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Some characterizations of Asian elephant (*Elephas maximus*) semen alpha-L-fucosidases

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Abstract

Alpha-L-fucosidase is an enzyme found in several organisms. Commonly, its function is the degradation of L-fucose containing biomolecules in lysosomes. Moreover, the enzyme is also found in sperm and seminal plasma. It plays an important role in sperm-egg binding. In this study, sperm and seminal plasma alpha-L-fucosidases of Asian elephants were characterized for the first time. The results showed that the average volume of semen was 27 mL, the pH was 7, the sperm motility was 20-50%, and the average concentration was 539 × 10^6 cells/mL. The localization of alpha-L-fucosidase was determined by the immunoperoxidase technique. The results revealed that the enzyme is localized at the plasma membrane of the sperm over the head and tail regions. Western blot results of the seminal plasma alpha-L-fucosidase showed three bands of 50.7, 24.3 and 12.6 kDa, whereas those of the sperm showed a band of 40 kDa. The optimal pH of seminal plasma alpha-L-fucosidase ranged between 3.2-6.6 with over 80% of maximal activity. Similarly, over 80% of the enzyme activity in sperm were found with a broad range of pH (3.4-6.6). Several isoforms of seminal plasma alpha-L-fucosidase were revealed by isoelectric focusing, including two major isoforms (pI 7.48, 7.98) and four minor isoforms (pI 3.33, 5.19, 8.43 and 8.80). This work reveals the presence of alpha-L-fucosidases in Asian elephant semen and helps us to better understand its properties, as well as providing information that may be useful in the development of reproductive biotechnology for breeding and the conservation of this endangered species.

Keywords: Alpha-L-fucosidases, Asian elephant, *Elephas maximus*, Semen

1. Introduction

The Asian elephant (*Elephas maximus*) is the national animal of Thailand and plays an important role in history, traditional culture as well as tourism. The Asian elephant is facing serious population decline throughout Asia. The main reasons for population decline include habitat degradation, illegal hunting, and human-elephant conflicts [1]. Since the Asian elephant is becoming extinct, the international union for conservation of nature (IUCN) has listed it as an endangered species. To protect and conserve Asian elephants, substantial amounts of work have focused on assisted reproductive biotechnologies, such as artificial insemination (AI) [2,3], identification of fertility biomarkers [4] and sperm sex-sorting [5]. However, an understanding of molecular reproduction is necessary to develop a specific AI protocol for this species.

The key event in fertilization is the sperm-egg binding. This step involves protein-carbohydrate interactions between the sperm and egg surfaces. There are various proteins on the plasma membrane of the sperm that specifically recognize the carbohydrate ligand on the egg surface. An alpha-L-fucosidase (EC 3.2.1.51) is one of those sperm related proteins. This enzyme is found on the sperm surface of both invertebrates and vertebrates, for example, bivalve mollusks [6], *Drosophila* [7], darters [8], toads [9], bulls [10], mice [11] and humans [12]. Usually, the enzyme exists in lysosomes, and it is responsible for the degradation of biomolecules containing L-fucose. In contrast, sperm-associated alpha-L-fucosidases involved in gamete recognition and the binding of various species [13-16]. Previous studies reported that bull seminal plasma alpha-L-fucosidases had the potential to induce the acrosome reaction of guinea pig sperm [17] and it is considered as a promising biomarker

for bull fertility [18]. Moreover, it has been reported that the catalytic and binding sites of the enzyme in bacteria, fungi, plants, and metazoans were highly conserved [19].

This study is the first report of the presence of alpha-L-fucosidases in both Asian elephant sperm and seminal plasma. We also analyzed some characteristics of the enzyme. The findings could lead to a better understanding of the molecular basis of fertilization and leading to improvements in assisted reproductive technologies for the conservation of Asian elephants.

2. Materials and methods

2.1 Animals and semen collection

Asian elephants were housed at the National Elephant Institute (NEI) and used as semen donors. The semen collection was performed by veterinary staff using a manual manipulation technique. Semen of four healthy elephants ranging from 35 to 42 years of age were collected only once. Four ejaculates were immediately evaluated for volume, sperm motility, pH, and sperm concentration. After that, the semen samples were kept at 4°C while brought to the laboratory.

2.2 Semen sample preparation

Each semen sample was gently mixed with phosphate-buffered saline (PBS, pH 7.4) at a ratio of 1:2 and centrifuged at 500 × *g* for 10 min to separate the sperm and seminal plasma. The supernatant (seminal plasma) was transferred to new 1.5-mL tubes and stored at -20°C for further studies. The pellet (sperm) was resuspended in cold PBS and recentrifuged at 500 × *g* for 10 min to eliminate seminal plasma contamination. After the supernatant was discarded, the sperm pellet was washed two more times and stored at -20°C for further analysis.

2.3 Immunolocalization of sperm alpha-L-fucosidase

The sperm suspension with a concentration of 20-40 × 10^6 cell/mL was smeared onto glass slides, air-dried, and fixed with 70% ethanol. Endogenous peroxidase activity was blocked by adding 0.25% hydrogen peroxide for 15 min, followed by washing three times with PBS. The smears were incubated for 1 h with primary and secondary antibodies using a polyclonal goat anti human liver alpha-L-fucosidase antibody (1:50) and a horseradish peroxidase (HRP) conjugated rabbit anti goat IgG antibody (1:100, SeraCare, USA), respectively. The signal was developed in 3,3'-diaminobenzidine (DAB) for 30 min and observed by a light microscope. The primary antibody was omitted in the negative control group.

2.4 Seminal plasma protein preparation

The dialysis tubing (Spectrum Laboratories, USA) was activated by boiling in 2% $NaHCO_3$ and 1 mM EDTA before use. The activated bag was filled with the seminal plasma and subjected to dialysis in 10 mM sodium phosphate buffer (pH 5.5) at 4°C. The buffer was changed every day for three days. After that, the Vivaspin® 6 centrifugal concentrator (GE Healthcare, USA) was used to concentrate the dialyzed seminal plasma proteins. The retentate solution was stored at -20 °C until further analysis.

2.5 Sperm protein preparation

The washed sperm pellet was resuspended with cold PBS to a concentration of 100 × 10^6 cells/mL. The stock Triton X-114 solution was mixed with the sperm suspension to get 2% (v/v) of final concentration. The mixture was incubated for 1 h on a refrigerated incubator shaker at 75 rpm to allow sperm protein extraction, followed by centrifugation at 4°C (10,000 × *g* for 20 min). Subsequently, the supernatant was incubated for 3-5 min at 37°C. After that, the clear supernatant changed to turbid. The turbid solution was subjected to phase partitioning by centrifugation at 1,000 × *g* for 5 min at room temperature. The aqueous (clear) and detergent (turbid) phases appeared at the upper and bottom of the tube, respectively. The upper solution was carefully collected and stored at -20°C until further analysis.

2.6 pH optimum of sperm and seminal plasma alpha-L-fucosidases

Determination of optimal pH used three buffers with overlapping pH values, including 0.1 M oxalic acid/sodium oxalate buffer (pH values 2.0-3.4), 0.1 M citric acid/sodium citrate buffer (pH values 3.2-6.4) and 0.1 M NaH_2PO_4/ Na_2HPO_4 buffer (pH values 6.4-8.8). Each reaction contained 25 µL of sample, 25 µL of buffer, and 50 µL of 1 mM *p*-nitrophenyl-alpha-L-fucopyranoside (Sigma, USA). The reaction mixture was

incubated at 37°C for 10 min and a glycine buffer (pH 9.75) was added to stop the reaction. After that, the absorbance of the solution was measured at 400 nm. The assays were duplicated. The pH activity curves of both sperm and seminal plasma alpha-L-fucosidases were constructed.

2.7 SDS-PAGE and Western blot

The sperm and seminal plasma proteins were quantified by Bradford assay. The protein samples were separated on 10% SDS-PAGE and then semi-dry transferred to a polyvinylidene difluoride (PVDF) membrane. An unoccupied site on the membrane was blocked by incubating with a blocking agent (GE Healthcare). After washing three times with a tris buffered saline buffer containing 0.1% Tween 20 (TBS-T), the PVDF membrane was incubated with primary (1:50,000) and secondary (1:250,000) antibodies (the same as the immunolocalization part). For detection, enhanced chemiluminescence (ECL) (GE Healthcare, USA) was added onto the membrane after three time washing with TBST. After ECL incubation for 3-5 min, the membrane was exposed to film (GE Healthcare, USA) and the film was manually processed with GBX developer and fixer (Kodak, USA).

2.8 Isoelectric focusing (IEF) of seminal plasma alpha-L-fucosidase

IEF was performed in a 40 mL-vertical glass column which connected with a cooling bath circulator. The column was filled with sucrose and 2% (v/v) ampholytes (pH 5-7) (Bio-rad, USA) to create a pH gradient. After loading the seminal plasma to the column, the electrofocusing was carried out at 600 V for 20 h. Each fraction was collected in a volume of 0.3-0.4 mL to determine pH and detect alpha-L-fucosidase activity. The IEF profile was constructed by plotting alpha-L-fucosidase activity against isoelectric point (pI).

3. Results and discussion

3.1 Characteristics of fresh semen

A total of four ejaculates were collected from four elephant bulls and was evaluated for semen quality. The data (Table 1) showed that the semen volume differed considerably between individuals and the mean of the total ejaculate volume was 27.8 ± 21.8 mL. The sperm motility percentages obtained from different individuals were also variable, ranging from 0 to 50%. The average sperm motility was 20.0 ± 18.7%. However, the pH values and sperm concentrations of each sample were quite similar. The mean pH value and sperm concentration was 7.4 ± 0.5 and 538.8 ± 51.1 × 10^6 cell/mL, respectively.

Table 1 Characteristics of fresh Asian elephant semen.

Seminal characteristics	Elephant				Mean ± SD
	1	2	3	4	
Total volume (mL)	60.0	12.0	35.0	4.0	27.8 ± 21.8
Total sperm motility (%)	50.0	20.0	10.0	0.0	20.0 ± 18.7
pH	6.5	8.0	7.5	7.5	7.4 ± 0.5
Sperm concentration (×10^6/mL)	582.5	577.5	475.0	520.0	538.8 ± 51.1

In the present study, we found that most semen parameters showed a high standard deviation. It may be the result of variance among individuals. According to previous studies, Asian elephants have differences in seminal characteristics between ejaculates collected from both different elephant bulls and the same elephant bull. It indicates intra- and inter-individual variations in semen quality [20-22]. Various factors have been suggested that may affect these variations, such as age, seasonal changes [22], reproductive variance [21] as well as the semen collection method [23]. However, only a small amount of semen (four ejaculates) was available for this study.

3.2 Immunolocalization of sperm alpha-L-fucosidase

The localization of alpha-L-fucosidase on Asian elephant sperm was detected by the immunoperoxidase technique. The control group and experimental group were treated identically, but the primary antibody was omitted for the control group. After DAB staining, the sperm in the experimental group showed the brown precipitates on the plasma membrane of the sperm head and tail regions (Figure 1B), but there was no staining

observed in the control group (Figure 1A). Moreover, some sperm cells in the experimental group displayed faint staining at the anterior but intense staining at the posterior of the head region (see arrows, Figure 1B).

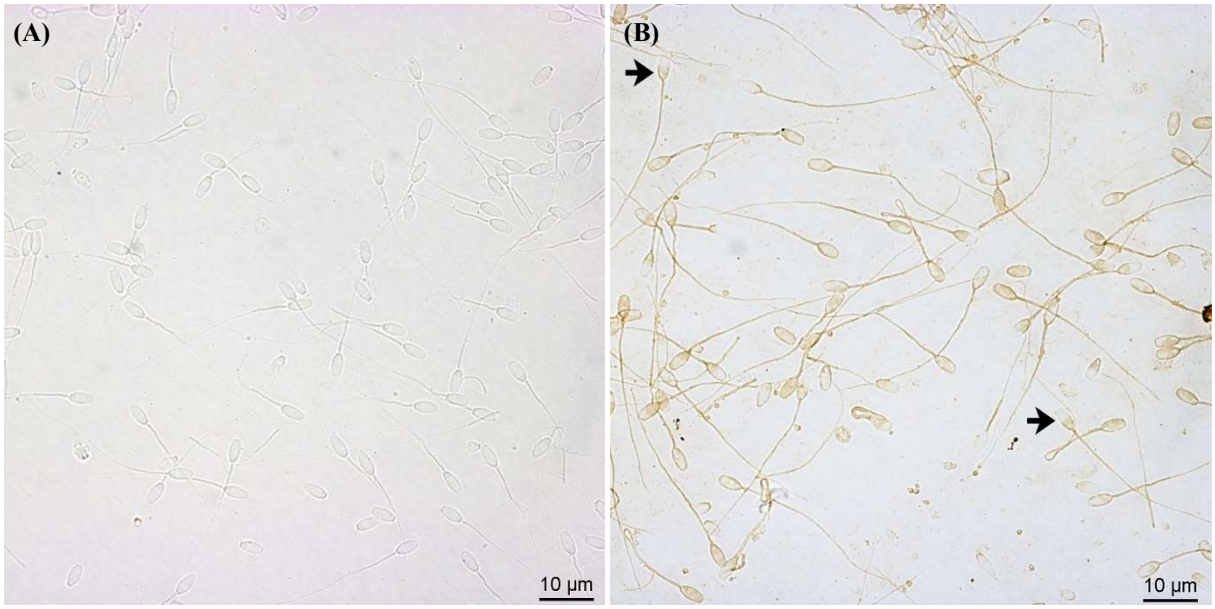

Figure 1 The immunostaining patterns of Asian elephant sperm in (A) control group and (B) experimental group.

This study provided the first evidence showing the existence of alpha-L-fucosidases in Asian elephant sperm. According to the immunolocalization study, we found the precipitated DAB which indicated the localization of the enzyme on the plasma membrane of the sperm head and tail regions. Sperm-associated alpha-L-fucosidases were found in other mammals, for example, the enzyme in human sperm was mainly found in the post-acrosomal or posterior head region and a slight amount was found in the anterior head region, neck, and midpiece of the plasma membrane [12,24]. Moreover, different immunolocalization patterns of the enzyme in human sperm were reported that it depended on the membrane integrity and the acrosome status of the sperm. The enzyme was distributed over the outer surface of the sperm with intact membranes. However, in the capacitated and acrosome-reacted sperm, the enzyme was mostly found in the acrosome and equatorial segments [25]. Similarly, the enzyme associated with mouse sperm was found in the apical part of permeabilized sperm and the equatorial region of acrosome-reacted sperm [11]. The current study also found the staining in the posterior head region of some sperm cells which may be the result of spontaneous acrosome reactions [25]. In addition to vertebrates, alpha-L-fucosidase was found in the invertebrate spermatozoa. For instance, the plasma membrane of the acrosome and tail regions of the *Drosophila* spermatozoa were covered with the enzyme [7].

3.3 Characterization of semen alpha-L-fucosidases

The pH-dependent activity of the enzyme was determined by using three different types of buffers at different pH values. The results showed that both seminal plasma and sperm alpha-L-fucosidases had a broad range of optimal pH. The enzyme in seminal plasma had maximal activity over 80% at pH 3.2-6.6, and the highest activity was at pH 6.2 (Figure 2A). Similarly, over 80% of maximal activity in sperm occurred at pH range between 3.4 and 6.6, and the greatest enzyme activity was at a pH of 5.8 (Figure 2B).

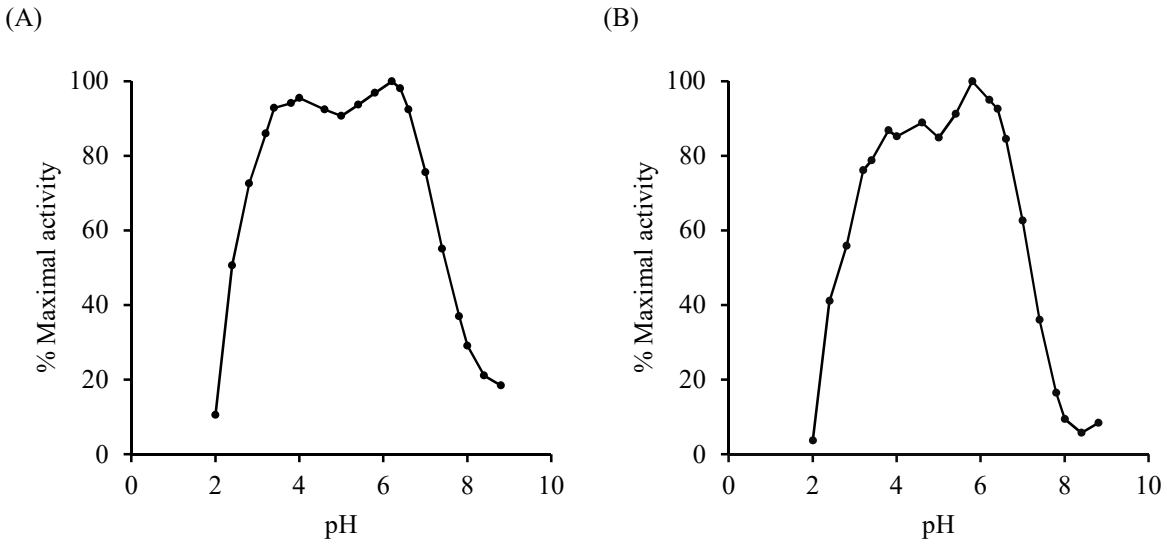

Figure 2 The pH-activity profiles of Asian elephant (A) seminal plasma and (B) sperm alpha-L-fucosidases.

The isoelectric focusing profile of Asian elephant seminal plasma alpha-L-fucosidase is illustrated in Figure 3. The enzyme in seminal plasma contained two main isoforms (pI 7.98 and 7.48) which had over 80% of maximal activity and four minor isoforms (pI 3.33, 5.19, 8.43 and 8.80).

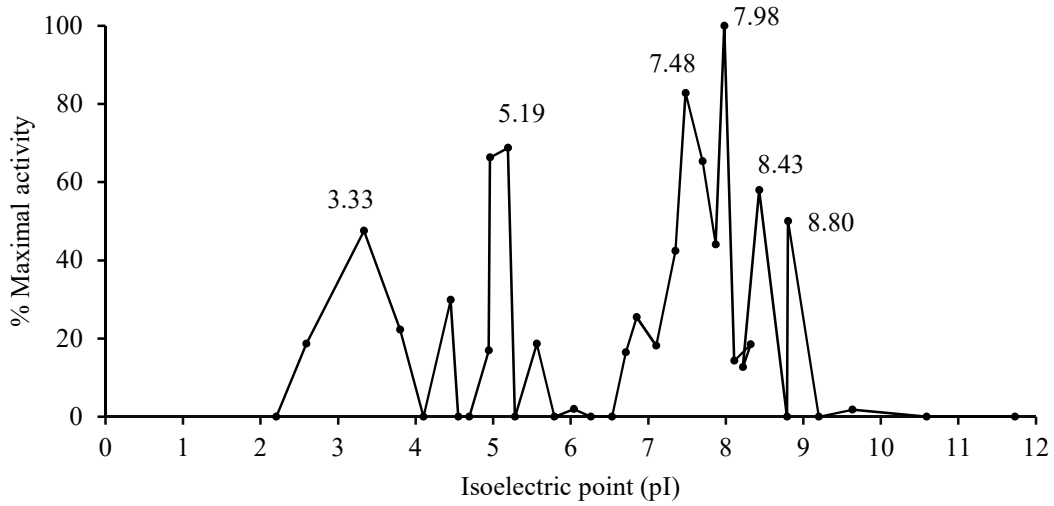

Figure 3 The isoelectric focusing profile of Asian elephant seminal plasma.

The western blot results of Asian elephant seminal plasma and sperm alpha-L-fucosidases is shown in Figure 4. Purified human liver alpha-L-fucosidase (lane 2) was used as a positive control and gave a single band of 57.3 kDa. In seminal plasma (lane 3), three immunoreactive bands were found on the film with molecular weights of 50.7, 24.3 and 12.6 kDa. A single band with a molecular weight of 40 kDa appeared on a blot of sperm extract (lane 4). The results indicated that the molecular weight of alpha-L-fucosidase in seminal plasma was higher than that in sperm.

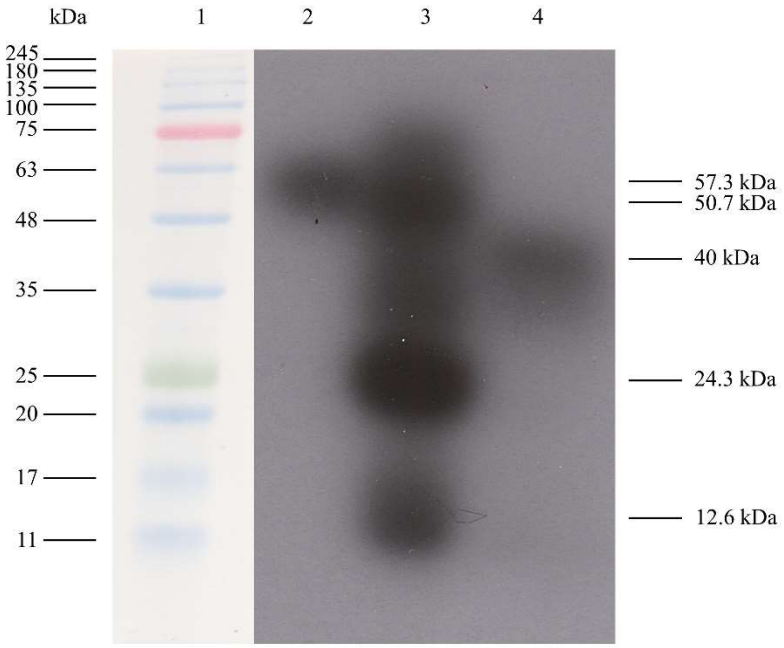

Figure 4 Western blot result of Asian elephant semen alpha-L-fucosidases. Lane 1: protein molecular weight markers; lane 2: purified human liver alpha-L-fucosidase; lane 3: seminal plasma alpha-L-fucosidase; lane 4: sperm alpha-L-fucosidase.

The biochemical study of alpha-L-fucosidases in Asian elephant semen showed similar and different results in comparison with other organisms. The present study found an activity curve of seminal plasma alpha-L-fucosidase in a broad pH range with the highest activity in an acidic condition. The previous report found that the optimal pH for human seminal plasma was also in the acid range but slightly lower [26]. The Asian elephant sperm alpha-L-fucosidase also had a broad range of optimal pH but the maximal activity was found at an acid pH value, like those of *Drosophila* which had two optimal pH values in the acid range, but different from those of humans which had maximal activity at a neutral pH [7,12]. The Asian elephant seminal plasma contained several isoforms of alpha-L-fucosidase. The results revealed that the pI values of the two main isoforms were slightly alkaline (pI ~ 7.5 and 8.0), and minor isoforms had both acidic and basic pI values (pI 3.0-8.8). Similarly, the IEF of the enzyme in human seminal plasma showed isoforms with different pI values of 5.0-7.0 [26]. The Western blot was performed with the appropriate positive control to determine specificity. The result showed that Asian elephant sperm alpha-L-fucosidase had a single band (40 kDa) with a molecular weight quite similar to those of humans (51 kDa), but the enzyme in seminal plasma showed three bands (50.7, 24.3, and 12.6 kDa) which differed from humans (56 kDa) [12,26]. The two bands with lower molecular weights for seminal plasma alpha-L-fucosidase could be the result of protein degradation because the molecular weights of these bands are lower than the typical range for mammalian alpha-L-fucosidase. The previous study reported that the *FUCA1* gene encodes the enzyme in leukocyte, placenta, liver, spleen, brain, urine, and semen [27]. The product size of the human *FUCA1* gene is 53.7 kDa (466 amino acids). In mammals, SDS-PAGEs of purified alpha-L-fucosidases usually show two bands that migrate closely with molecular weights of approximately 50-60 kDa [28]. The large form of Asian elephant seminal plasma alpha-L-fucosidase could be degraded by protease activity that can lead to the two truncated proteins containing epitopes for polyclonal antibody recognition. Protein degradation may also affect sperm samples. However, the amount of human sperm enzyme is very low [29]. Therefore, the small number of degraded products may not be detected by primary antibodies and may not be seen on the blot. However, the two smaller bands of the enzyme in Asian elephant seminal plasma may be the enzyme subunits or isoforms which are found only in some species or tissues as reported in a few studies. The study of human placenta alpha-L-fucosidase found that the purified enzyme was separated into three bands on SDS-PAGE (55, 51.4, and 25.3 kDa) and suggested that the smallest form is structurally associated with the larger form [30].

4. Conclusion

In conclusion, the present study provides evidence proving the presence of alpha-L-fucosidases in Asian elephant semen. It could be another key to successful artificial insemination for the conservation of an

endangered species. However, further investigations are necessary to understand the role of the enzyme in the sperm-oocyte binding of Asian elephants.

5. Ethical approval

Permits for the semen collection used in this study were provided by the National Elephant Institute (NEI), Lampang, Thailand. The research was carried out under the approval of the Animal Usage and Ethics Committee, Kasetsart University (ACKU 01858).

6. Acknowledgments

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