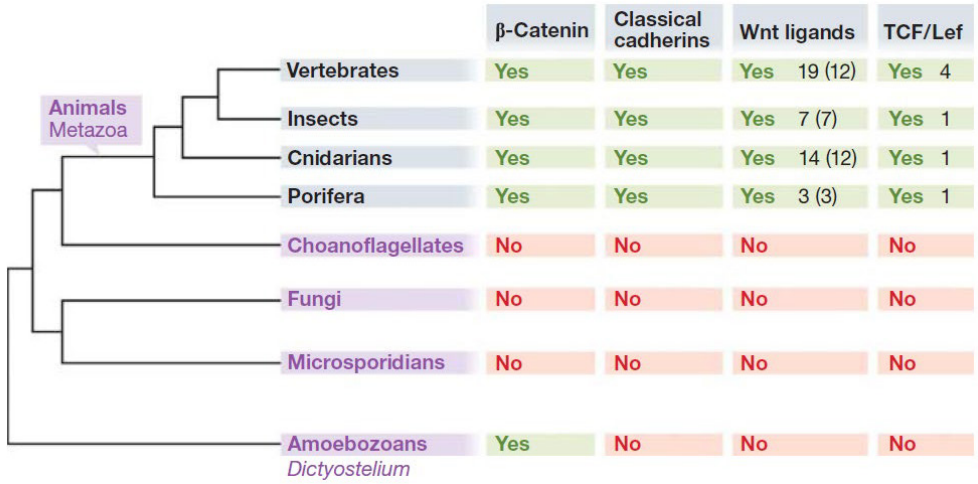

Figure 9 Schematic evolutionary tree showing the relationships between *Amoebozoa* and metazoa, as well as the diversity of signalling components. The presence of b-catenin and key components of adherens junctions (classical cadherins—containing an intracellular domain binding to b-catenin) and of canonical Wnt signalling (Wnt ligands, TCF/Lef transcription factors) is indicated to the right. In the case of Wnt ligands, the first number indicates how many different Wnt ligands were determined, the second number in brackets indicates how many Wnt subfamilies were determined in a particular group. The number in the case of TCF/Lef refers to how many different TCF/Lef proteins were found. Yes means presence, no absence. The following animal species were compared: Porifera (Sponges): Amphimedon queenslandica, Cnidaria: Nematostella vectans, Insects: Drosophila melanogaster; Vertebrates: Mus musculus [37].

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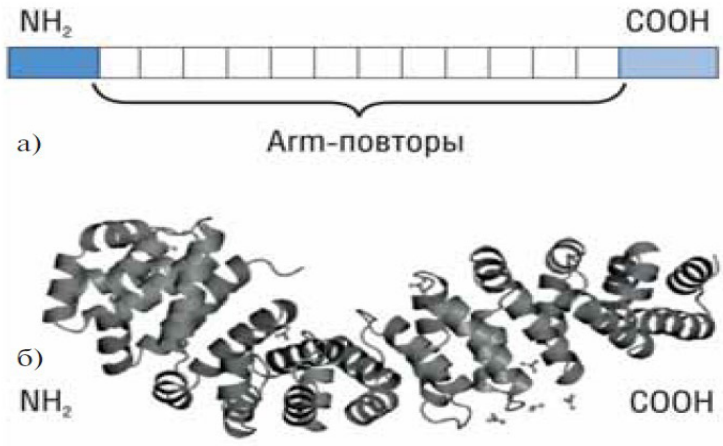

Figure 10 a) β-catenin protein primary structure of central domain consisting of 12 MER repeats; b) Ribbon structure of β-catenin [33].

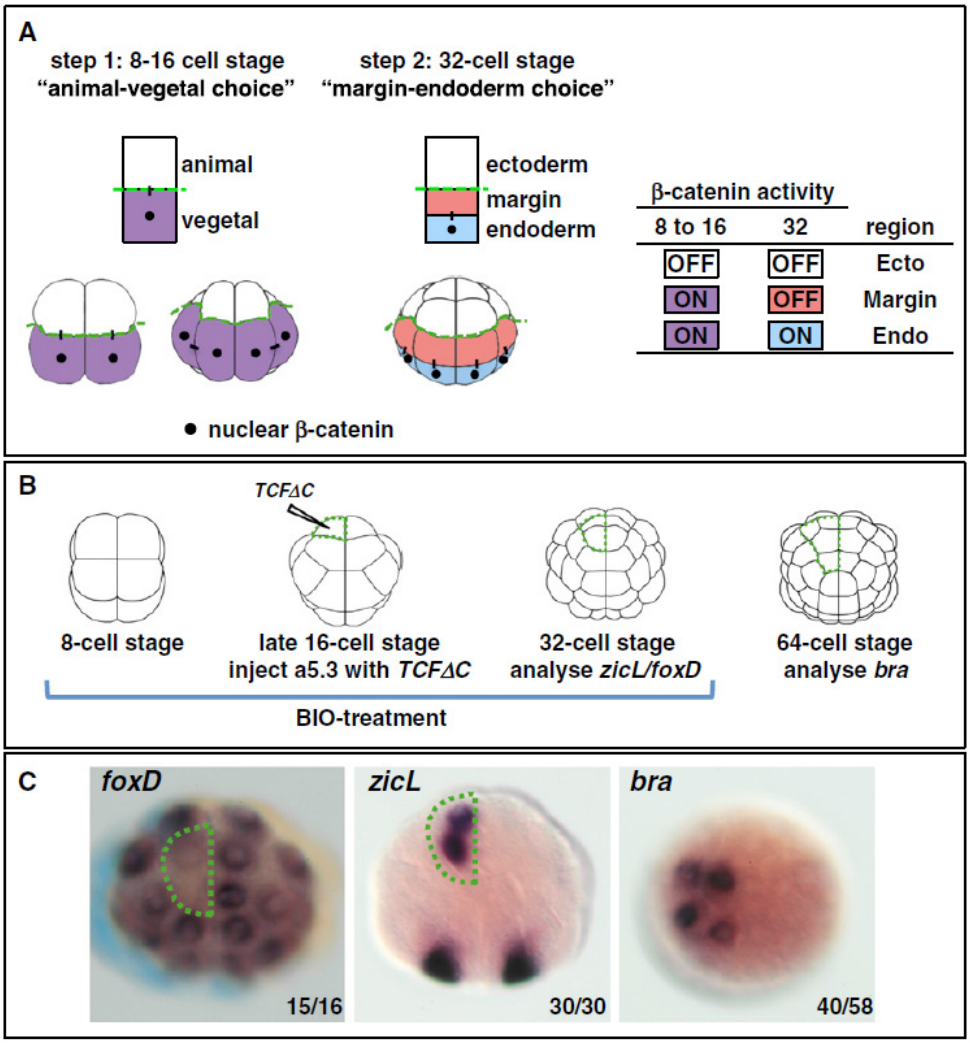

Figure 11 The Role of b-Catenin during Germ Layer Segregation in Ciona. A) Model. B) Experimental procedure generating a b-catenin ON-to-OFF sequence in a subset of animal cells. C) foxD, zicL, and bra expression after the procedure in (B). The number of embryos (shown in animal pole view) that the panel represents is indicated. foxD is activated in animal cells after BIO treatment and inhibited in the injected cells by TCFDC. zicL expression was detected in two (28/30) or one (2/30) and bra in four (15/58) or one to three (25/58) animal cells [37].

three fractions of different molecular weights: one fraction has a molecular weight of 100,000 or more, and thus stays at the origin, while the other two fractions migrate into the gel and have molecular weights of approximately 20,000 and 8,000. The chemical analysis of these ascidian polysaccharides indicates the presence of amino sugars, hexoses, and sulfate [50].

Seventy-two percentage of the total free AA [51] in fresh meat of *S. plicata* consisted of proline, alanine, glutamic acid, glycine and serine. Encompasing 82 % of the total free AA in the integument, while the amount of free AA in the integument was about 1\10 of that fresh meat. The function of these polysaccharides in the tunic of the ascidian is a matter of speculation. Since the tunic of ascidians is an external supportive and protective skeleton [50,52] and these polysaccharides occur in concentrations which resemble the great quantities of the glycosaminoglycans that are characteristic of cartilages [53-60], it may be that the ascidian polysaccharides are essential for maintaining the structural integrity of the tunic, resembling the structural function of the glycosaminoglycans in the connective tissues [50].

Conclusion

Bioingredients can be obtained from marine and terrestrial sources. This study bring the light into the Tunicates as source of nanocellulose called as T-complex, due to their unique properties and composition spectra from the cellulose to protein portion. The importance of ingredients with bioactive substances has been well recognized in connection with health promotion, disease and risk reduction in health care costs, food and packaging material applications. Special in the tunicate nanocellulose case, the protein portion felt in a category of special proteins related to anti-cancer activity in humans. Because of its unique functional properties, these compounds are widely used in food industries, pharmaceutical preparations, cosmetic and material industries. Thus the cellulose-protein moieties exploration remarks a starting step in this study.

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