

Isolation and Detection of PLC ζ (Sperm Factor Protein) in Testes of *Rattus rattus diardii* Using One-Step RT-PCR

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Abstract

The fertilization process in many species studied involves induction of Ca²⁺ oscillations in eggs. Prolonged time period of calcium oscillations is essential for regulating various kinds of events that will occur after fertilization. PLC zeta (PLC ζ) has been discovered as a novel isoenzyme of PLC which is responsible in promoting intracellular Ca²⁺ oscillation in oocytes. Until now, PLC ζ has been identified in many mammalian species. Although PLC ζ has been identified in the rodent family, such as the laboratory mice, rats and hamsters, no research has been carried out to identify PLC ζ in house rats. This study was carried out in order to identify the existence of PLC ζ in testes of house rats (*Rattus rattus Diardii*), one of the major pests in houses. Testes from ten male rats were subjected to RNA extraction using easy-BLUETM Total RNA Extraction Kit. One-step RT-PCR technique was chosen to identify PLC ζ using forward and reverse primers from published sequence of PLC-zeta. PCR amplicons were extracted from Agarose gel, purified and further subjected to sequencing. Sequences obtained were aligned using NCBI Blast search at www.ncbi.nlm.nih.gov/blast to check for identification from published PLC-zeta sequences. Results from this study showed that PLC ζ from the testes of house rat was successfully amplified by using One-step RT-PCR technique. The sequence also showed strong homology to published sequence of *Rattus norvegicus* PLC ζ . We hope that with the understanding of molecular properties of PLC ζ in house rats could lead to development of contraceptive vaccines, an alternative approach to control the rat population.

Keywords: PLC ζ , sperm-factor, *Rattus rattus diardii*.

Introduction

Oil palm and rice have been the most widely grown crops in Malaysia and South East Asia (Wood and Fee, 2003). These two crops are attacked by rats which are adaptable and fast reproducing animals and can be fully active all year round in a tropical environment (Wood and Fee, 2003). In Malaysia, at least five of 18 species of *Rattus* have become serious pests (Payne *et al.*, 1985). The *Rattus* genus is believed to have originated in Southeast Asia and consists of about 66 species (Judith *et al.*,

2007). *Rattus rattus* or Malayan black rat, Malayan house rat or roof rat is a common mammal living in a wide range of habitats in the world (Tosihide *et al.*, 1971). Roof rats are usually dark brown intermixed with black or grey in colour. They are shorter about 16-20cm but with bigger eyes and ears compared to the Norway rat. The tail is also relatively longer than its body (Harrison, 1966). The *Rattus rattus diardii* also known as house rat was found occasionally near houses, but from 1983, it occurred quite commonly in oil palms (Wood and Fee, 2003). In the first two years

in the field, the rats are able to bore into the bases of young palms, evidently to get at the buds causing the fronds to collapse leading to palm death. Rats can significantly cause serious and persistent losses, and there is evident need to improve understanding of their reproductive physiology as a basis for efficient control. Phospholipase C- ζ (PLC- ζ) is a sperm-specific enzyme that initiates the Ca^{2+} oscillations in mammalian eggs that activate embryo development (Nomikos et al., 2007). More recent studies showed that reducing sperm PLC ζ protein by transgenic RNA interference (RNAi) approach significantly perturbed the Ca^{2+} oscillations (Knott et al., 2005), thus not effecting embryonic development. Our approach here is to prevent the overpopulation of this rat by controlling the sperm factor Phospholipase C-zeta (PLC ζ). This study was carried out to detect and isolate PLC ζ from testes of *Rattus rattus diardii* rats using one step RT-PCR technique. This method was chosen base on the ease of preparation and sound qualitative results with every extraction carried out (Kelleher et al., 2001).

Materials and Methods

Animals

Rattus rattus diardii were bought from MARDI Bumbung Lima which traps these rodent pests on a daily basis. The animals also attacked paddy plantations and could be trapped through various escape routes around the bunds surrounding the paddy fields. Once captured, animals were identified, marked and placed in cages and brought back to Universiti Putra Malaysia where they were sacrificed with chloroform overdose.

Isolation of RNA

Testes from *Rattus rattus diardii* were collected and flash-frozen in liquid nitrogen. The testes were then quickly fractured by conventional grinding method and subjected to RNA extraction using easy-BLUE™ Total RNA Extraction Kit. The concentration and purity of the purified RNA were determined by a spectrophotometer.

PLC ζ amplification by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse transcription (RT) and PCR amplification of the target sequence were performed by using QIAGEN One-Step RT-PCR Kit. Both RT and PCR amplification reactions were performed in 50 μ l volume containing 33 μ l RNase-free water, 10.0 μ l 5x QIAGEN One-Step RT-PCR buffer, 2.0 μ l dNTP mix (10mM each dNTP), 1 μ l forward primer, 1 μ l reverse primer, 2.0 μ l QIAGEN One Step RT-PCR Enzyme Mix and 1 μ l template RNA ($\leq 2\mu$ g/reaction). The primers used (forward primer 5'-CGGGATCCATGGCTGACAGTTACCAT-3' and reverse primer 5'-CGGAATTCACCTCGACTTTAGAAT-3') were chosen based on published sequences by Fujimoto et al. (2004). The primers were synthesized by Research Biolabs (Singapore). Amplifications were carried out in the Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) according to the following program: 30 min at 50°C (RT), 15 min at 95°C (initial PCR activation), 34 cycles of 30 sec at 94°C, 45 seconds at 49°C (annealing temperature) and 45 sec at 72°C. A final extension step at 72°C for 4 min was included. The amplification products were separated and analyzed by electrophoresis through 1.5% agarose gel. 100bp DNA ladder (New

England Biolabs) was used as DNA size marker. The gel was stained with ethidium bromide before visualization under UV light using Alpha ImagerTM 2200 (Alpha Innotech, USA).

Gel extraction and sequencing

Gel extraction was performed by using HiYield Gel/PCR DNA Fragments Extraction Kit (Yeastern Biotech, Taiwan). The purified DNA fragments were sent to First-BASE laboratories for further confirmatory assay like sequencing. The sequence result obtained was aligned using NCBI Blast search at www.ncbi.nlm.nih.gov/blast to check for identification.

Results and Discussion

Results of gel electrophoresis as shown in Figure 1 demonstrates the successful amplification of PLC ζ from 3 different *Rattus rattus diardii* testes which showed clear bands while another seven rats showed faded bands (data not shown). At the annealing temperature of 49°C, a single specific bright band was observed at the estimated location of 417-bp. As a housekeeping gene, Actin was chosen to serve as a control for the *Rattus rattus diardii* testes sample and was used to normalize the expression of PLC ζ . Figure 2 illustrates the alignment of the obtained *Rattus rattus diardii* PLC ζ sequence with *Rattus norvegicus* PLC ζ sequence from the NCBI Blast Database. Almost 86% homology was found when PLC ζ sequence from *Rattus rattus diardii* in this study was blasted into the NCBI Blast Database and compared with the *Rattus norvegicus* PLC ζ sequence.

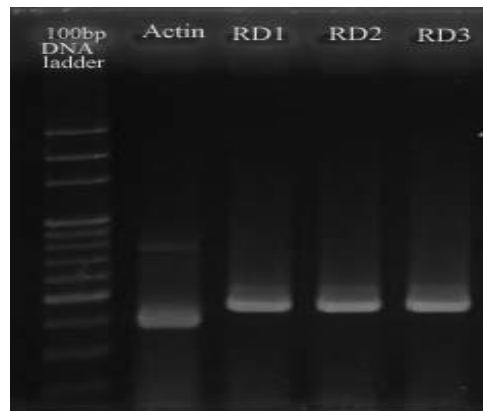


Figure 1: RT-PCR amplification of PLCzeta from *Rattus rattus diardii* testes. First lane- 100bp DNA ladder. Lane Actin- control (housekeeping gene) Lane RD1 to RD3- *Rattus rattus diardii*. Arrow shows 471 bp.

Compared to traditional methods of RNA analysis, the RT-PCR procedure has provided a technique that is more specific and sensitive which becomes the method of choice for studying gene expression since it can be performed with small number of total

RNA. We have chosen One-step RT-PCR rather than 2-step RT-PCR because the accuracy of the two-step method procedure will be affected as the total number of manipulations is greater, thereby increasing the chance of pipetting errors.

NCBI Blast Sequence

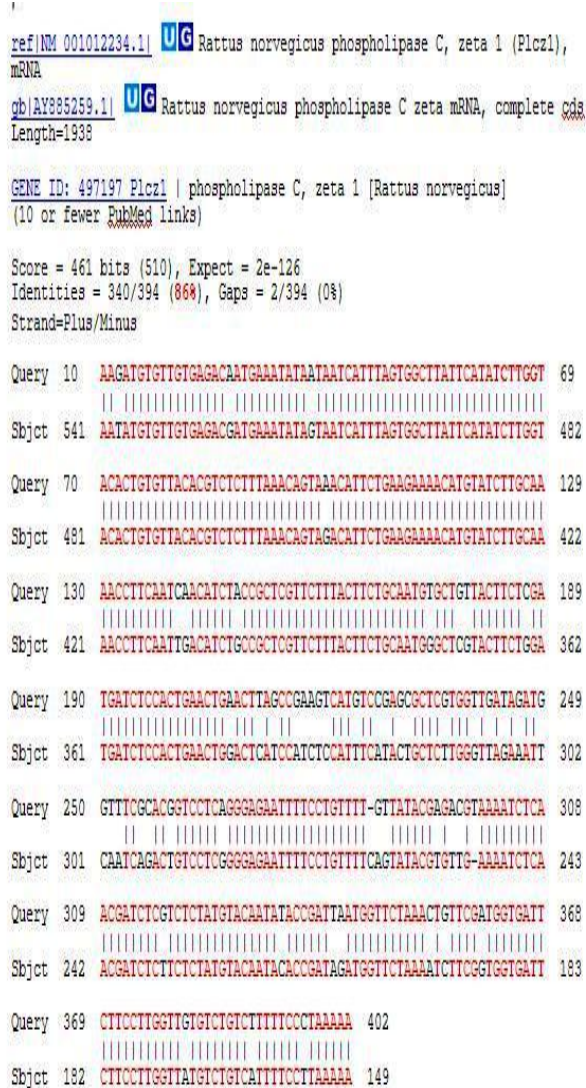


Figure 2: Alignment of the *Rattus rattus diardii* PLCζ sequence with *Rattus norvegicus* PLCζ sequence from NCBI denoting 86% homology. The black nucleotides show the difference in pairing.

In mammals, extensive studies on PLC ζ have been carried out as early as 2002 by Saunders *et al.* (2002), right up to 2012 by Nomikos *et al.* (2012). Nevertheless whether PLC ζ represents the sole Ca²⁺ oscillation-inducing factor in mammalian sperm and how its absence has an impact on male fertility has not been conclusively recognized. Therefore, this study may serve as an initial step for more related research in understanding the molecular properties of PLC ζ and its potential in further development of alternative approaches to control pest population.

Conclusions

PLC ζ from testes of *Rattus rattus diardii* was successfully amplified by using One-step RT-PCR technique. Approximately 86% homology was observed when the *Rattus rattus diardii* PLC ζ sequence in this study was blasted into the NCBI Database and compared with *Rattus norvegicus* PLC ζ . Therefore, the amplified fragments visualized by agarose gel electrophoresis were confirmed to be PLC ζ . The identification of PLC ζ from the testes of house rats, can serve as a platform for the development of contraceptive vaccine in order to control the population of this pest. Lastly, the success of the detection of PLC ζ sequence in the house rat will become useful as basic knowledge in the study of fertilization process and hopefully, more related research will be carried out in order to improve the understanding of the characteristic of PLC ζ in the future.

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