Dogfish egg case structural studies by ATR FT-IR and FT-Raman spectroscopy

V.A. Iconomidou[1], M. Georgaka[1], G.D. Chryssikos[2], V. Gionis[2], P. Megalofonou[3] and S.J. Hamodrakas[1]

[1]Department of Cell Biology and Biophysics, Faculty of Biology, University of Athens, Panepistimiopolis, Athens 157 01, Greece e-mail: shamodr@biol.uoa.gr, veconom@biol.uoa.gr
[2]Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, Athens 116 35, Greece e-mail: gdchryss@eie.gr, vgionis@eie.gr
[3]Department of Zoology and Marine Biology, Faculty of Biology, University of Athens, Panepistimiopolis, Athens 157 01, Greece e-mail: p.megalo@biol.uoa.gr

Abstract

The dogfish eggcase is a composite structure that combines mechanical tensile strength, toughness and elasticity, with high permeability to small molecules and ions. Presumably, it provides both a protective and a filtering role for the egg/embryo contained within it. In this work, we performed structural studies of the G. melastomus eggcase at two different developmental stages, utilizing ATR FT-IR and FT-Raman spectroscopy. Combining data from both types of vibrational spectroscopy we deduce that:

(a) G. melastomus eggcase is a complex, composite structure which consists mainly of an analogue of collagen IV, in close analogy to that of the related species S. cunicula. This protein appears to have a uniform secondary structure in the egg case.

(b) An asymmetrical distribution of protein components, especially rich in tyrosine, was found in the eggcase, with the outermost layer being exceptionally rich in these proteins. Also, the innermost layer is rich in glucosaminoglycans.

(c) Didulphide bonds do not appear to play a significant role in cross-linking. However cross-links involving tyrosine residues appear to sclerotize the eggcase. It is proposed that the intensity of the Raman band at ca. 1615 cm^{-1}, which is due to ring stretching vibrations of Tyr, might be a useful indicator of the sclerotization status of a certain proteinacous tissue, when tyrosines are involved in sclerotization mechanisms.

Introduction

The selachian egg case appears as an ovoid, elongate, leathery capsule ca. 50mm by 20 mm. It is a remarkable composite structure that combines mechanical tensile strength, toughness and elasticity, with high permeability to small molecules and ions. Presumably, it provides both a protective and a filtering role for the egg/embryo contained within it[1]. The egg case of the spotted dogfish, *Scyliorhinus canicula*, is constructed largely from collagen-containing fibrils with a unique crystalline arrangement[2-3]. The collagen in these fibrils is secreted in a clearly defined zone (the D-zone), which forms the bulk of the nidamental gland[4], and it is an analogue of the mammalian collagen Type IV, on the basis of similarities at molecular level .[3]

The egg case of the spotted dogfish *Scyliorhinus canicula* appears to consist of four different layers (L_1-L_4).[2] The outermost layer (L_1), 25 μm thick, contains hydrophobic protein granules 2 μm in diameter, exceptionally rich in tyrosine content (ca. 20%), surrounded by radial arrangements of collagen fibrils.[5] The second layer (L_2), 225 μm thick, contains collagen, secreted mainly by the D-zone, as mentioned above, and comprises the main bulk of the egg case wall, in a unique plywood (helicoidal) arrangement.[1] The third layer, (L_3), 30 μm thick, contains also collagen but appears more homogeneous than the previous ones. Finally, the innermost layer, (L_4), extremely thin, 4-5 μm, contains mainly glycosaminoglycans and presumably collagen as well.[2] The presence in the spotted dogfish egg case of dopamine oxidase, DOPA, peroxidase and of the proteins exceptionally rich in tyrosine, in the hydrophobic granules, suggest some kind of extensive oxidative phenolic cross-linking but details of the sclerotisation mechanisms are largely unknown.[1-2]

The structure of the collagen assemblies in the egg case of the dogfish *Scyliorhinus canicula* have been studied previously using electron microscopy and 3-D reconstruction[3] and X-ray diffraction.[8] From these studies, the collagen forming the bulk of egg case appears to assemble in layers of various thickness, in which oriented quasi-crystalline fibers aggregate in approximately orthogonal directions systematically from one layer to an other.[1-2] It is organised into a regular 3-dimensional network with varying degrees of order.[6] The molecules of this sheet-forming collagen at the molecular level are approximately 45 nm long (ref. 1 and references therein) and are packed in a body-centred cubic lattice in the egg case. The molecules kink systematically within the crystal lattice, giving rise to a crankshaft arrangement.[6]

In this work we studied the wall of the egg case of another affined oviparous species, the blackmouth catshark, *Galeus melastomus* Rafinesque, 1810, which is classified under the Family Scyliorhinidae, like the species *Scyliorhinus canicula*, of the Class Elasmobranchii. ATR FT-IR spectroscopy and FT-Raman spectroscopy were used to study two specimens of the egg case of *Galeus melastomus*, at different developmental stages, which could be discerned by the different colouration of the specimens: white is

the colour of a newly formed egg case as is secreted by the nidamental gland,[1] whereas brown is the colour of the egg case in the oviduct. We tried to apply the two different but related vibration spectroscopies in order to detect similarities/differences with the corresponding egg case of *Scyliorhinus canicula*, by studying the secondary structure of its protein components and we were also interested to find out whether differences exist in the molecular structure of its protein components at different developmental stages and perhaps to detect details of the cross-linking mechanisms.

Materials and Methods

Materials

Two egg cases at different developmental stages have been investigated. They were obtained from a single female shark of the species *G. melastomus*. The egg cases were flattish rectangular pouches of some 6cm × 3cm. We define hereinafter as inner surface their surface in contact with the egg and the developing embryo. One had white colour, representing a newly formed egg case in the nidamental gland and the other had a brown colour, which apparently results from oxidative phenolic crosslinking[1], and it was obtained from the oviduct.

Attenuated Total Reflectance Infrared Spectroscopy (ATR FT-IR)

For ATR FT-IR measurements, small pieces (5mm × 5mm) of both white and brown egg case walls were laid wet on a front coated Au mirror. The IR beam was directed perpendicular to the surface (inner or outer) of the specimens.

Infrared spectra were obtained at a resolution of 4 cm^{-1}, utilizing an IR microscope (IRScope II by BRUKER OPTICS) equipped with a Ge Attenuated Total Reflectance objective lens (20x) and attached to a Fourier-transform spectrometer (Equinox 55 by BRUKER OPTICS). Ten 32-scan spectra were collected from each sample and averaged to improve the S/N ratio. The spectra are shown in the Absorption mode after correction for the wavelength-dependence of the penetration depth ($d_p \propto \lambda$).

Fourier Transform (FT)- Raman spectroscopy

Sample preparation for Raman measurements involved pressing pieces (2mm × 2mm) of white and brown egg case walls into the 2 mm cavity of a standard aluminum holder, which were measured one at a time.

The laser beam was directed perpendicular to the surface (inner or outer it does not matter since the laser beam passes through the rather thin, ca. 300 μm, walls) of the specimens). Raman spectra were obtained on a Fourier transform instrument (Bruker RFS 100) employing for excitation ca. 400 mW of the Nd: YAG 1064 nm line in a backscattering geometry. The resolution was 4 cm^{-1} and the total acquisition time was ca. 10000 scans. The spectra were finally averaged and the standard deviation (σ) was calculated.

Results

ATR FT-IR spectroscopy

The ATR FT-IR spectra of the white and brown egg case wall specimens taken from their outer and inner surfaces are shown in Figures 1a, 1b and 1c, 1d respectively.

The data from both types of specimens, white and brown, and from both surfaces, inner and outer, suggests that the structure of the component proteins of the egg case wall is very similar to a collagen IV type of structure.

The strong amide I band at ca. 1633 cm^{-1} and the amide II and III bands at 1547 and ca. 1235 cm^{-1} respectively, are most probably due to protein(s) with a collagen IV type of structure. This is indicated by the similarity of the spectra to those obtained from aqueous solutions of pure collagen IV and other samples containing collagen IV.[9-11] It is interesting to note that both specimens white and brown and also both surfaces outer and inner, exhibit similar spectra in the amide I, II and III regions.

The presence of Tyr in significant amounts in these and/or other component proteins of the egg case wall is also indicated by the presence of a very strong band at ca. 1515 cm^{-1} in all the ATR FT-IR spectra.

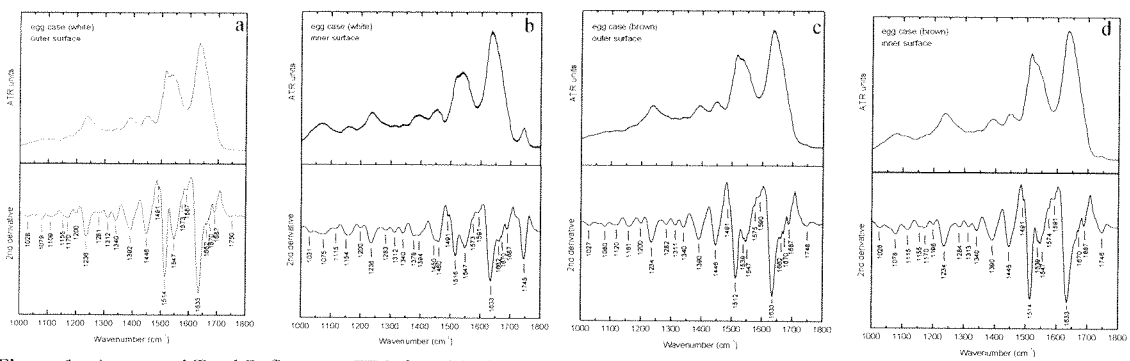

Figure 1. Attenuated Total Reflectance FT-Infrared (ATR FT-IR) spectra of: **(a)** *Galeus melastomus* white egg case, outer surface **(b)** *Galeus melastomus* white egg case, inner surface, **(c)** *Galeus melastomus* brown egg case, outer surface **(d)** *Galeus melastomus* brown egg case, inner surface.. The 2nd derivative spectra are included. Errorbar equals 1σ.

This band is probably due to ring stretching vibrations of Tyr.[12] Although it is difficult to judge from the intensity of this band relative amounts of Tyr in the proteins of both surfaces, outer and inner and both

specimens white and brown, since this band partially overlaps with the amide II band, it is worth noting that in the brown, presumably cross-linked specimen, both surfaces inner and outer show a pronounced band of Tyr at ca. this wavenumber, whereas in the white, non-cross-linked specimen, this is not true: actually, this band is more pronounced in the spectra from the outer surface of the white specimens, which agrees to the finding that the hydrophobic granules, containing rich in Tyr proteins are mostly found in the outermost layer L_1 of the egg case wall (see Introduction and refs. therein).
Of special interest is the 1000-1100 cm^{-1}, so-called polysaccharide region, where C-O, and C-C stretching modes of polysaccharides are usually observed.[9-10] The spectra of collagen IV show two bands at ca. 1030 and 1078 cm^{-1}, which are assigned to proteoglycans associated with collagen IV.[9-10] A close study of Figures 1a, 1b and 1c, 1d shows that in this region bands of appreciable intensity appear in the inner sides of both white and brown specimens at ca. 1030 and 1078 cm^{-1} and can be assigned to the glucosaminoglycans known to be abundant in the inner zone L_4 of the egg case wall.[2]

FT-Raman specroscopy

The Raman spectra obtained from the white and brown specimens of the egg case wall specimens are shown in Figures 2a and 2b, respectively.

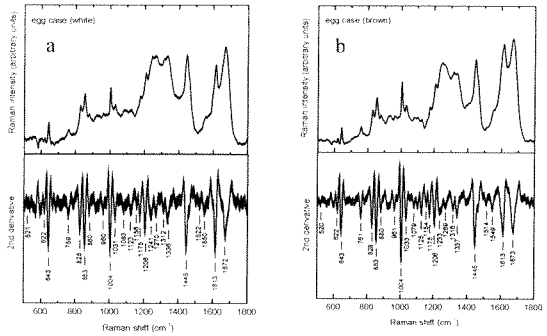

Figure 2. FT-Raman (500-1800 cm^{-1}) spectra of **(a)** *Galeus melastomus* white egg case **(b)** *Galeus melastomus* brown egg case. 2nd derivative spectra are included. Errorbar equals 1σ.

In the amide I region we find sharp Raman bands centered at 1672 and 1673 cm^{-1} for the white and brown specimens, respectively, and in the amide III region a doublet at 1241 and 1270 cm^{-1} for the white and at 1233 and 1269 cm^{-1} for the brown egg case wall specimen, respectively. This split of the amide II band into two components, which are assigned to triple helical (3_1 helix) parts of the collagen I type of molecules is probably related to the biphasic nature of the triple helix of collagen and the two bands are attributed to the proline-poor (polar) and proline-rich (non polar) regions distributed along the triple helix.[13,14]
The shifts of the amide I position from 1670 cm^{-1} as seen in collagen I (with an apparent shoulder at 1642 cm^{-1})[13] to 1672 and 1673 cm^{-1} in our two types of specimens, white and brown, might be related to structural differences which exist between collagen I and collagen IV. It is well known that collagen IV, (a) is not a straight triple-helical type of molecule, but it rather has kinks along the triple helix, most probably due to disruptions of the regular Gly-X-Y repeats along its sequence, and (b) it contains C-terminal globular non-collagenous domains (NC1 domains), whose structure has been solved near atomic resolution (1.9 Å), where β-sheet structure prevails.[15] Unfortunately, there have been no reports of Raman spectroscopic studies of collagen IV in the literature, for direct comparison.
The FT-Raman spectra of both white and brown egg case wall specimens exhibit several bands which are clearly due to side chain vibrations of aminoacid residues, especially to the aromatic ring-containing Phe, Tyr and Trp (Figures 3a, 3b). More specifically, the bands at 622, 1004 and 1031 cm^{-1} are ascribable to Phe, whereas the bands at 643, 828, 853, 1175, 1206 and 1613 cm^{-1} are most likely due to Tyr.[16,17] The intensity ratio of the tyrosine doublet at 853 and 828 cm^{-1}, $R = I_{853}/I_{828}$, is sensitive to the nature of hydrogen bonding or to the state of the ionization of the phenolic hydroxyl group, and has been used to identify "buried" and "exposed" Tyr moieties.[18] In our case it was found that R~1.3 indicating that the phenolic hydroxyl group may act as donor and acceptor of moderate hydrogen bonds (ref. 18 and refs. therein). The 643 and 1206 cm^{-1} bands may also involve contributions from vibrations of Phe. Also, the band at 1613 cm^{-1} may hide components due to Phe and Trp. A band at 761 cm^{-1} is ascribable to Trp.[16,17] The absence of a band at 1361 cm^{-1} suggests presumably that the Trp side chains are not "exposed"[17].
Bands in the 500-550 cm^{-1} region are typically associated with the S-S stretching mode of the C-C-S-S-C-C structural unit of disulphide bonds, usually at 510, 525 and 540 cm^{-1}.[16,17] Some minor features are seen in this region in the Raman spectra of both specimens, white and brown, but they are within the limit of experimental error and do not appear exactly at these wavenumbers. We shall not attempt to assign them with certainty to specific S-S stretching modes, despite the fact that there are some claims that disulphide bonds might play a minor role in cross-linking the proteins of the egg case (ref. 1 and references therein).
Of particular interest is the observation that in the brown egg case specimen there is a significant increase in intensity of the 1613 cm^{-1} band due to Tyr, compared to the intensity of the corresponding band in the newly formed white egg case specimen. This band is probably due to ring stretching vibrations of Tyr[12]

and it may be related to the formation of di-tyrosine and tri-tyrosine bonds which may be formed by oxidative phenolic tanning reactions, cross-linking the egg case proteins, involving the action of peroxidase(s) or more complex tanning mechanisms (ref. 1 and references therein).

Discussion

The egg case of dogfish and related species is a complex composite with extraordinary mechanical and functional properties (see Introduction) and it is largely made of an analogue of the mammalian collagen Type IV, 45 nm long, compared to the 400 nm long mammalian collagen type IV (ref. 1 and references therein; ref. 7 and references therein). The egg case collagen terminates, at both ends, with two globular domains about 4 and 2 nm in diameter, which are hydrophobic in character (ref. 1 and references therein; ref. 7 and references therein) and it appears capable of kinking, presumably due to an interruption of the collagen triple helix (ref. 7 and references therein). In this study, we have shown rather conclusively (see Results), utilizing the ATR FT-IR and FT-Raman spectroscopies that the *Galeus melastomus* egg case collagen is also an analogue of mammalian collagen IV. To our knowledge, it is the first time that an FT-Raman spectrum has been reported for an analogue of collagen IV. Therefore this might be used as a reference for studies of the medically important mammalian collagen IV of the basal lamina.[10,11] However, intriguing remains the profound similarity of the relevant spectra (Figures 2a, 2b), with spectra obtained from proteins, both fibrous and globular, with pure β-sheet structure.[19] This similarity most probably arises from contributions to the spectra from the globular non-collagenous domains, which it has been documented to contain abundant β-sheet structure at least for mammalian collagen IV.[15]

The observations from this ATR FT-IR study of *G. melastomus* egg case fully confirm the observations from EM studies and biochemical data on the related species *S. canicula* eggcase, about the asymmetrical distribution of components of the egg case wall: It clearly shows the preponderance of proteins rich in Tyr in the outermost surface layers of the egg case and the presence of glycosaminoglycans in the innermost layers (see Results section and ref. 1). Furthermore, with the help of the observations of the FT-Raman study, it suggests that the main protein component of the egg case is a protein with a secondary structure similar to that of collagen IV, which apparently has the same structural features from the outermost layers to the innermost layers.

Concerning the findings about cross-linking mechanisms, (a) no indications were found from the FT-Raman data, at least with certainty, that disulphide bridges are playing a role in cross-linking the eggcase, and (b) the data suggest that tyrosines are cross-linked, either by the action of peroxidase(s) for the formation of di-tyrosine and tri-tyrosine cross-links or by more complex mechanisms involving the action of other enzymes.[1,20] Therefore, we suggest that FT-Raman spectroscopy can be used as an easy means of identifying sclerotization mechanisms, involving tyrosine residues, by observing the Raman band at ca. 1615 cm^{-1} and its variations, at different stages of developing tissues, since similar observations were also found to be true for insect cuticle sclerotization (Iconomidou, Chryssikos, Gionis, Willis and Hamodrakas, unpublished data). Future, more refined, biochemical and structural, work may reveal in detail these mechanisms.

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