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Microstructure, mechanical, and biomimetic properties of fish scales from *Pagrus major*

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Abstract

The fish scale of *Pagrus major* has an orthogonal plywood structure of stratified lamellae, 1–2 μm in thickness, consisting of closely packed 70- to 80-nm-diameter collagen fibers. X-ray diffraction, energy-dispersive X-ray analysis, and infrared spectroscopy indicate that the mineral phase in the scale is calcium-deficient hydroxyapatite containing a small amount of sodium and magnesium ions, as well as carbonate anions in phosphate sites of the apatite lattice. The tensile strength of the scale is high (~90 MPa) because of the hierarchically ordered structure of mineralized collagen fibers. Mechanical failure occurs by sliding of the lamellae and associated pulling out and fracture of the collagen fibers. In contrast, demineralized scales have significantly lower tensile strength (36 MPa), indicating that interactions between the apatite crystals and collagen fibers are of fundamental importance in determining the mechanical properties. Thermal treatment of fish scales to remove the organic components produces remarkable inorganic replicas of the native orthogonal plywood structure of the fibrillary plate. The biomimetic replica produced by heating to 873 K consists of stratified porous lamellae of *c*-axis-aligned apatite crystals that are long, narrow plates, 0.5–0.6 μm in length and 0.1–0.2 μm in width. The textured inorganic material remains intact when heated to 1473 K, although the size of the constituent crystals increases as a result of thermal sintering.

Keywords: Fish scale; Microstructure; Mechanical strength; Biomimetic replica

1. Introduction

The elasmoid scales of teleost fish are composed of calcium-deficient hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and extracellular matrix, mainly type I collagen fibers, which together form a highly ordered three-dimensional structure. Each scale consists of two distinct regions: an external (osseous) layer and an internal fibrillary plate (Onozato and Watabe, 1979; Zylberberg and Nicolas, 1982). In the upper external layer, collagen fibers are randomly arranged and embedded in a proteoglycan matrix. Within the fibrillary lower layer, in contrast, the collagen fibers are co-aligned and organized into lamellae that are superimposed to produce an orthogonal and/or a double-twisted plywood pattern (Nicolas et al., 1997; Oslon and Watabe, 1980; Zylberberg and Bereiter-Hahn, 1991). The collagen fibers are produced within the fibrillary layer by scleroblasts located at the base of the scales (Onozato and Watabe, 1979; Zylberberg et al., 1992). The fibers are organized through the cooperative involvement of microtubules and actin microfilaments that are subjected to consecutive alterations during the formation of plies of the basal plate (Byers et al., 1980; Zylberberg et al., 1988, 1992; Zylberberg and Bereiter-Hahn, 1991). In general, the spatial organization of collagen fibers is of key importance for the mechanical properties of different connective tissues (Weiner et al., 1999).

Mineralization of fish scales occurs continuously throughout the life of the organism. The external layer is initially mineralized with matrix vesicles, and then the

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internal layer is developed (Onozato and Watabe, 1979; Zylberberg and Nicolas, 1982). Needlelike or flaky crystals of apatite in random orientation are observed in the outer layer (Onozato and Watabe, 1979; Oslon and Watabe, 1980). Calcification of the internal layer, in contrast, occurs in the absence of matrix vesicles (Schönbörner et al., 1979), and the orientation of the crystallographic *c*-axis of apatite crystals parallel to collagen fibers in bony fish (*Leuciscus cephalus*) has been recently determined using small-angle X-ray diffractometry (XRD) (Bigi et al., 2001).

In the present study, the microstructure of fish scales extracted from sea bream, *Pagrus major*, is reported using transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDX), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. To the best of our knowledge, the mechanical properties of fish scales have not been studied, and we therefore present some preliminary investigations on the tensile strength and mechanism of failure under strain of these biomineralized tissues. We also show that thermal removal of the organic components at high temperature produces inorganic replicas with biomimetic structures resembling the plywood texture of the native scales. Because such materials are not available by conventional synthesis methods, the bioderived architectures should be of general interest in biomaterials research.

2. Materials and methods

The fish scales were extracted from hatchery sea bream, *P. major* (0.5–1.0 kg). The fresh scales were fixed by immersion in 3% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h followed by postfixation using 1% OsO_4 solution in the same buffer for 1 h at room temperature. The fixed samples were dehydrated in a graded series of ethanol concentrations (50, 70, 90, and 100%), and then embedded in spurr resin (Spurr, 1969) and polymerized at 70 °C for 12 h. Ultrathin sections (80–100 nm in thickness) were cut perpendicular to the scale surface using a diamond knife, and examined in a JEM-1230 TEM.

Samples of fresh fish scales and scales heated at 873 and 1473 K were studied as follows. Inorganic and organic contents were determined by thermogravimetric analysis (Thermo plus TG 8120, Rigaku) of 10-mg samples using a heating ratio of 20 K/min in static air. Fresh scales were cut to a size < 1 mm with a razor and studied by XRD (PW1700, Philips) and diffuse-reflectance FTIR spectroscopy (FTIR8000, Perkin-Elmer) using the KBr method to identify the calcium phosphate mineral phase. Morphological analysis was undertaken using a JEOL-5600LV SEM at an accelerating voltage of 20 kV. Elemental analysis was undertaken by EDX using a JEOL-JED2200. The samples were coated with tungsten for EDX analyses or platinum for morphological observations with Elionics-E101.

Demineralized scales were obtained by immersion of fresh scales in 0.5 M EDTA (1:25 of weight ratio) for 2 days after removal of surplus proteins by washing with 10 wt% NaCl solution for 1 day. All these procedures were done at 277 K to inhibit collagen fragmentation. The resultant scales were washed thoroughly with distilled water three times. Complete demineralization was confirmed by a weight loss of 100% using TG analysis up to 1473 K.

The tensile strengths of mineralized and demineralized scales (3×20 mm) were determined using a texture analyzer (TA-XT2i; EKO) with a gauge length of 10 mm. The tensile test was conducted for 10 wet samples under a crosshead speed of 0.1 mm s^{-1} at room temperature. The Young's modulus was determined from the stress–strain curves at a strain of 0.001 by extrapolation of the initial linear part of the curves. Fracture surfaces of the broken mineralized fish scales were studied by SEM.

3. Results

3.1. Microstructure and characterization of fish scales

SEM images of whole mounts of *P. major* fish scales showed characteristic growth rings (Fig. 1) with an upper osseous layer and lower fibrillary plate that were clearly demarcated in TEM images of thin cross sections of stained samples (Fig. 2). The osseous layer was 3–4 μm in width and consisted of randomly packed collagen fibers (Fig. 3). In contrast, the fibrillary plate was composed of stratified lamellae, 1–2 μm in width, which contained tightly packed co-aligned collagen

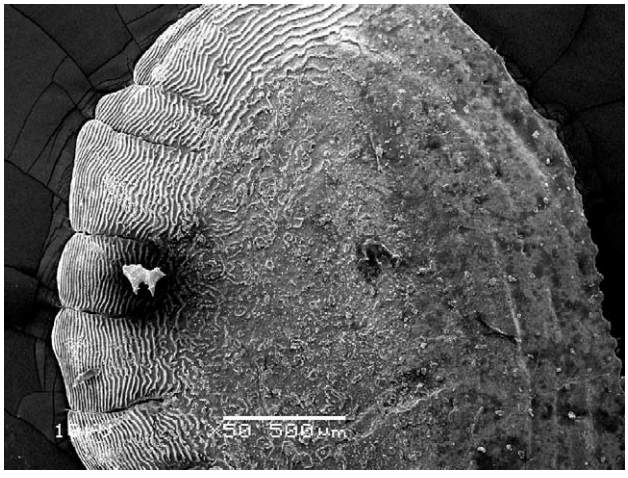

Fig. 1. SEM micrograph of whole mount of a *P. major* fish scale showing growth rings.

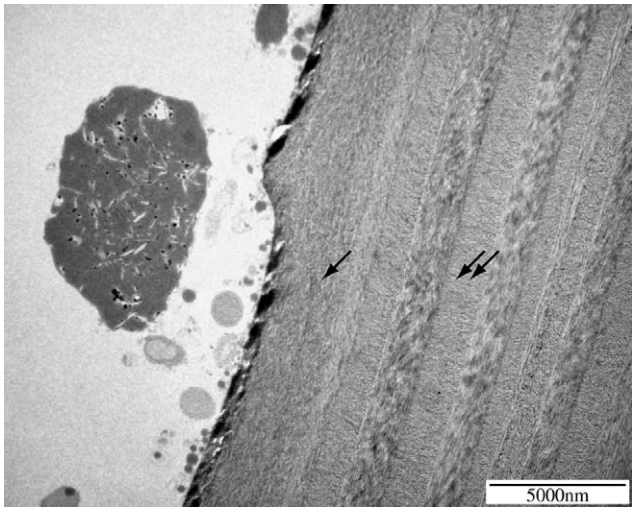

Fig. 2. TEM image of a transverse section of a *P. major* fish scale stained with osmium tetroxide. The single and double arrows show the external osseous layer and internal fibrillary plate, respectively.

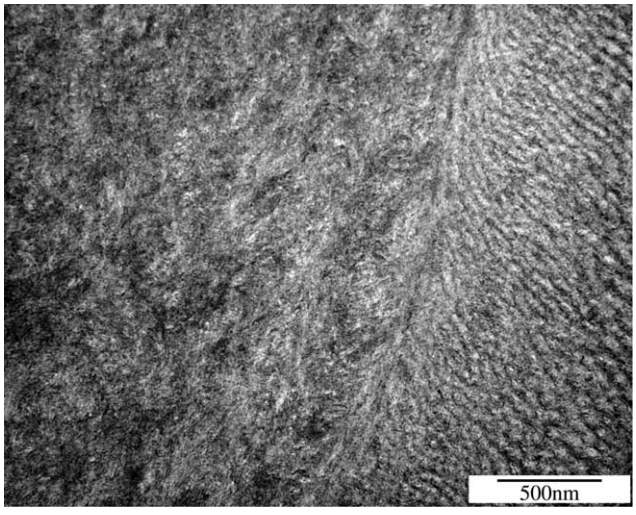

Fig. 3. TEM image showing stained collagen fibers in the external osseous layer of a *P. major* fish scale. The left side of the image is nearest the surface of the scale and shows randomly organized collagen fibers. The interface with the ordered internal fibrillary layer is shown toward the right side of the image.

fibers with a diameter of 70–80 nm (Fig. 4). The fiber alignment alternated by ~90° between adjacent lamellae to produce a plywood-like structure. Other types of sheetlike structure were not observed (Onozato and Watabe, 1979). The characteristic stripe pattern of type I collagen with 65-nm periodicity was observed in stained samples viewed at high magnification. However, the orientation of apatite crystals within the matrix could not be determined by TEM, possibly because of dissolution of the crystals during sample preparation. Overall, the ultrastructure of the scales of *P. major* was very similar to that reported for *Poecilia reticulata* (Zylberberg and Bereiter-Hahn, 1991).

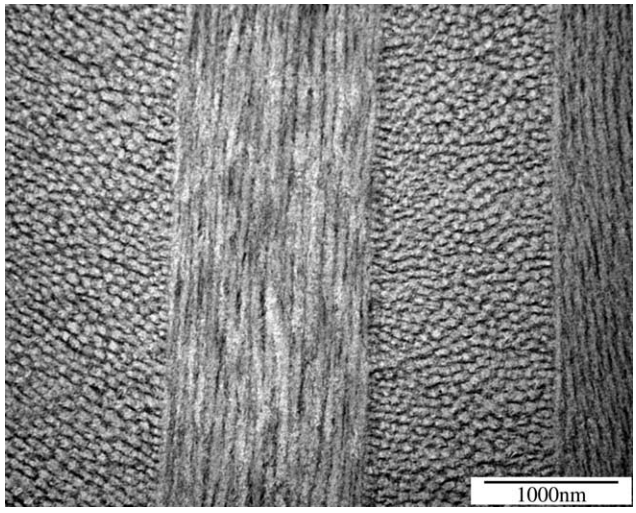

Fig. 4. TEM image of the internal fibrillary plate showing stained 70- to 80-nm-thick oriented collagen fibers within individual lamellae of a *P. major* fish scale. The collagen fibers are co-aligned within each sheet, which rotate alternately through an angle of ~90°.

XRD patterns showed broad reflections, with *d* spacings of 0.343, 0.280, 0.225, 0.194, and 0.172 nm, corresponding to the apatite structure (Fig. 5). No other calcium phosphates or calcium carbonates were observed. The broad diffraction peaks indicated that the crystals were small or structurally disordered, or both. Quantitative EDX analysis gave an inorganic composition of P_2O_5, Na_2O, MgO, and CaO with 41 ± 2, 3 ± 2, 2 ± 1, and 54 ± 2 wt%, respectively, and a Ca:P ratio of 1.67. TG-DTA measurements indicated that weight ratios of water, organic, and inorganic

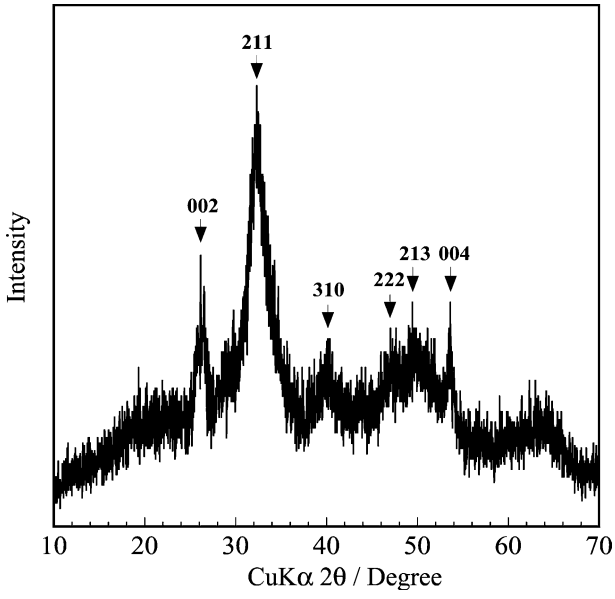

Fig. 5. X-ray diffraction pattern of native fish scales. Broad reflections corresponding to the (0 0 2), (2 1 1), (3 1 0), (2 2 2), (2 1 3), and (0 0 4) of hydroxyapatite are shown.

(apatite) components were 13, 41, and 46%, respectively. Absorbed water was degassed exothermically at 473 K, and thermal decomposition of the collagen fibers and other biopolymers (polysaccharides) occurred exothermically at 648 K and was complete below 873 K. FTIR spectra showed strong absorption bands at ~ 600 and $1000\,cm^{-1}$, corresponding to phosphate groups in the apatite lattice, and peaks at 873, 1420, and $1447\,cm^{-1}$, corresponding to carbonate anions substituted for phosphate ions in the apatite lattice (Fig. 6). Similar data have been reported for human bone and tooth biomaterials (Elliott, 1994). In addition, three FTIR bands attributed to amides I, II, and III of type I collagen were observed at 1657, 1520, and $1227\,cm^{-1}$, respectively. The results indicate that the fish scale is a nanocomposite consisting of type I collagen and calcium-deficient apatite containing carbonate ions.

Mechanical measurements of the tensile strength of *P. major* fish scales gave an average value of 93 ± 1.8 MPa ($n = 10$). The tensile stress–strain curve (Fig. 7) was initially linear with a corresponding Young's modulus (stress/strain) of 2.2 ± 0.3 GPa. This value corresponds to a relatively low stiffness due to the small mineral content of the fish scales (46%) compared to that of red deer (~50%, 6.1 GPa) and axis deer (~80%, 31.6 GPa); the relationship between mineral contents and stiffness (Young's modulus) has been described by Currey and Brear (1990) and Mann (2001). At high stress values, the tensile stress–strain curve showed considerable plastic yielding before fracture. Corresponding SEM images of the fracture surface indicated that sliding of the collagen lamellae and pulling out of individual collagen fibers, 2–3 μm in thickness, were responsible for the plasticity close to the yield point (Fig. 8). Demineralization of the fish scales considerably reduced the average tensile strength and Young's modulus to values of 36 ± 8.4 MPa and 0.53 ± 0.06 GPa ($n = 10$), respectively, although the fracture behavior was essentially the same as for the mineralized tissue.

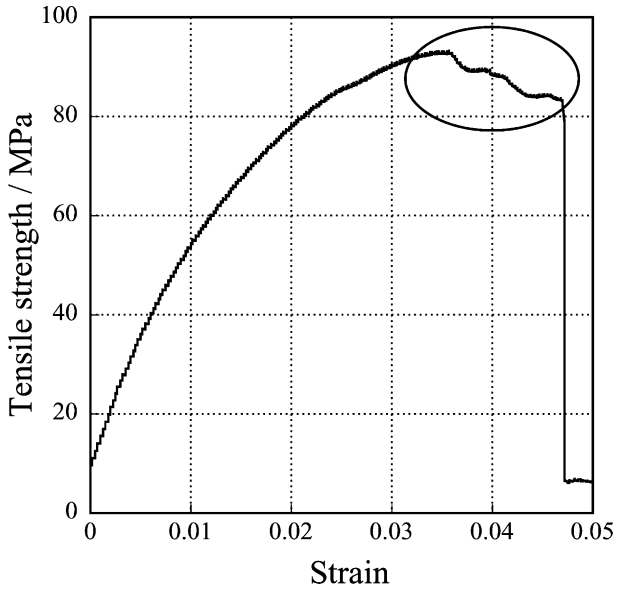

Fig. 7. Tensile stress–strain curve for fish scales ($n = 10$). Outlined area shown at high strain values corresponds to the plastic yield associated with sliding and pulling out of collagen fibers within the lamella structure.

3.2. Thermal processing and biomimetic replicas

XRD and FTIR spectroscopy indicated that the inorganic products prepared by heating fish scales at 873 or 1473 K were well-defined hydroxyapatite or a mixture

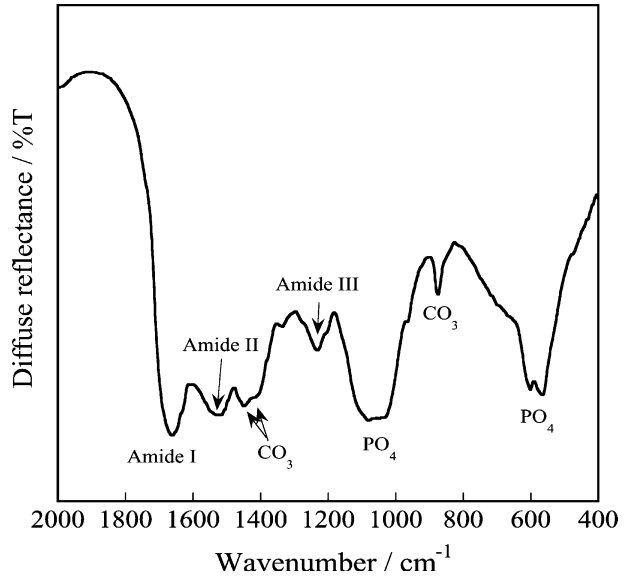

Fig. 6. Infrared spectrum of native fish scales showing amide I, II, and III bands from collagen fibers, and absorbances for phosphate and carbonate ions in the apatite lattice.

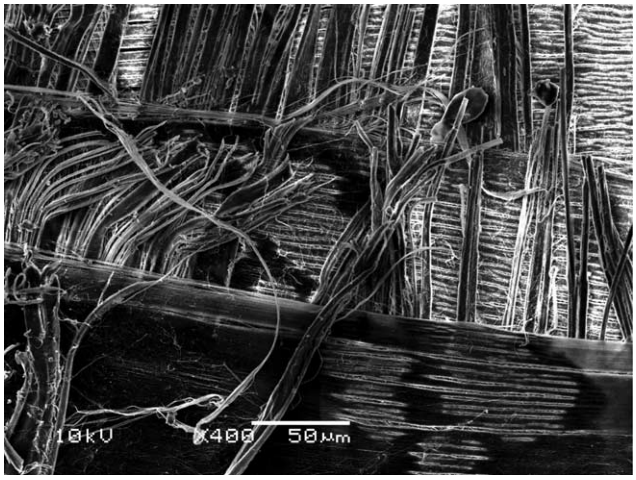

Fig. 8. SEM image of a fractured section of a fish scale showing sliding of the collagen lamella structure and pulling out and breakage of individual collagen fibers.

of hydroxyapatite and tricalcium phosphate (TCP; $Ca_3(PO_4)_2$), respectively (data not shown). The phase transformation to TCP at high temperature is a characteristic property of calcium-deficient apatite. SEM images of the surface of the osseous layer after heating at 873 or 1473 K showed sintering and aggregation of the calcium phosphate crystallites to produce porous replicas with an interconnected granular texture (Fig. 9). Typical domain sizes were 0.1–0.2 μm and 1–2 μm, respectively. Small crystals of TCP with sizes of 0.1–0.2 μm were also observed at the surface of samples prepared at 1473 K. Corresponding SEM images of the fibrillary plate after thermal processing at 873 or 1473 K showed inorganic replicas with remarkable preservation of the native plywood structure (Fig. 10). In both cases, intact sheets consisting of 2- to 4-μm-wide parallel strips of calcium phosphate were stacked up such that the alignment alternated by ~90° between adjacent lamellae. The strips of calcium phosphat e had smooth flat surfaces and side edges, and were separated by a spacing of 1–2 μm. Each sheet was structurally stabilized as an open structure by thin branchlike connections between the adjacent strips. No such connections were observed between adjacent sheets, which sometimes readily peeled away at the surface of the heated fibrillary plate. High-magnification SEM images of samples heated at 873 K indicated that the parallel strips of calcium phosphate consisted of long, narrow platelike apatite crystals, ~0.5–0.6 by 0.1–0.2 μm in dimension, that were co-aligned parallel to the strip axis. Increasing the temperature to 1473 K increased the particle size by sintering of the apatite crystals but had minimal effect on the spatial separation or organization of the calcium phosphate striated structure. The results suggest that the preferred crystallographic orientation of the apatite particles along the collagen fibers is highly replicated within the calcined product.

4. Discussion

The results presented in this paper indicate close similarities between the microstructure and composition of fish scales of the sea bream, *P. major*, and other species studied previously. The mineral phase is a calcium-deficient carbonated hydroxyapatite, and in contrast with investigations on the fish scales of *Carassius auratus* (Onozato and Watabe, 1979; Zylberberg and Nicolas, 1982), no carbonate minerals were detected. Although TEM studies of the sectioned scales were inconclusive in determining the location of the mineral phase within the collagen matrix, the heated samples indicated that the crystals were long, narrow, and platelike in morphology and suggested that they were aligned parallel to the collagen fibers within individual lamella. This is consistent with previous studies on other species, which reported apatite crystals in both the external osseous and internal and fibrillary layers (Onozato and Watabe, 1979; Schönbörner et al., 1979; Zylberberg and Nicolas, 1982).

It is generally accepted that mineralization of the thin osseous layer of teleost scales occurs before calcification of the fibrillary plate. Several studies have indicated that there are distinct microstructural differences between these layers (Fouda, 1979; Schönbörner et al., 1979; Yamada and Watabe, 1979; Zylberberg and Nicolas, 1982), which involve the organization of collagen fibers of the organic matrix. The external layer consists of randomly oriented collagen fibers, whereas the fibrillary plate is constructed from lamellae composed of highly ordered collagen fibers. The TEM results reported here are consistent with this structural model of the fish scale, and indicate that the orthogonal plywood lamella structure of the *P. major* scale is very similar to that of *P. reticulata* (Zylberberg and Bereiter-Hahn, 1991) but different from the double-twisted plywood structure

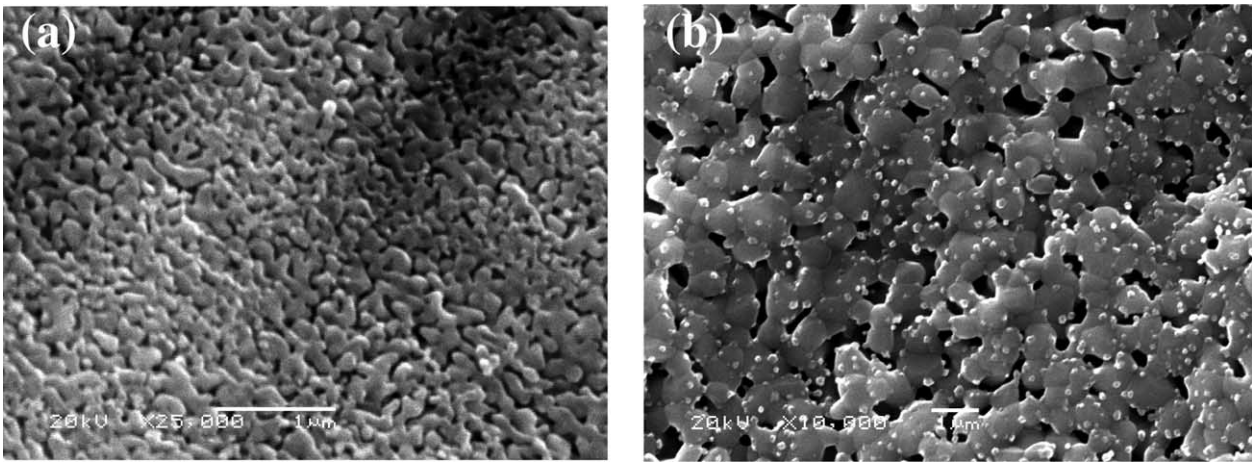

Fig. 9. SEM images of the surface morphology of the osseous layer of a fish scale heated at (a) 873 K and (b) 1473 K showing porous granular texture of calcium phosphate crystals.

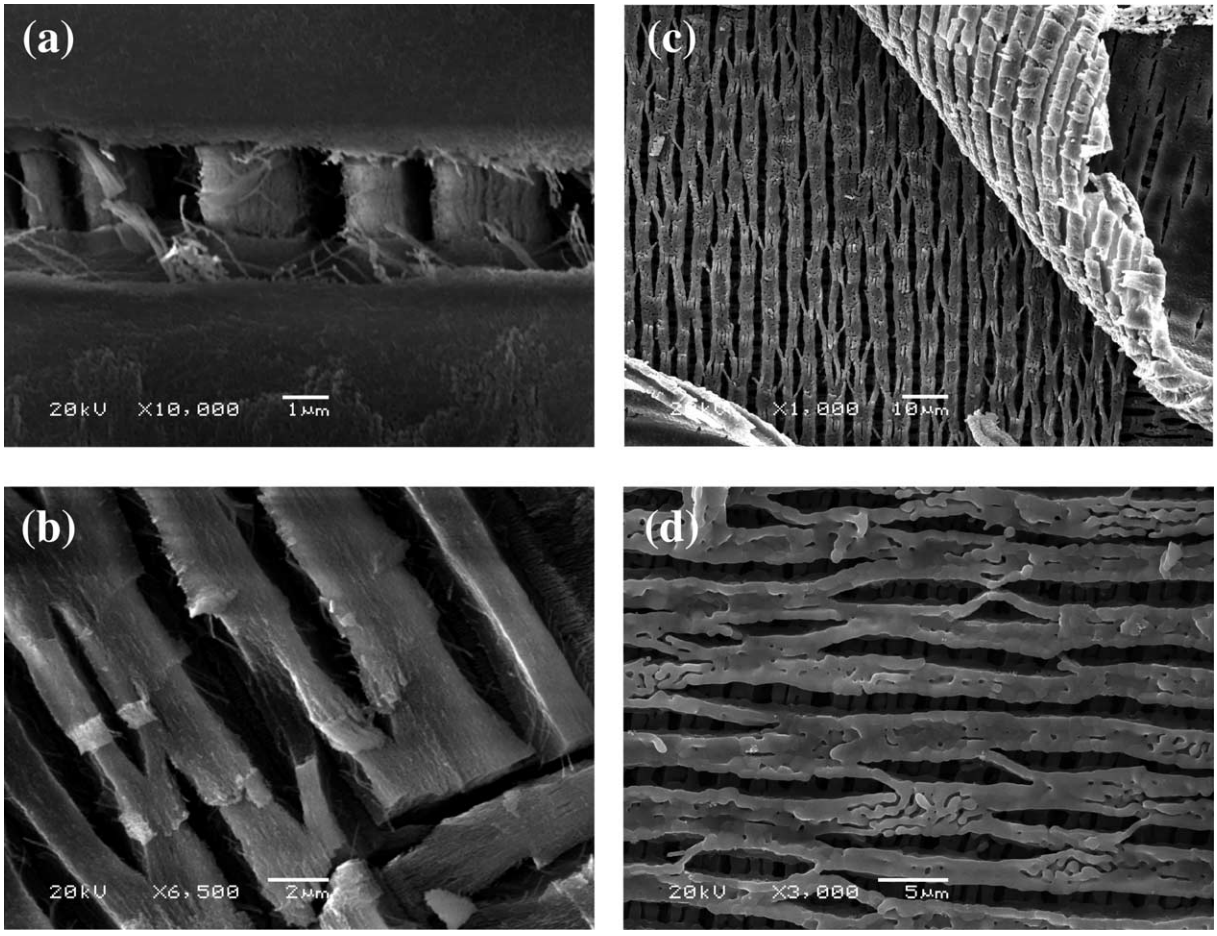

Fig. 10. SEM images of the fibrillary layer of fish scales after heating at (a, b) 873 K and (c, d) 1473 K. (a, b) Long, narrow platelike apatite crystals showing preferential orientation parallel to the native lamella collagen structure. (c) Patterned replica showing plywood stacking of porous calcium phosphate sheets with striated texture. (d) Higher magnification image of individual calcium phosphate strips and interconnecting bridges.

described for *C. auratus* (Onozato and Watabe, 1979; Zylberberg and Nicolas, 1982).

The mechanical properties of fish scales have not been described before. Meunier (1984) suggested that that collagen layers in the fish scale are stiffened by mineralization, and this is consistent with the substantial lowering in tensile strength that we observe after demineralization of the tissue. The high tensile strength of the native scale is therefore attributed to the highly ordered collagen fibers in association with long, narrow platelike apatite crystals that are aligned along the crystallographic *c*-axis parallel to the collagen fibers. Alignment of apatite in ordered collagen biomatrices is well established (Bigi et al., 1996; Birk and Trelstad, 1986; Ørvig, 1968) and not only serves as an inorganic binder to strengthen the tissue but also provides functional advantages based on mechanical anisotropy. Moreover, we observe a substantial amount of plastic yielding in the fish scales of *P. major*, suggesting that the mineral deposits invade the interfibrillary spaces but do not penetrate substantially the intrafibrillary matrix, particularly those regions containing thick collagen fibers (Moss, 1961; Schönbörner et al., 1979).

Finally, we note that calcium phosphate replicas of the fibrillary plate can be readily prepared by heat treatment of the native scales. Although such structures are not of direct biological relevance, they could be of considerable interest in the field of biomimetic materials research. Further work will investigate the potential of fish scales as a novel route to porous inorganic materials with complex architectures for uses in separations technology, catalysis, and biomedical applications.

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