Identification of dugong (*Dugong dugon*) tissues using isozymes

KONGKIAT KITTIWATTANAWONG, KANJANA ADULYANUKOSO and PANTARAK NA BUAKAEW
Phuket Marine Biological Center P.O. Box 60, Phuket 83000
Email: kongkiat_k@hotmail.com

ABSTRACT
Two tissue specimens, suspected of being dugong were tested by analyzing isozymes. The first tissue specimen was collected from Ban Paklok, Phuket on 26th of October, 2000 and the second one was collected from a fresh market in Phuket town on 29th of October, 2000. The suspected tissues were compared with known tissues of five dugongs (*Dugong dugon*), three cows (*Bos taurus*), three pigs (*Sus scrofa*), three chickens (*Gallus domesticus*) and one finless porpoise (*Neophocaena phocaenoides*). The study employed seven enzymes namely dihydrolipoamide dehydrogenase, glucose-6-phosphate isomerase, lactate dehydrogenase, isocitrate dehydrogenase, malate, mannose-6-phosphate isomerase and phosphoglucomutase.Using the zymograms of these seven enzymes, the first tissue specimen was identified as dugong tissue, while the second tissue specimen was not. Due to incompatibility of protein patterns, we were unable to assign the second tissue specimen to any of the compared organisms. An electrophoretic analysis of isozymes has proven to be an effective tool for recognition of dugong tissue and shows potential for identification of other conserved organisms that are poached.

INTRODUCTION

In Thailand, dugong (*Dugong dugon*) is one of the protected sea animals by the Fisheries Act 1947 and under CITES in 1983. Hunting and possession of dugong including its remains are illegal (Adulyanukosol, 2001). In the past, dugongs contributed to the diet of villagers along both coastlines of the Andaman Sea and the Gulf of Thailand. However, the chance of catching dugong is very low because of the present small numbers. Although dugongs have not been hunted, they are caught after becoming entangled in fishing gear especially gill nets (Boonprakob *et al.*, 1983; Chantrapornsyl and Adulyanukosol, 1994; Adulyanukosol 1995 and 1999).

Most of the dugong's muscle systems resemble closely those of manatees, except the shoulder muscles that are quite different (Domning 1977 in Nishiwaki and Marsh, 1985). Although fresh meat of dugong and the appearance of both texture and pink-reddish color are very similar to pork, the dugong's meat has lesser fat than pork (Adulyanukosol, *unpublished information*). Furthermore, pork seems to decompose easier than dugong's flesh and generally pork is smellier than dugong. However without skin, it is quite difficult to distinguish the two species. Soon after the local villagers find a dead dugong, sometimes they illegally sell the meat on the black market or alternatively distribute meat inside the village.

Recently in late October 2000, we received two samples of unknown tissues, which were suspected to be dugongs' meat. This paper evaluates electrophoresis of isozymes as a tool for identification of dugong samples.

MATERIALS AND METHODS

The tissues

The two tissues, suspected of being dugong, were collected from Ban Paklok and a fresh market, Phuket province on 26th and 29th of October, 2000, respectively. These two tissues were suspected as dugong material because of the pink-reddish color, which is the normal color of dugong muscle. The tissues were sent to the electrophoresis laboratory at PMBC in fresh condition. The suspected tissues were compared with five known species tissues samples, *i.e.* five dugongs, three cows (*Bos taurus*), three pigs (*Sus scrofa*), three chickens (*Gallus domesticus*), and one finless porpoise (*Neophocaena phocaenoides*) (Table 1).

Electrophoresis

The analysis was carried out on 7-8th of November 2001. A total volume of 30-60 ?l of 1 % polyvinyl pyrrolidone (PVP) was added to the tissue samples together with a small amount of cleaned sand before homogenizing. After centrifuging at 4,105 g for 5 minutes, the supernatants were absorbed onto paper wicks, which then were inserted into starch gels. Starch gels were prepared with 12% starch

(Sigma S-4501) in tris-citrate buffer pH 7.0 (Benzie, 1993). The proteins were separated at 600 volts and 80 mA for 3 hours. Staining was done with seven enzyme staining recipes i.e., dihydrolipoamide dehydrogenase (DDH or diaphorase DIA, EC 1.8.1.4), glucose-phosphate isomerase (GPI, EC 5.3.1.9), lactate dehydrogenase (LDH, EC 1.1.1.27), isocitrate dehydrogenase (IDH, NADP EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), phosphoglucomutase (PGM, EC 5.4.2.2). The staining recipes were described by Harris & opkinson (1976) and Manchenko (1994).

Table 1. Collecting sites and condition of the known-species tissues used as references for identification of suspected tissues. Field numbers are identification codes for specimens kept at Phuket Marine Biological Center.
Type: M=muscle, L=liver

Species	Field No.	Type	Collecting place	Tissue condition
Dugong dugon (5)	Du- 119	M	Toloyai Island, Trang	Decomposed, 9-month frozen
	Du- 120	M	Laem Yong Lam, Trang	Semi fresh, 8-month frozen
	Du- 048	M	Kam Island, Ranong	Semi fresh, 56-month frozen
	Du- 074	M	Wean Island, Trang	Semi fresh, 33-month frozen
	Du- 084	M	Port of Phuket, Phuket	Fresh, 26-month frozen
Suspected tissue 1	-	M	Paklok, Phuket	Fresh, 1-week frozen
Suspected tissue 2	-	M	Fresh market, Phuket	Fresh, 1-week frozen
Neophocaena phocaenoides (1)	FINP 166	M	Phangnga	Fresh, 13-month frozen
Bos taurus (3)	-	M, L	Fresh market, Phuket	Fresh, not frozen
Sus scrofa (3)	-	M, L	Fresh market, Phuket	Fresh, not frozen
Gallus domesticus (3)	-	M, L	Fresh market, Phuket	Fresh, not frozen

Zymogram interpretation

A zymogram is defined as a strip or band of electrophoretic medium showing the pattern of enzymes or isoenzymes after their separation by electrophoresis (Harris and Hopkinson, 1976). Band separation is mainly due to the net charge, size and shape of protein. A protein with negative charge will run to the anode while proteins with positive charges will run to the cathode. The higher the charge a protein contains, the faster it can run. In contrast, the bigger the molecular size of a protein, the slower it can move along an electric field. A zymogram derived from each enzyme-specific staining method can possess more than one locus or system. Within a locus, each individual possesses one to several bands depending on whether it is heterozygote versus homozygote as well as on forms of proteins e.g. monomere, dimere and trimere (Richardson *et al.,* 1986). These bands are phenotypes of alleles or genes. However, not every band is counted as an allele. Bands of alleles at each locus can be designated numerically with the fastest anode-migrating band denoted "1", the second fastest "2", and so on. The tissue specimens were assigned as or differentiated from dugong tissues by visual comparison of the bands' locations with those of other organisms. As a general rule, zymograms of conspecific organisms tend to share the same locations, while the ones of different species tend to locate distinctly (Hartl and Clark, 1989). However, it has to be emphasized that identical migration rate does not necessarily indicate identical amino acid composition of the proteins.

RESULTS

Zymograms of seven-enzymatic stains are shown as Figure1. Bands of suspected and dugong tissues were developed well for all enzymes except IDH. Most enzymes exhibited both anodic and cathodic migrating bands except GPI which possessed only protein bands with positive charges. Referring to MDH, DDH, LDH, MPI, and PGM, the first suspected tissue (U1) was assigned as dugong tissue whereas the second one (U2) could not be assigned to any of the compared organisms.

MDH: This enzyme was one of the best enzymes to differentiate dugong tissues from other compared organisms. Two zones were observed, probably representing two loci. The first locus (anodic migrating, line 1-6) had six alleles and the second one (cathodic migrating, line 7-10) had four alleles. For the first locus, band of U1 located at the 6th line was common with the ones of dugong tissues while it differed from other compared organisms. U2 was not compatible with any of compared organisms. D3 did not show any bands. The second locus showed pale bands but was still possible to score. At this locus, U1 was located on the same line as dugong tissues (line 7), while differentiated from other organisms.

DDH: Two zones were observed, probably representing two loci. However, there were only heterozygotes in hens' muscles in the second loci (cathodic migrating, line

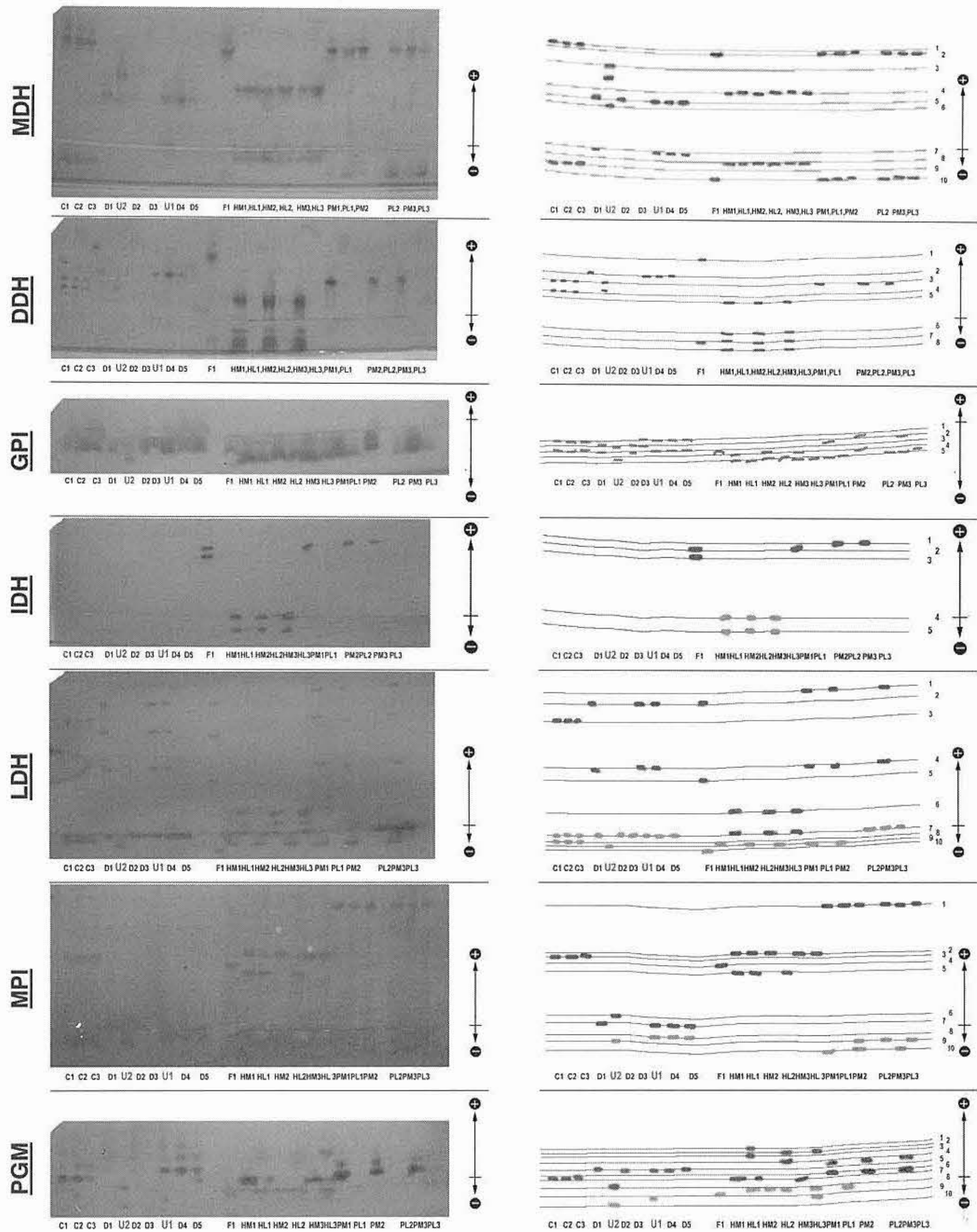

Fig. 1. Electrophoretic comparisons of two unknown specimens (U1 and U2) to known specimens of 5-dugong muscles (Dugong dugon, D1=Du-074, D2=Du-048, D3=Du-119, D4=Du-128, D5=Du-120), 1-finless porpoise muscle (Neophocaena phocaenoides, F1), 3-cows (Bos taurus, C1-3), 3-pigs (Sus scrofal, P1-3) and 3-chickens (Gallus domesticus, H1-3). L and M are liver and muscle tissues respectively. Minus and plus signs indicate the charges of electric field and the horizontal mark in between indicates the starting line. The figures on the left panel show original zymograms, while on the right pane are zymogram interpretations.

6-8). In the first locus (anodic migrating, line 1-5), U1 was located in the same line (line 2) with the other dugongs (D4 and D5). D1 had very pale band, while D2 and D3 were absent. U2 possessed very pale bands, which were located similar to cow tissues (C1-3). In chickens and pigs, the bands only developed in muscle tissues but not in liver tissues.

GPI: One zone was observed, probably representing locus (anodic migrating, line 1-5). All the muscle tissues exhibited heterozygotes except the liver tissues, which were homozygote. The known dugong tissues, as well as

U1, had 3 alleles located at line 1, 2 and 3. These dugong alleles resembled the ones of cows. However, they were significantly different from alleles of chickens and pigs. U2 had two alleles. The first allele was close to line 2, while the second one was between line 4 and 5.

IDH: Two zones were observed, again most likely representing two loci (anodic migrating locus, line 1-3 and cathodic migrating locus, line 4-5). No activities were obtained from suspected tissues or from dugong tissues.

LDH: Two zones were observed, again most likely representing two loci (anodic migrating locus, line 1-7 and cathodic migrating locus, line 7-10) In locus 1, heterozygotic alleles of U1 resembled the ones of dugong tissues (line 2 and 4) and differentiated from other organisms. In locus 2, dugong tissues as well as U1 shared the same alleles with the ones of cows, and pigs (line 7). U2 appeared only in locus 2 and shared the same position (line 9) with chicken tissues.

MPI: Two zones were observed, again most likely representing two loci (anodic migrating locus, line 1-7 and cathodic migrating locus, line 8-10). In locus 1, U1 was on the same line as dugong tissues (line 7) and could be distinguished from other compared organisms as well as U2. In locus 2, the bands were pale and located close to each other. This locus was not employed for the determination.

PGM: Two zones were observed, again most likely representing two loci (anodic migrating locus, line 1-7 and cathodic migrating locus, line 8-10). The locus 1, U2 revealed a band located on the same line as the ones of compared dugong tissues (line 5) and could be distinguished from other compared organisms including U2. In locus 2, the bands were pale and not used for determinations.

CONCLUSIONS

Isozyme electrophoresis has been proven to be an effective tool in detecting the suspected dugong tissue. Our study successfully employed five out of seven enzymes staining recipes to assign the suspected tissue as dugong tissue and to differentiate it from other compared organisms i.e. finless porpoise, cows, chickens, and pigs. Among five enzymes, MDH revealed the best result in both band intensity (easy to detect) and separating potential (good separation from other compared organisms). However, in most cases, the authors suggest employing all five enzymes for accurate detection.

Variation of band intensity reflects the activity of enzymes contained in tissues, the fresher the original sample, the higher intensity. The dugong tissues used in this study varied in their initial freshness (fresh to decomposed) and also terms of duration kept in a -20oC freezer (8-56 months). The study demonstrated that fresh-collected dugong tissue frozen for 24 months as well as semi-fresh-collected samples frozen for up to 33 months, are sufficient for isozyme electrophoresis. Longer preservation of semi-fresh tissue may not give useful results, while samples stored storing at -20oC for 56 months showed no activity in most enzymes. The decomposed dugong tissue (Du-119) proved almost useless for isozyme analysis.

ACKNOWLEDGEMENTS

We are grateful to villagers of Ban Paklok and Mr. Chatchai Sarikhapan, staff of PMBC for providing the samples from Ban Paklok and fresh market, respectively. Mr. Niyom Lohakarn, chief of Phuket Provincial Fishery Office, and Mr. Thongchai Pathanakul, head of the conservation of Fishery Resources Section, the Phuket Provincial Fishery Office, are acknowledged for their co-operation in dugong research and conservation in Phuket province. We also appreciate the editing assistance of Vibeke Simonsen at Aauhus University, Babara Brown at New Castle University, as well as Ellen Hines, Yvette Alva, and Frank Cipriano at San Francisco State University.

REFERENCES

Adulyanukosol K., 1995. The status and tendency of declination of dugong population in Thailand. 1995. (in Thai). Proc. of The Seminar on Fisheries in 1995, Department of Fisheries. 385-392.

Adulyanukosol K., 1999. Dugong, dolphin and whale in Thai waters. Proc. of the 1st Korea Thailand Joint Workshop on Comparison of Coastal Environment: Korea-Thailand, 9-10 September 1999, Seoul, Korea. 5-15.

Adulyanukosol K., 2001. Dugong in Thailand. (in Thai). Technical Paper. Phuket Marine Biological Center. 15 pp.

Benzie, J. A. H., 1993. Genetics of giant clams: an overview. - ACIAR Proceedings 47: 7-13.

Boonprakob U. *et al.*, 1983. Occurrence of dugong (Dugong dugon) in coastal waters of Phuket Island and attempt to keep dugongs in captivity. A Symposium on Marine Mammals of the Indian Ocean, 22-25 February 1983, Colombo, Srilanka. 13 pp.

Chantrapornsyl S. & K. Adulyanukosol, 1994. Occurrence of dugongs (*Dugong dugon*) and status of the dugong in Thailand. (in Thai). Proc. of the Seminar of the Department of Fisheries in 1994, Bangkok, Thailand. 380-385.

Hartl D. L. & A.G. Clark, 1989. Principles of population genetics. Sinauer associates, Inc. Sunderland. 682 pp.

Harris H. & D. A. Hopkinson, 1976. Handbook of enzyme electrophoresis in human genetics. North-Holland, Amsterdam. 259 pp.

Manchenko G. P., 1994. Handbook of detection of enzymes on electrophoretic gels. CRC Press, London. 341 pp.

Nishiwaki, M. & H. Marsh, 1985. Dugong Dugong dugon (Muller, 1776). Handbook of Marine Mammals Vol 3. Academic Press Inc. (London) Ltd. : 1-31.

Richardson, B. J. *et al.*, 1986. Allozyme electrophoresis: A handbook for animal systematics and population studies. Academic Press, Inc., New York. 410 pp.