Leatherback Turtle (*Dermochelys coriacea*) after Incubation Eggshell in Andaman Sea, Thailand Study: Microanalysis on Ultrastructure and Elemental Composition

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Abstract**—**There are few studies on eggshell of leatherback turtle which is endangered species in Thailand. This study was focusing on the ultrastructure and elemental composition of leatherback turtle eggshells collected from Andaman Sea Shore, Thailand during the nesting season using scanning electron microscope (SEM). Three eggshell layers of leatherback turtle; the outer cuticle layer or calcareous layer, the middle layer or middle multistrata layer and the inner fibrous layer were recognized. The outer calcareous layer was thick and porosity which consisted of loose nodular units of various crystal shapes and sizes. The loose attachment between these units resulted in numerous spaces and openings. The middle layer was compact thick with several multistrata and contained numerous openings connecting to both outer cuticle layer and inner fibrous layer. The inner fibrous layer was compact and thin, and composed of numerous reticular fibers. Energy dispersive X-ray microanalysis detector revealed energy spectrum of X-rays character emitted from all elements on each layer. The percentages of all elements were found in the following order: carbon (C) > oxygen (O) > calcium (Ca) > sulfur (S) > potassium (K) > aluminum (Al) > iodine (I) > silicon (Si) > chlorine (Cl) > sodium (Na) > fluorine (F) > phosphorus (P) > magnesium (Mg). Each layer consisted of high percentage of $CaCO_3$ (approximately 98%) implying that it was essential for turtle embryonic development. A significant difference was found in the percentages of Ca and Mo in the 3layers. Moreover, transition metal, metal and toxic non-metal contaminations were found in leatherback turtle eggshell samples. These were palladium (Pd), molybdenum (Mo), copper (Cu), aluminum (Al), lead (Pb), and bromine (Br). The contamination elements were seen in the outer layers except for Mo. All elements were readily observed and mapped using Smiling program. X-ray images which mapped the location of all elements were showed. Calcium containing in the eggshell appeared in high contents and was widely distributing in clusters of the outer cuticle layer to form $CaCO_3$ structure. Moreover, the accumulation of Na and Cl was observed to form NaCl which was widely distributing in 3 eggshell layers. The results from this study would be valuable on assessing the emergent success in this endangered species.

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Keywords**—**Leatherback turtle (*Dermochelys coriacea*), SEM (SEI/EDX), turtle eggshell.

I. Introduction

THEConvention of International Trade in Endangered Species (CITES) was then released and declared leatherback turtle as Critically Endangered Wildlife Species Type 1 [1]. Moreover, leatherback turtle also the one species of sea turtle is covered under the program in nature of The Royal Thai Navy for conservation. The navy was conducting a research on the biology, spawning, migration and nourishment [2]. Then, they set up the sea turtle exhibition to broadcast and promote the spectacular life for those who are interested. Nevertheless, there are few reports have been documented on eggshell morphology and structure in captive and free-living of sea turtles in Thailand [3], [4]. So, the study of microanalysis on ultrastructure and elemental composition of leatherback turtle eggshells will be valuable to promote knowledge of this conservation program. This research is mainly focused on the morphology ultrastructure and component elements in both quantitative and qualitative sense. We conducted the research with the scanning electron microscope (SEM) by 2 detectors (SEI and EDX). The results could be applied to categorize and model of eggshell from the Andaman Sea Shore. It will be expect to improve and promote the understanding of sea turtle for conservation.

II. Materials and Methods

Leatherback turtle eggshells were collected from Andaman Sea Shore, Thailand during the nesting season. Eggshells were washed with distilled water for 5min. Then they were dried at room temperature, and stored at 30^{o}C in desiccator before SEM microanalysis. All leatherback turtle eggshells were analyzed under SEM (JOEL JSM 6400 LV) using SEI and EDX detectors and operating at 15kV. Elemental mapping was also done using Smiling program X-ray images which mapped the elemental composition and distribution of all elements. The elemental mapping was showed by means of color coding for each element from 3 layers. The data was done by SPSS for windows package. Concentrations of various trace elements in eggshell samples were statistically analyzed by using a one-way ANOVA. If Lenene's test and classified by Duncan's test indicated heteroscedasticity ($P<0.05$), a nonparametric Kruskal-Wallis test was performed.

III. Results

The result from SEI demonstrated that eggshell layer of leatherback turtle was the outer cuticle layer or calcareous layer, the middle layer or middle multistrata layer, and the inner fibrous layer. The outer cuticle layer was thick and porosity which various shapes and sizes forming of branching needle-like crystals unit. The loose attachment between these units resulted in numerous spaces and openings in the range of 5-15µm (Fig. 1). The middle layer was compact thick with several multistrata of $CaCO_3$ fibers (range from 0.3-2.0µm) that were firmly forming as network thorough the layer. This layer contained numerous openings connecting to both outer cuticle layer and inner fibrous layer (Fig. 2 A, B). It also consisted of organic amorphous material with crystals predominantly of NaCl. The inner fibrous layer was compact and thin, and composed of numerous reticular fibers (Fig. 2 B). The thickness of outer cuticle, middle, and the inner fibrous layer of leatherback turtle were 83.67±17.15, 144.20±2.20, and 9.69±2.53 micrometer, respectively.

Energy dispersive X-ray microanalysis detector revealed energy spectrum of X-rays character emitted from all elements on each layer. The percentages of all elements were found in the following order: carbon (C) > oxygen (O) > calcium (Ca) > sulfur (S) > potassium (K) > aluminum (Al) > iodine (I) > silicon (Si) > chlorine (Cl) > sodium (Na) > fluorine (F) > phosphorus (P) > magnesium (Mg). Each layer consisted of high percentage of $CaCO_3$ (approximately 98%) implying that it was essential for turtle embryonic development. A significant difference was found in the percentages of Ca and Mo in the 3 layers. Moreover, transition metal, metal and toxic non-metal contaminations were found in leatherback turtle eggshell samples. These were palladium (Pd), molybdenum (Mo), copper (Cu), aluminum (Al), lead (Pb), and bromine (Br). The contamination elements were seen in the outer layers except for Mo (Table I).

All elements were readily observed and mapped using Smiling program. X-ray images which mapped the location of all elements were showed. Calcium, carbon and oxygen containing in the eggshell appeared in high percentage and widely distributed in clusters of the outer cuticle layer to form $CaCO_3$. The accumulation of Na and Cl was observed to form NaCl which was widely distributing in 3 eggshell layers. Meanwhile, Al and S showed a low content and widely distributed in all layers (Fig. 3).

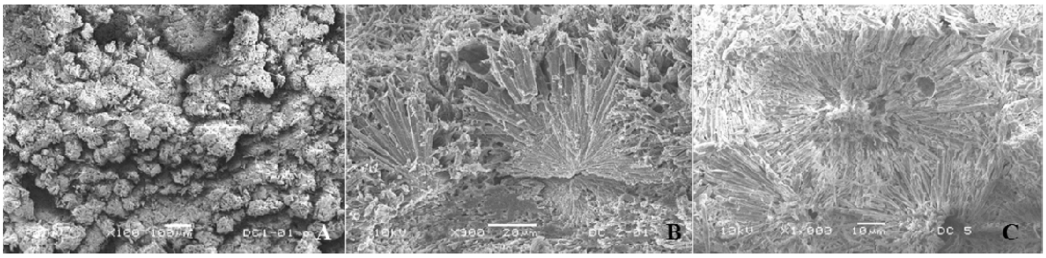

Fig. 1 Micrographs of SEM showed forming of branching needle-like crystals on the outercuticle layerofleatherback turtle eggshell (A-B). Numerous pores which connect to both outer cuticle layer and inner fibrous layer were showed (C)

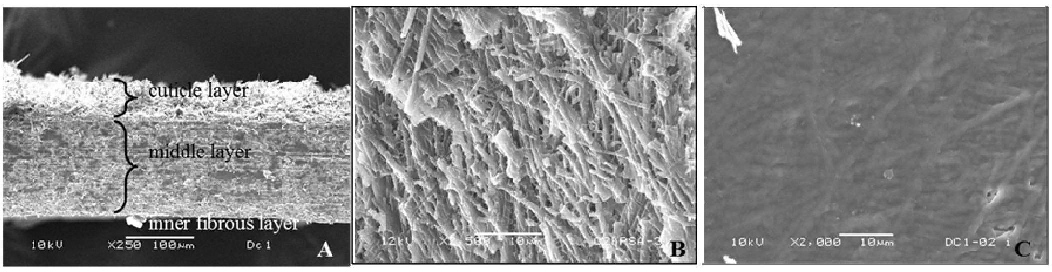

Fig. 2 Micrographs of SEM showed the cuticle layer, the middle layer, and the inner fibrous layer on lateral side of leatherback turtle eggshell (A). At high magnification showed the reticulate fiber of middle layer of (B) and inner fibrous layer of leatherback turtle eggshell (C)

TABLE I
PERCENTAGE OF ELEMENT COMPOSITION IN 3 LAYERS OF LEATHERBACK TURTLE EGGSHELL

Elementals	Percentage of elemental compositions (Mean ± SD)			
	The outer cuticle layer	The middle layer	The inner fibrous layer	Total
C	47.19±22.54	47.29±14.15	50.32±15.33	48.46±18.05
O	37.18±8.96	41.52±9.25	43.80±13.74	40.70±11.34
Ca	14.44±15.13 b	8.35±6.75ab	3.09±5.24a	8.42±11.29
S	1.81±1.26	2.15±1.70	1.95±1.48	1.93±1.42
Br	0.39±0.30	-	-	0.39±0.30
Pd	0.31*	-	-	0.31*
K	0.30±0.28	0.07±0.05	0.07±0.06	0.12±0.16
Al	0.29±0.85	0.02±0.01	0.07±0.06	0.16±0.57
Pb	0.29±0.10	-	-	0.29±0.10
Mo	0.25±0.23a	0.45±0.14b	0.57±0.29b	0.41±0.28
I	0.23±0.12	0.21±0.10	0.13±0.08	0.19±0.10
Cu	0.22±0.22	-	-	0.22±0.22
Si	0.15±1.78	0.11±0.79	0.23±0.15	0.18±0.15
Cl	0.13±0.29	0.05±0.03	0.06±0.05	0.09±0.20
Na	0.12±0.09	0.1±0.05	0.13±0.10	0.12±0.08
F	0.12±0.07	0.23±0.13	0.34±0.25	0.25±0.19
P	0.09±0.10	0.03*	0.06±0.05	0.07±0.07
Mg	0.04±0.03	0.04±0.02	0.08±0.05	0.06±0.04

* The statisticallyanalysiscannotberesolveddueto the smallquantitativeofdata
- Nodata

IV. DISCUSSIONS

Leatherback turtle eggshell contains 3 layers; outer cuticle layer, middle layer or middle multistrata layer, and inner fibrous layer. This finding is similar to the report of green turtle eggshell during pre and post hatching periods [5]. The outer cuticle layer of leatherback turtle eggshell consists of branching needle-like crystals unit. The center of the unit was attached to the top of middle layer with pores. The middle layer is fibrous reticulum with sodium chloride crystal. Meanwhile, the inner layer consists of hundreds of fiber tightly matting. These characters also found in green turtle eggshell [3], [4]. The thickness of outer cuticle, middle, and the inner fibrous layer of leatherback turtle were 83.67±17.15, 144.20±2.20, and 9.69±2.53 micrometer, respectively. It could be used as a characteristic on turtle taxonomy and there are some report can identify the species of animal by the thickness of their eggshell [6].We found several trace element distributions in 3 layers of leatherback turtle eggshell. The percentages of all elements were found in the following order; C > O > Ca > S > K > Al > I > Si > Cl > Na > F > P > Mg. Each layer consisted of high percentage of $CaCO_3$ (approximately 98%) which essential for juvenile sea turtle development [3]-[5]. In addition, transition metal, metal and toxic non-metal elements were found in some examples such as Pd, Mo, Cu, Al, Pb, and Br. Similar results were reported the levels of trace elements in green turtle eggs collected from Hong Kong [7]. The contamination elements were seen in the outer layers except for Mo. However, the contamination is relatively small compare to other components [3], [4], [8]. This project reviewed the merit of SEM with SEI/EDX detectors and Smiling program. X-ray images. It is a powerful and comfortable to analyze percentage of trace elemental accumulation and distribution. Moreover, this finding could be applied to categorize and model the eggshell that was collected from the Andaman Seashore. The result is expected to improve and promote the understanding of sea turtle for conservation.

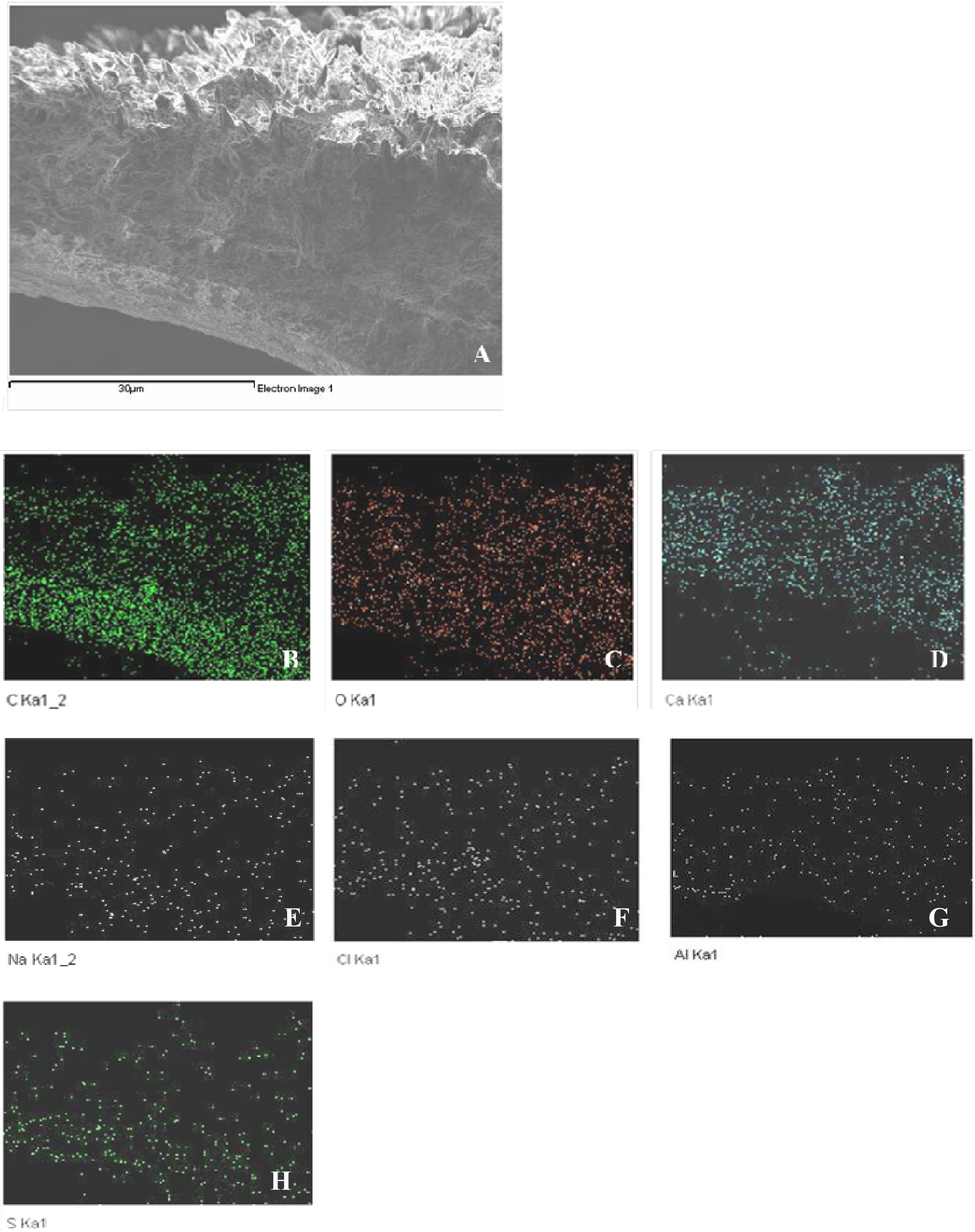

Fig. 3 Micrographs of SEI detector showed outer cuticle layer and middle layer of leatherback turtle eggshell (A). X-ray images reviewed the location map of C, O, Ca, Na, Cl, Al, and S (B-H)

V. Conclusions

Ultrastructure of leatherback turtle eggshell showed 3 layers of outer cuticle layer, middle layer, and inner fibrous layer. The leatherback eggshell thicknesses were83.67±17.15, 144.20±2.20, and 9.69±2.53 micrometer, respectively. EDX reviewed high percentage of $CaCO_3$ (approximately 98%) and contamination of metals and nonmetals in outer layers (Pd, Mo, Cu, Al, Pb and Br). While the contamination of Mo was found at all layers of turtle eggshells. This finding would be the fundamental data for further work to management of sea turtles conservation.

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REFERENCES

[1] K.L. Eckert, K.A. Bjorndal, F. Alberto Abreu-Grobois,and M. Donnelly (Eds). *'Research and Management Techniques for the Conservation of Sea Turtle*, IUCN/SSC Marine Turtle Specialist Group Publication no. 4, Consolidated Graphic Communications, Blanchard, Pennsylvania,232 pages, 1999.

[2] T. Chuen-Im, M. Areekijseree, S. Chongthammakun, and S.V. Graham,"Aerobic bacterial infections in captive juvenile green (*Cheloniamydas*) and hawksbill (*Eretmochelysimbricata*) sea turtles from Thailand", *Chelonian Conservation and Biology*, vol. 9, no 1, pp. 135-142. DOI: 10.2744/CCB-0808.1., 2010.

[3] M. Areekijseree, S. Nuamsukon, T. Chuen-Im, and N. Narkkong, "Microanalysis on ultrastructure and elemental composition of green turtles (*Cheloniamydas*) eggshells". *Journal of the Microscopy Society of Thailand*, vol.24,no 2, pp. 78-82, 2010.

[4] S.Nuamsukon, T. Chuen-Im, S. Rattanayuvakorn, K. Panishkan, N.Narkkong, and M. Areekijseree, "Thai marine turtle eggshell: Morphology, ultrastructure and composition". *Journal of Microscopy Society of Thailand*, vol. 23, no 1, pp. 52-56, 2009.

[5] S. N. Al-Bahry, I.Y. Mahmoud, I.S. Al-Amri, T.A. Ba-Omar, K.O. Melgheit, and A.Y. Al-Kindi, " Ultrastructural features and elemental distribution in eggshell during pre and post hatching periods in the green turtle, *Cheloniamydas* at Ras Al-Hadd, Oman. *Tissue and Cell*, vol. 41, pp. 214-221, 2009.

[6] J. D. Winkler, "Testing phylogenetic implications of eggshell characters in side-necked turtles (Testudines: Pleurodira)". *Zoology*, vol. 109, pp.127-136, 2006.

[7] J. C. W. Lam, S. Tanab, S.K.F. Chan, M.H.W. Lam, M. Martin, and P.K.S. Lam, "Levels of trace elements in green turtle eggs collected from Hong Kong: Evidence of risks due to selenium and nickel" *Environ. Pollut*. vol. 144, pp. 790-801, 2006.

[8] S. Nuamsukon, T. Chuen-Im, P. Peng-Pan, S.Rattanayuvakorn, and M. Areekijseree, "Juvenile sea turtles conservation: Analysis of structure, element of eggshell, microbiological, histological investigation", In the 9th International Congress on Cell Biology, 7-10 October 2008, Seoul, Korea.