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DETECTION THE PROTEIN INITIATES STARFISH EGG ACTIVATION DURING FERTILIZATION

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Abstract:
The role of an interaction between PLCγ and the proteins during fertilization is still unclear. To recognize whether fertilization includes PLCβ- Ca2+ and PLCγ release mechanisms, GST-SH2 fusion protein was designed and injected into the egg of *Asterina miniata* sp. In this study, the mixture of SH2-pGEMT-Easy vector was combined with the component cell of DH5α, the results proved the success of SH2 domains insertion into SH2-pGEMT-Easy vector using AmPLCγ plasmid. AmPLCγ was treated with EcoR1 to make a GST-SH2 fusion protein. The designed new SH2 primer including EcoR1 and Xho 1 digestion site and clone them into pGEX vector system, could be directly detect the interaction protein with SH2 domain with an easy purification.

Keywords: Fertilization, Starfish , PLCγ, Asterina miniata sp

INTRODUCTION

When fertilized, the eggs experience a free cytoplasty of Ca2 +, which is required to stimulate embryogenesis. In the eggs of starfish, studies consumed inhibitors prepared versus vertebrate proteins have described that this Ca^{2+} increase demands in egg Src family kinase (SFK) that indirectly or directly initializes phospholipase C-gamma (gamma-PLC) to yield IP3, that leads the release of Ca^{2+} from the reticulum of egg's endoplasmic (ER) [reviewed in Semin. Cell Dev. Biol. 12 (2001) 45]. To test in more features the parameters of endogenous in eggs of starfish that are demanded for the release of Ca^{2+} at fertilization, an oocyte cDNA encoding gamma-PLC was separated from the starfish Asterina miniata.

This cDNA, specified Am-gamma PLC, encodes a protein with 49% identity to mammalian gamma-PLC1. A 58-kDa Src family kinase reacted with recombinant Am-gamma PLC Src homology 2 (SH2) scopes in a particular, the responsive manner for fertilization. Sea urchin egg gamma-PLC immunosuppressively directed to an antimonopoly directed toward am-gamma PLC discovered gamma-PLC fertilisator-dependent gamma-PLC phosphorylation. Injecting the eggs of starfish with the tandem SH2 scopes of Am- gamma PLC (which prevents of the activation of gamma-PLC) specifically prevented release of Ca^{2+} at fertilization. These results refers that an endogenous the egg of starfish gamma-PLC activates with a SFK egg and intercede release of Ca^{2+} at fertilization via a gamma-PLC SH2 scope-intercede technique.

The gamma-PLC in fertilization has been indispensable over the years of researches. The fertilization and the insight interactions of the human beings, plants and animals started from the beginning of human life. Cell signal transduction refers to cells during intracellular receptors or the cell membrane feelings informational molecules stimulation by intracellular

signal transduction system conversion, thereby affecting cell biological learning function of the process. (Ramadan et al., 2012; Shimohama et al., 1995; Park et al., 1994).

During fertilization the sperm triggers a chain of activities in the egg to cause mitosis and development. Besides the sperm delivers its gene, the egg also needs a signal to start the development (K Swann et al. 2004). The significant observed signal in the egg during fertilization is the increased concentration of calcium in egg cytoplasm (Stricker 1999). The activation of egg keeps still if no changed concentration of calcium in the egg while the sperm fusing with the egg (Kline JT & Kline D1992). Hence, the increased calcium signal in the egg is the key element during the beginning of fertilization. The initial calcium rise from egg's endoplasmic reticulum is caused by inositol 1,4,5- trisphosphate (IP3) that is the construction of phospholipase Cγ (Ian K. Towny et al. 2009).

Phospholipase Cγ is the enzyme that hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5- bisphosphate (PIP_2), to produce inositol 1,4,5- trisphosphate (IP3) and diacylglycerol (DAG). Phospholipase Cγ contains many domains from C terminal to N terminal - PH domain, two SH2 domain, domain of X catalytic, SH3 domain, domain of Y catalytic and domain of C2. When growth factor binds to the receptor, it triggers autophosphorylation of the receptor on tyrosine residue, which has interaction with NH_2^+-terminal SH2 domain. This action leads $COOH^-$-terminal SH2 domain and PH domain to companion with the membrane and start to hydrolyze PIP_2 [1].

Aspect of PLC gamma in fertilization
During the fertilization, the sperm initializes an egg to reenter the cycle of cell and forming a new zygote. An intracellular calcium that release from the endoplasmic reticulum can induce egg activation. PLC molecules able to detect the fertilization-calcium association in the new generation via IP3 [2] . The Ca^{2+} important in egg activation was released between 1920s and 1930s. Whereas, the study about molecular signaling pathway that involved in sperm and egg fusion is continuous until today[3] Study the regulation of IP3 receptor and calcium signaling has important role on cell function such as concentration of smooth muscles, fertilization, immune response and gene expression[4]. The interaction of molecular mechanisms help to create different calcium signaling that mediated by IP3[5].

The levels of intracellular calcium help to regulate IP3R by store the depletion, conformational changes in IP3 receptor. Also, this interaction can regulate some various signals molecules that effect many physiological processes[6].

METHODS AND MATERIALS

Bioinformatics
To design the SH2 primer, Am PLCγ full line cDNA sequence and SH2 domian were obtained from NCBI . To design SH2 forward primer-5'-ACCCGGAACTCTACATGGAC-3' and Am PLCγ SH2 reverse primer-5'-CTAGTAG CGTTGACTCGGGG-3'.

pGEMT-Easy vector sequence that contain EcoR1 restriction site SH2 domain and the primer aattcgatt acccggaac tctacatgga cccaaaccag tttgtaccaa aggtgacagt gaaagccctg tatgactaca gagc ccaacg agacgatgag ttgacattct gcaagcacgc catcatcacc aatgtagata agcaagacct tggctggtgg agoga gact acggcggcaa gaagaacatg tggttcccct ccaactacgt ggaagagacc caaccaaatg acaacagccc cgagtcaacg ctactag aatcactagtg were downloaded. To choose pGEX6p-1 as the subclone

vector, pGEX6p-1, pGEX6p-2, and pGEX6p-3 cDAN sequence were downloaded from NCBI using ORF Finder [7].

Cell culture and Plasmid Extraction

In this research, two ways of plasmid extraction were used: QIAGEN kit (Qiagen,MD,USA and traditional method . The quality of DNA was assessed via agarose gel electrophoresis. While the yield and purity of extracted gDNA was assessed using a NanoDrop spectrophotometer (NanoDropND-1000) recording nucleic acid content at 260 nm, protein contamination at 280 nm and various contaminants at 230 nm. The ratios (260:280 and 260:230) give an indication of purity with values as close to 2.0 as possible.

Plasmid DNA was resumed from overnight cultures using a plasmid miniprep extraction kit (Qiagen, UK) according to the manufacturer's guidelines . Plasmid PLCγ was purified using QIAGEN plasmid miniprep kit. A single colony of *E.coli* was grown with AMP on LB broth (10 ml) at 37°C while being shaken 200 rpm overnight. The culture was centrifuged (8000 rpm; 3 min; 25°C) [7].

PCR and Gel electrophoresis

The full length of Am PLCγ cDNA was used as a template and the bioinformatics method was used to design SH2 primers. Am PLCγ SH2 forward primer is 5'-ACCCGG AACTCTACATGGAC-3' and Am PLCγ SH2 reverse primer is 5'-CTAGTAGCGTTGACTCG GGG-3'. The DNA template was amplified using the full length of Am PLCγ. PCR Mastermix, two primers and ddH_2O in the PCR tube. Run 25 cycles with annealing temperate 58℃ and then run 1% gel to confirm the PCR product .The DNA sample was loaded on 0.1 agarose with ethidium bromide and ran for 1 hour at 125 volt [7].

Transformation

The mixture of SH2-pGEMT-Easy vector was combined with the component cell of DH5α (1:50) and incubated in ice for 45 min and then heat shock at 35 ℃ bath water for 45 seconds. Then it incubated on the ice for 2 min and then shake with S.O.C medium for 1 hour [7].

X-gal blue-white selection

Spread 100ul 20mg/ml X-gal and 40ul 100mM IPTG on each pre-made agar plate and then wait 30 minutes until the plates dry. Pour each transformation tube on two plates for 400ul and 600ul. Incubate these plates at 37℃ overnight [7].

Restriction Digestion and Ligation

The plasmid was treated with EcoR1 at 37 ℃ for 1 hour. For ligation SH2 fragment , pGEX6p-1, and pGEMT-Easy vector were mixed (3:1) with T4 DNA ligase at 4 ℃ overnight. Then the plasmid was extracted and digested using the selected enzyme and analysed by agarose gel (0.7%) electrophoresis to confirm its integrity [7].

Gel Purification

pGEX6p-1 plasmid and SH2 domain were run in 0.7 % gel electrophoresis; pGEMT-Easy plasmid treated with EcoR1. pGEX6p-1 and SH2 domains gels were cut into two individuals tubes. QIAGEN kit was used for purification. Two tubes were weighted and filled with QG buffer (3:1). Vortex and heat were used until the gel dissolved. Isopropanol was used and

centrifuge for 1 minute at 10000rpm. PE buffer was used to wash the sample and re-centrifuge. EB buffer was added to elute the DNA into new tube [7].

Dephosphorylation

pGEX6p-1 was incubated with 5 µl of 0.01u/µl CIAP at 37℃ for half hour. The CIAP was re-added and incubated at 37 ℃ for 30 minutes. To stop the reaction, stop buffer (200mM NaCl, 1mM EDTA, 10mM Tris-HCL ,and 0.5% SDS) and CIAP were added. . To remove the supernatant into the new tube, phenol:chloroform:isoamyl alcohol were added at ratios 25:24:1, respectively. Ammonium acetate and 100% ethanol were used to wash it and then dry the pellet. Add EB buffer to save the sample[7].

RESULTS AND DISCUSSION

The Sperm at fertilization starts a spreading increase of Ca2 + in the cell, that is focal to the production of the Cell [8] . In echinoderm as well as the eggs of vertebrate, the increase in outcomes of Ca^{2+}, at minimal in a huge part for the release of Ca^{2+} from the response of endoplasmic reticulum to an increase in inositol trisphosphate IP3 [9] . However, the description of how the IP3 is produced at fertilization is not mentioned.

The phosphatidylinositol bisphosphate (PIP2) generate IP3, by the work of a phospholipase C [10]. This enzyme's family contains δ, γ and β isoforms. The initialization of PLCβ is activated by G proteins, while the initialization of PLCγ is activated by tyrosine kinases. PLCδ regulation is badly grasp, although the initialization for enzymatic of all 3 PLC isoforms can be stimulated by a raise in Ca^{2+} [11]. Very probably the production of IP3 at the findings of fertilization from the initialization of one of these isoforms of phospholipase C.

Both PLCγ and PLCβ path ways are offer in eggs. Exogenous receptors known to release Ca2+ by a G protein/PLCβ pathway like Expression in mammalian, frog and starfish eggs, and allows the release of Ca^{2+} in eggs when the corresponding agonists are used like muscarinic m1or serotonin 2c receptors [11]. This refers that corresponding G proteins and functional PLCβ are offer. Likewise, expression in frog eggs and starfish of exogenous receptors recognized to release Ca^{2+} by a PLCγ/ tyrosine kinase pathway, like receptors for PDGF or EGF, allows release of Ca^{2+} to these agonists response [12]. The receptors of point-mutated that do not activate PLCγ do not cause release of Ca^{2+} [12]. These results refers that a PLCγ functional is offer. In such experiments the existence of PLCγ has been described by immunoblotting but have not been implemented in mammalian eggs [13]. In this research, SH2 domain of phospholipase Cγ showed interaction with a specific protein (unidentified) during the early stage of fertilization and calcium release. SH2 domain localization was detected through cloning SH2 into cloning pGEX6p-1 vector to make a GST-SH2 fusion protein. The fusion protein has ability to improve the expression and the solubility of the target protein. GST fusion protein is a high-quality purification protein with few protease cleavage sites [14].

Furthermore, maltose binding protein (MBP)-SH2 fusion protein could be used to promote the solubility of SH2 domain or GFP-SH2 fusion protein to detect the position of SH2 domain in the cell [15].

Design SH2 of PLCγ PCR primer

SH2 primer was used to amplify SH2 domain of AmPLCγ . Figure 1- presented the full-length of AmPLCγ cDNA sequences using the NCBI primer design, the AmPLCγ sequence

was used to design forward and reverse SH2 primers using primer design program: 5'-ACCCGGAACTCTACATGGAC-3'F and 5'-CTAGTAG CGTTGACTCGGGG-3'R.

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ORIGIN
        1 cttcagaatg gccaccaaca gcctctacaa gaagaagctg acgccccagg aggtggccag
       61 cgtcaccaag atgctgaaaa tgggcaccgt cctgacgcgc ttctacggca aacgacgacc
      121 ggaaaggagg tcgttcgaaa tctgcatgga gacgcggcag atactgtgga ggcgacagac
      181 tgggcggaca gacggagcag ttaaaattcg tgagataaaa gagattcgtc ccggtaagaa
      241 ctcacgagac ttcgagaggt ggccggatga agccaagaag tatgatacct cgctctgtct
      301 tgtcatatgc tacggtgccg agttcagact caagagcttg tccgtcgttg ccggcaatgc
      361 cgatgaacga cacaagtgga tcgtcggcct caactggcta gtggaagacc ataaaatctc
      421 aagttaccca agcagactag aatggtggtt acgacgggag ttctacgcca tggggaaaac
      481 aaagaatgat acggtgtcac ttagggacat gaagtcattc atgccatacg tcaacctgaa
      541 aatgaacaca aaggacctta aagaatattt caatgaagtg gatcggtgga acaagcaaga
      601 aattggattt gacggctttg tccagctcta ccacaacctc atcttccaga gggaggtcgc
      661 tgacagattc aaagagtaca tcgatgaacg aaatctggtg actgtgaatg gcatgattcg
      721 cttcctagcc caagaacaaa aggacaccac ggcaaacaat cctatcgcag tgaaggccat
      781 gatggaatcg tttctgacgg acctcggccg accgtgccag gagtcggacc ccaaattcac
      841 agtcccagag tttctgctgt acttgttctc gccggataat gagatctggg acaagaaatt
      901 tgatgagatt gtggacgact tggaccaacc gctctgtaac tacttgatag cttcctctca
      961 caatacatac cttaccgggg atcagatcgc tagtgagtca tcgtgtgagg cgtacgtccg
     1021 ttgtttacgc atgggctgcc gatgtctgga acttgactgt tggggacggg cggaaggaat
     1081 gcccattatc taccatggcc tgacactgac ttcaaaaatc aaattcatgg acgttttgaa
     1141 gacgataaag gagcacgctt gggtgcagtc agatttgccc atcattttat caatcgagaa
     1201 ccactgcaca ctgatacagc aacgcaacat ggcctcatca ttccaggaag tctttggaga
     1261 ctccctttg acgcagccag tggatcgcga tgccaccata ctgccgacgg tcaaccagct
     1321 aagaaagaag atcatactca agcacaagaa gctgatgggc accaatgaaa cctttagcgt
     1381 acccactgaa gaaatgtctg gtcttgattt gaggaactct ctgaagaacg gaatcctcaa
     1441 gatcgaagac cccatggata atgaatgggt cccacattac tttgtgctga ccacggagaa
     1501 gctcttctac tgcgagcaaa cccaaaacct tgtcaatcaa gatgacgatg acgacacgac
     1561 tagccaattg gacatgcaag ccacgcccaa tgacgagctg cacttctccg agccctggtt
     1621 ccacggcaag ctgaacagca acagcgacgt cacgcccagg atgcttgccg agcagctgct
     1681 caaccaatac cagaagggcg acggcacttt cctggcccga gagagcgaaa ctttcaaggg
     1741 ggactactcc ttgtcattct gggcgcgagg caaggtgaac cactgccgca tccggtacaa
     1801 gctagaccag agccggccca agtacttctt ggtggagcac acgtgcttcg atagtctcta
     1861 cagcctcatc tcccactacc gccagtgccc cttgcgtagc cgggggctcg agctcctact
     1921 gacggagccc gtgccacaac ccctcagcca cgagggaaaa gattggtttc ataagaagtt
     1981 aagccggcct caggcagagg aaatgctgaa acgagtccac caggatggct cgttcctggt
     2041 caggaagaga gagcagggag acgactccta cgccatatca ttcagggcgg agggtaagat
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Figure 1: cDNA sequence of AmPLCγ and SH2 domain. The full length of AmPLCγ was obtained from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/AY486068).

Amplify SH2 domain of AmPLCγ

DH5α cells were grown overnight on LB broth, and the full length of AmPLCγ plasmid was used as PCR template. The result shows that the size of AmPLCγ plasmid is 3816 bp (Figure 2).

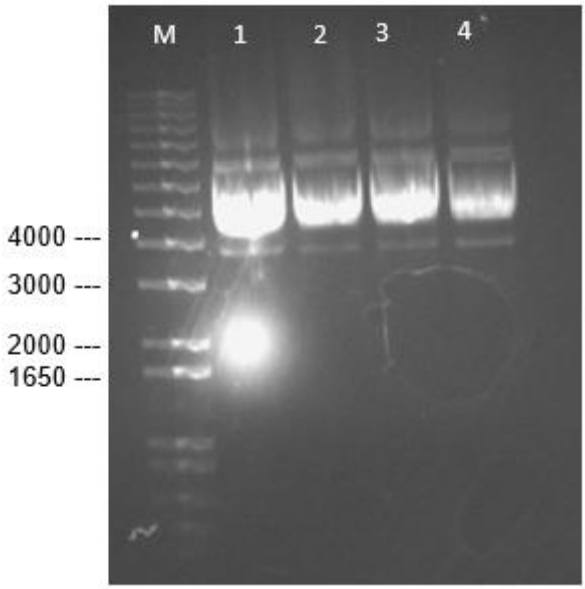

Figure 2: Agarose gel (0.7%) electrophoresis of the PCR product of AmPLCγ plasmid (3816 bp) on 125 volt for 1 hour. Line M: DNA marker (1kb); lines 1- 4 PCR- positive results. Continuously, in figure 3 the size of SH2 domain product was 199bp.

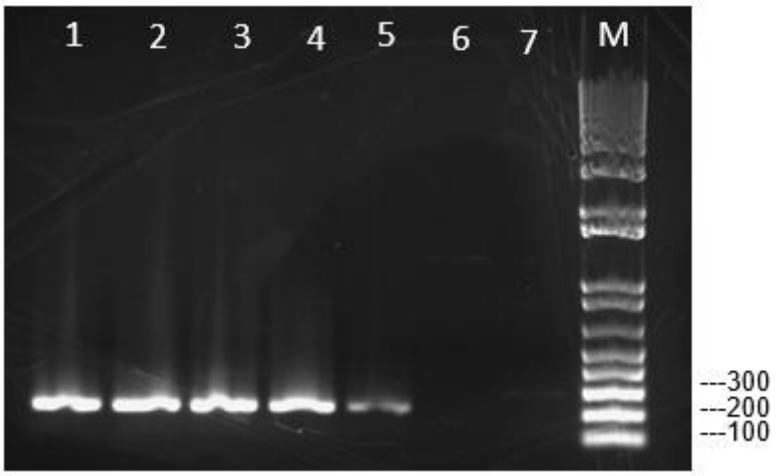

Figure 3: Agarose gel (0.1%) electrophoresis of the PCR product of SH2 product by AmPLCγ plasmid (199 bp) on 125 volt for 1 hour. Line M: DNA marker (1kb); lines 1-4 are samples . line 5 without template DNA(control1); line 6 without forward SH2 primer (control 2); line 7 without reverse SH2 primer (control 3). 2 µl of DNA template, 12.5 µl PCR master Mix, and 2 µl if each primer with dH_2O to total 25 volume. The annealing temperature was set to 58 ºC and run 25 cycles. 20 µl of DNA and 4 µl loading dye were loaded per each well.

Clone PCR product to pGEMT -Easy vector

To confirm the digestion of EcoR1 at GAATTC two sites of SH2 domain using pGEMT-Easy vector, T4 DNA ligase kit (Promega,UK) was used. SH2 domain was inserted into SH2-pGEMT-Easy vector at 4ºC. SH2- pGEMT-Easy vector was transformed to DH5α cell (1:50). pGEMT-Easy contains *lacZ* that use in X-gal blue-white selection to detect bacterial plasmid and SH2 domain. Overnight incubation (white clones), and plasmid DNA extraction using EcoR1 and run on 1% agar gel. Figure 4 presented only line2 and line9 contain SH2 domain. Hence, save No.2 and No.9 tubes to do sub-clone.

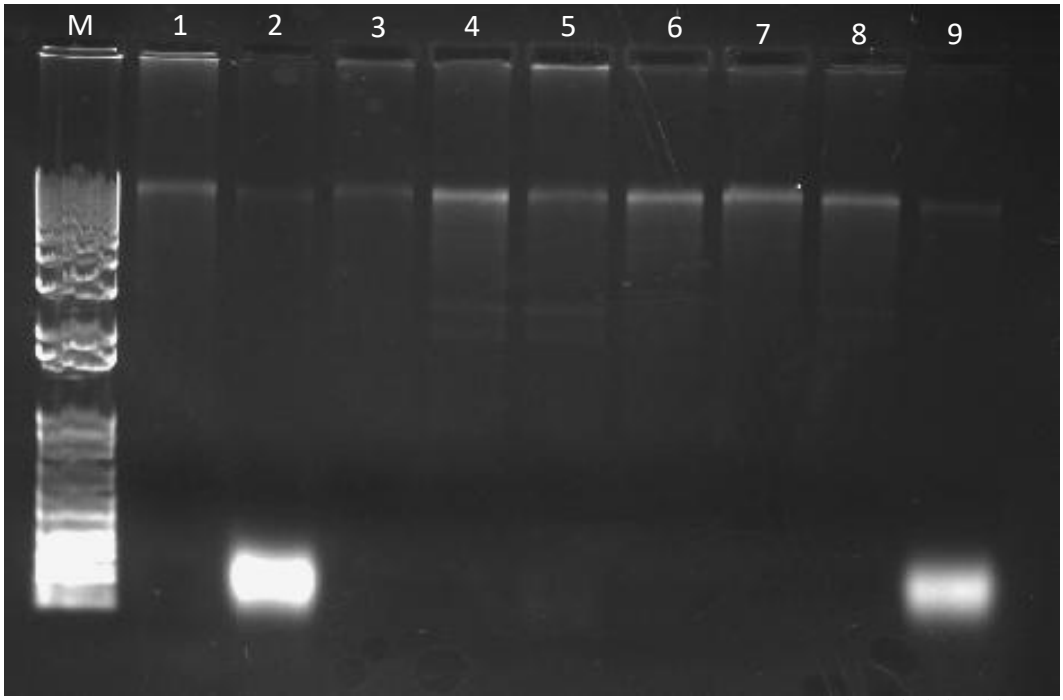

Figure 4: Agarose gel (1%) electrophoresis of gel of SH2-pGEMT-Easy plasmid DNA digestion. Mix 3 µl of plasmid DNA, 2 µl of 10X buffer, 1 µl EcoR1 and 14 µl dH_2O at 37 ºC for 1 hour. Load 20 µl DNA and 4 µl dye per well .

Sub-clone SH2 domain in pGEMT-Easy into pGEX6p-1 vector

pGEX6p-1 plasmid and SH2-pGEMT-Easy plasmid (tube No. 2 and 9)were treated with EcoR1. Then 0.7% agar gel was used to run SH2 domain and pGEX6p-1.; after that pGEX6p-1 was treated with phosphatase. T4 DNA ligase was used to insert SH2 domain into pGEX6p-1 at 4 ºC overnight. To confirm the transformation, the plasmid DNA was extracted and digested using these enzymes and analysed by agarose gel electrophoresis to confirm its integrity. Figure 5 does not show no SH2 domain in pGEX6p-1 vector.

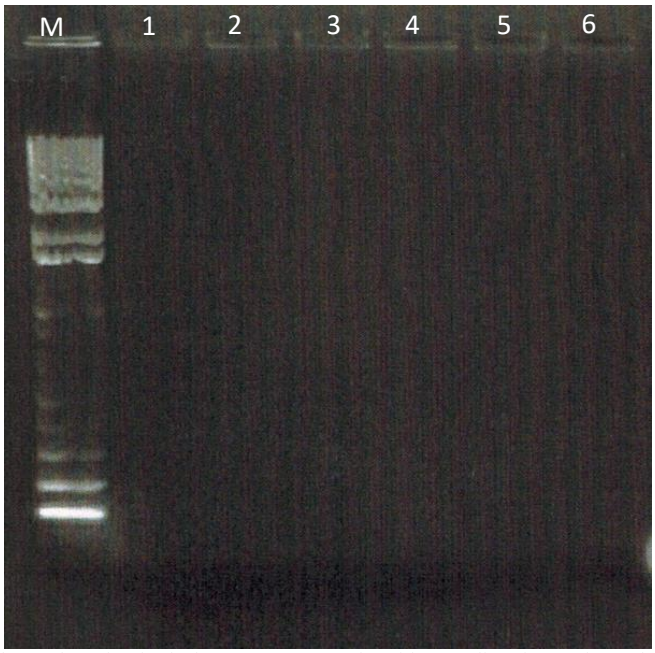

Figure 5: Agarose gel (1%) electrophoresis of gel of SH2-pGEX6p-1 plasmid DNA digestion. Mix 3 µl of plasmid DNA, 2 µl of 10X buffer, 1 µl EcoR1 and 14 µl dH_2O at 37 ºC for 1 hour. Load 20 µl DNA and 4 µl dye per well.

Re-pick clone from X-gal blue-white choice plate

Due to the negative results of the earlier experiment, 12 clones were picked from X-gal blue-white selection plate and grown in LB broth overnight. The SH2-pGEMT-Easy plasmid DNA was extracted and treated with EcoR1. Gel electrophoresis of purified plasmid gave many bands without pointed to SH2 domain (Fig.6). Due to the low efficiency of EcoR1, PCR was used to examine the presence of SH2 domain in the SH2-pGEMT-Easy plasmid. SH3-pGEMT-Easy plasmid was used as a template for SH2-pGEMT-Easy plasmid to run PCR. The results proved the success insertion of SH2 domains into The SH2-pGEMT-Easy vectors (Fig. 7).

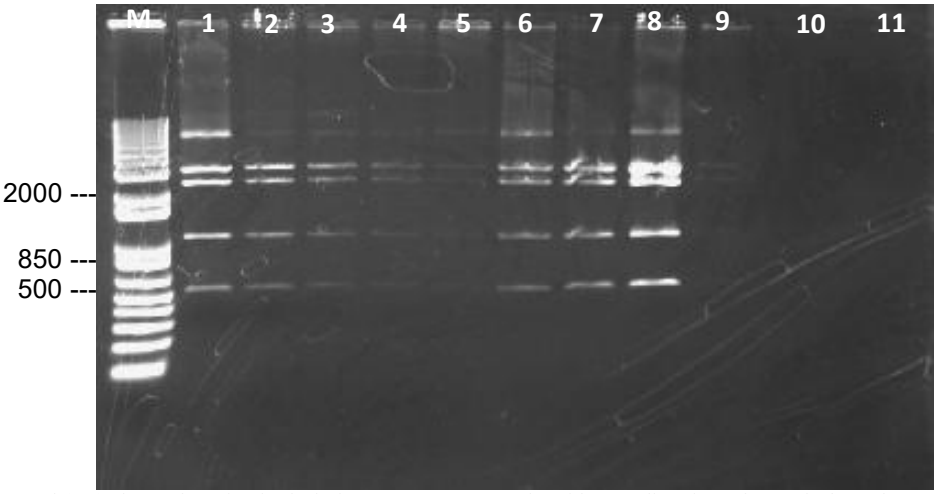

Figure. 6: Agarose gel (1%) electrophoresis of gel of of SH2-pGEMT-Easy plasmid DNA digestion. The result showed many bands without pointed to SH2 domain. Mix plasmid 3µl plasmid DNA, 2µl 10X buffer, 1µl EcoR1 and 14µl dH_2O and incubate 37℃ for 1 hour.

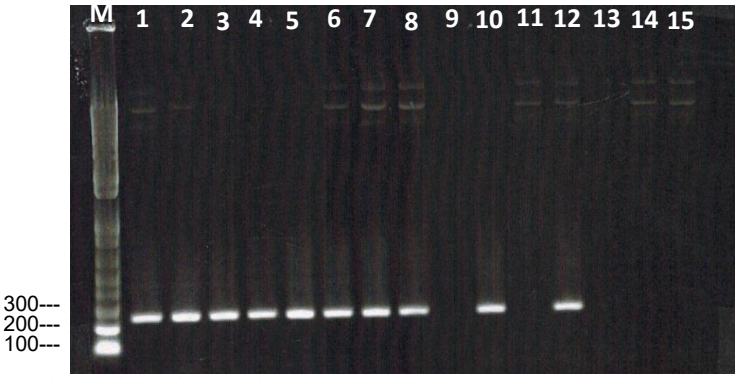

Figure. 7: Agarose gel (1%) electrophoresis of gel of SH2 domain product by PCR using SH2-pGEMT-Easy template.

The forward SH2 primer is 5'-ACCCGGA ACTCTACATG GAC-3' and the reverse SH2 primer is 5'-CTAGTAGCGTTGACTC GGGG-3'. 1 µl of DNA template, 12.5 µl PCR Master Mix, and 2 µl if each primer with dH_2O to total 25 volume. The annealing temperature was set to 58 ºC and run 25 cycles. 20 µl of DNA and 4 µl loading dye were loaded per each well. The gelelectrophpresis was run at 125 volt for 1 hour. Lines 1-12 are samples; line 13 without DNA template(control 1); line 14 without forward SH2 primer (control 2); and line 15 without reverse SH2 primer (control 3).

The insertion of SH2 domain into pGEMT-Easy vector was amplified using SH2-pGEMT-Easy plasmid DNA (Fig.7). This positive result proved the frustration of SH2 digestion with EcoR1 . The main goal of this process is to create GST-SH2 fusion protein and design a new SH2 primer including EcoR1 and Xho 1 digestion site and clone them into pGEX vector system. Furthermore, the GST-SH2 fusion protein could be directly detect the interaction protein with SH2 domain with an easy purification.

REFERENCES

[1] Wood, E.R., et al., A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. Cancer research, 2004. 64(18): p. 6652-6659.
[2] Tokmakov, A.A., et al., Calcium signaling and meiotic exit at fertilization in Xenopus egg. International journal of molecular sciences, 2014. 15(10): p. 18659-18676.

[3] Swann, K. and F.A. Lai, Egg activation at fertilization by a soluble sperm protein. Physiological Reviews, 2015. 96(1): p. 127-149.
[4] Mikoshiba, K., IP3 receptor/Ca2+ channel: from discovery to new signaling concepts. Journal of neurochemistry, 2007. 102(5): p. 1426-1446.
[5] Pchitskaya, E., E. Popugaeva, and I. Bezprozvanny, Calcium signaling and molecular mechanisms underlying neurodegenerative diseases. Cell calcium, 2018. 70: p. 87-94.
[6] Liao, Y., et al., Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. Proceedings of the National Academy of Sciences, 2008. 105(8): p. 2895-2900.
[7] Runft, L.L., et al., Identification of a starfish egg PLC-γ that regulates Ca2+ release at fertilization. Developmental biology, 2004. 269(1): p. 220-236.
[8] Coward, K., et al., Phospholipase Cζ, the trigger of egg activation in mammals, is present in a non-mammalian species. Reproduction, 2005. 130(2): p. 157-163.
[9] Jaffe, L.A., et al. Ca2+ signalling during fertilization of echinoderm eggs. in Seminars in cell & developmental biology. 2001. Elsevier.
[10] Liao, F., H. Shin, and S. Rhee, In vitro tyrosine phosphorylation of PLC-γ1 and PLC-γ2 by src-family protein tyrosine kinases. Biochemical and biophysical research communications, 1993. 191(3): p. 1028-1033.
[11] Guder, W.G. and B.D. Ross, Enzyme distribution along the nephron. Kidney international, 1984. 26(2): p. 101-111.
[12] Shilling, F.M., et al., Evidence for both tyrosine kinase and G-protein-coupled pathways leading to starfish egg activation. Developmental biology, 1994. 162(2): p. 590-599.
[13] Fernández, A.M., et al., Functional inactivation of the IGF-I and insulin receptors in skeletal muscle causes type 2 diabetes. Genes & development, 2001. 15(15): p. 1926-1934.
[14] De Marco, A., Two-step metal affinity purification of double-tagged (NusA–His 6) fusion proteins. Nature protocols, 2006. 1(3): p. 1538.
[15] Diakonova, M., et al., SH2-Bβ is a Rac-binding protein that regulates cell motility. Journal of Biological Chemistry, 2002. 277(12): p. 10669-10677.