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# Generation of a Novel high-Affinity Monoclonal Antibody on Luciferase from Lampyris Turkestanicus

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ABSTRACT

Firefly Lampyris turkestanicus is a widely used reporter enzyme since it catalyzes a bioluminescent reaction that can be directly monitored. Luciferase plays a crucial role in tumour imaging and metastasis detection.We harvested a series of monoclonal antibodies in order to apply in different laboratory detection systems like ELISA, western blotting and immunocytochemistry (ICC). To reach this goal, anti-luciferase mAb was harvested from the M2G11 hybridoma line generated through the fusion of mouse myeloma cells and splenocytes of Balb/c mouse which was immunized with firefly luciferase. In order to obtain proper amounts of purified mAb , hybridoma cells were injected intraperitoneally to some mice. The ascitic fluid was collected after two weeks and was passed through the protein G Sepharose affinity column. Specificity of mAb was confirmed by ELISA and western blotting. Enzyme, activity was measured by luminescence assay in presence and absence of mAb . which confirms luciferase activity in the presence of Mab.



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## INTRODUCTION

Luciferases are the enzymes that catalyze the light emitting reaction in bioluminescence organisms. FFL(Firefly luciferase) catalyzes the oxidation of luciferin in the presence of ATP, Mg2+ and molecular oxygen to produce light, oxyluciferin, CO2 and AMP [1]. Like other firefly luciferases Lampyris turkestanicus,the Iranian firefly luciferase, is a 62 kDa-monomer enzyme [2].

The scope of its applications include: the ultra-sensitive detection of ATP [3,4]; the detection of phosphatase activity (5); acting as a reporter gene in molecular biology (6,7)6. S.J. Gould and S. Subramani. Anal. Biochem. 175 (1988), p. 5. Abstract | View Record in Scopus | Cited By in Scopus (1; sequencing of the DNA (8); it is also a tool for monitoring in vivo protein folding and chaperonin activity [9]. The ultra-sensitive ATP detection property of this enzyme has led to its application in microorganisms' detection [10,11] , to estimate cell viability (12), to detect refined petroleum products in cell culture bioassays [13] and represents the major use of FFL in industrial, defense and public health applications [14].

Monoclonal Antibody (mAb) Production technology was developed in 1975 by kohler and Milstain [15]. Since its development, it has been very essential in the modern medical science in the diagnosis and therapy( [16], such as infection, cancer, research and even basic science (4).Antibodies are the main tools utilized by many researchers. [3].

MAbs recognizing FFL(Firefly luciferase) specificly were developed by obtaining hybridoma cell lines from the immunized animals injected with the firefly luciferase protein . These lines are produced by fusing B cells from the immunized animal with myeloma cells [17].

These molecules can be applied in studying the structure of FFLs and detection and comparison of its different variants found so far or completely novel FFLS. Addition This mab can be used for detection of luciferase on ELISA, western blot, IHC,IFC and... also, we can use it for detection inactive luciferase , luciferase antibody staining kits for detection of cells which expressed luciferase.

The kinetic ans structural properties of different species of firefly luciferases have been found different due to alteration in their 3-dimentional structures. The main goal of this work was production of mAbs recognizing FFL from Lampyris turkestanicus efficiently and specifically.

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*Methods and Matherial:*

*Prepration of luciferase:*

A single colony of transformed E.coli strain $BL_{21}$ with pET16b plasmid containing firefly luciferase was grown in LB medium and purified by Ni-Sepharose affinity chromatography, as reported earlier [18]. After that, concentration of luciferase was assessed by Bradford assay and BSA was used as standard sample. purified luciferase protein was utilized for electrophoresis on a 12.5% polyacrylamide gel , as was described by Laemmli(19). Enzymatic activity was examined by a luminometer(Berthld detection system). The activity was derived from the strength of the emitted light.

*Immunization:*

We immunized 5 balb/c mice with 50µg highly purified luciferase protein that was prepared for injection by mixing with complete fraunds adjuvant . Mice were immunized every 4 weeks. Next, titer of serum for antibody by ELISA method was measured. Blood samples were obtained from mice to measure the serum antibodies. mouse which had high antibody titer ,were boosted by injecting antigen without adjuvant intraperitoneally or intravenously(via the tail veins)3 days before the fusion but two weeks after the previous immunization.

*Fusion:*

Spleen cells of the immunized mouse were fused at 5:1 ratio with the previously prepared myeloma cells respectively using standard techniques (kohler and Milstein, 1975) by means of poly ethylene glycol 1500 (PEG). Hybridomas were distributed among 96 wells plates containing RPMI-1640 supplemented with 20% FBS with 1x HAT, containe of glutamine, penicillin at 100 U/ml, and streptomycin at 100 µg/ml antibiotic and feeder cells which were derived from saline peritoneal washes of mouse [20].

Supernatants were assayed by ELISA. Candidate clones were isolated by limiting dilution in RPMI-1640 containing 20% FBS .the single clones were screened by ELISA.

*ELISA Test:*

Indirect ELISA: Microtiter plate were coated with 1µg/ml(100ng/well) of purified luciferase and left overnight at 4 ℃ .Uncoated surfaces were blocked with 1% albumin in phosphate buffer saline (PBS) pH 7.4 for 1 h at 37℃. Sera or supernatant at appropriate dilution in PBS containing 0.1% albumin and 0.05% tween20 were then included and incubated for 1h at 37℃. After having been washed for three times, bound antibodies were detected by incubation with horseradish peroxidase anti-mouse Ig-G dilution 1/2000 and TMB as substrate .Colour enrichment was stopped with 100µl of 2M $H_2SO_4$ and the absorbence was read at 450nm.

Competitive ELISA: In this experiment a specific concentration of luciferase (1 µg/ml) was coated in microtiter plates and then a mixture of $M_2G_{11}$(with a definite concentration) and luciferase with varying concentration (15ng-500ng) were added into each well.

*Cloning of hybridoma:*

We employed limiting dilution method for cloning of hybridoma. For this purpose, when a positively screened well was approximately 75% confluent, we resuspended the cells with a pipet very gently, and moved them to a single well in a 24-well plate and fed them. After three dayes of healthy growth , we softly resuspended it again and removed nearly half of the cells for cloning. Cell count and viability was checked prior to cloning. We have done several dilutions to attain one cell per well statiscally. We repeated this work three times to be sure of the monoclonality of hybridoma.

Three mice were prepared and were injected 0.5 ml pristan [2,6,10,14] tetramethylpentadecane) intraperitoneally .Then after 7 days ,$1\times10^6$ hybridoma cells in 0.5 ml PBS , were injected to per mouse. About 16 days later, abdomen of mice enlarged. With use of 18 gage needles, their ascetic fluid was harvested and centrifuged at 12000 rpm for 5min,supernatant was collected for purification consequently.

*Monoclonal antibody purification:*

Packed protein G sepharose column was equilibrated with 10 column volumes of PBS buffer (ph=6.5) and passed 0.5 ml ascite that was diluted with 0.5 ml PBS and filtered on the column. The column was washed with 10 column volumes of PBS(PH=6.5) and then eluted with 0.2 M glycine(PH=2.5) to collect the Ig fraction. The protein concentrations were determined by measuring the absorbance at 280nm, and proteins were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis.

*Western blot analysis:*

10 µg of luciferase solubilized with 10µl of 5 mM tris-HCl buffer,pH 6.8, containing 5% SDS, 25% glycerol, 0.1% bromophenol blue. The sample was loaded on 12.5% acrylamid gel. Following electrophoresis, proteins were electroblotted onto a nitrocellulose membrane using the procedure described by Towbin(21) and the membrane was subsequently blocked with 3% BSA in PBS. The membrane was incubated with antibody

(diluted 1/4000 in PBS) followed by washing and incubating with anti mouse HRP-Ig (22). The binding of anti-luciferase monoclonal antibody to luciferase incubated the membrane with 0.5 mg/ml of diaminobenzidine containing 0.05% hydrogen peroxide.

*Results:*

*Purification of luciferase:*

After purification, the total luciferase extracted from 200 ml cell culture media was 3-4 mg.

SDS-PAGE analysis and activity of the eluted fractions showed that the enzyme was efficiently purified and that the corresponding fractions contained highly purified protein. (fig.1).

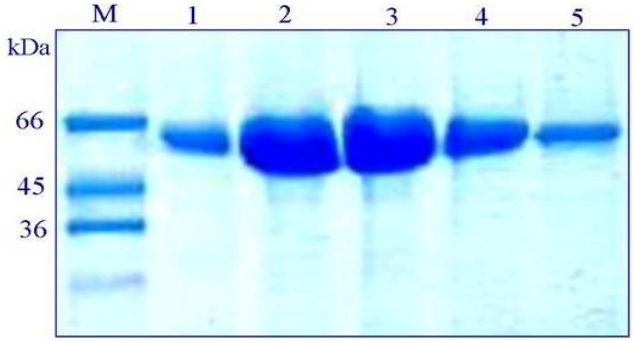


**Fig. 1:** SDS-PAGE gel electrophoresis of different fractions from Ni–Sepharose column. (M) Molecular weight marker; (1–4) fractions were obtained upon elution.

*Screening of hybridoma:*

After spending 15 days of fusion, the supernatants from 96-wells plates containing colonies were chosen for screening. The supernatants tested for the presence of anti-luciferase antibodies using luciferase coated on the microtiter plate and a total of 56 wells were identified with diverse dimensions of reactivity. An antibody-producing clone was further selected by limiting dilution, and supernatants were analyzed by ELISA . We dubbed this hybridoma $M_2G_{11}$.

*Purification of $M_2G_{11}$:*

The monoclonal antibodies were purified from the pooled ascites of hybridoma cells by protein G Sepharose column chromatography. The purification of the monoclonal antibody was investigated by 12.5% SDS-PAGE (under reducing condition). As shown in fig2, two bands corresponding to the heavy (about 50kd) and light chains (about 23 kd) of immunoglobulin were observed.

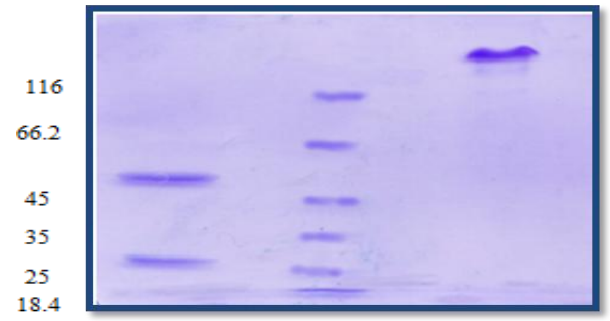


**Fig. 2:** SDS-PAGE of $M_2G_{11}$ in reducing condition in presence of mercaptoethanol, Marker, $M_2G_{11}$ in non-reducing condition respectively left to right.

*ELISA:*

Indirect ELISA: In this experiment a variation concentrations of luciferase was coated in microtiter plates and then $M_2G_{11}$(with a definite concentration) was added into each well(fig3).

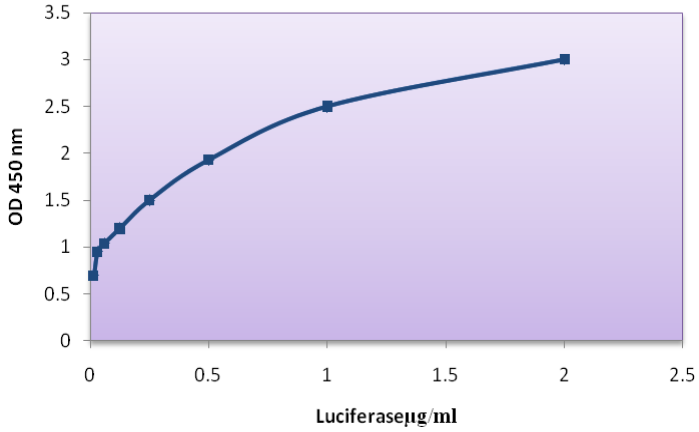


**Fig. 3:** Indirect ELISA.

Competitive ELISA: In order to indicate whether $M_2G_{11}$ is capable of recognizing its epitope while luciferase is free in the solution, the competitive ELISA was performed .The result presented here (fig4) is indicative of the ability of $M_2G_{11}$to recognize and react with soluble $M_2G_{11}$.

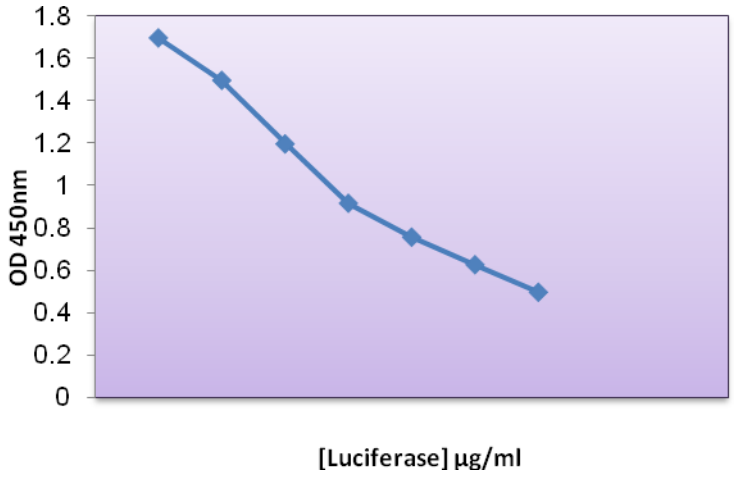


**Fig. 4:** In this experiment a specific concentration of luciferase (1 µg/ml) was coated in microtiter plates and then a mixture of $M_2G_{11}$(with a definite concentration) and luciferae with various concentration (15-500 ng) were added into each well.

*Western blot:*

As illustrated in fig.4, $M_2G_{11}$ showed prominent reactivity toward 62 kD bond.

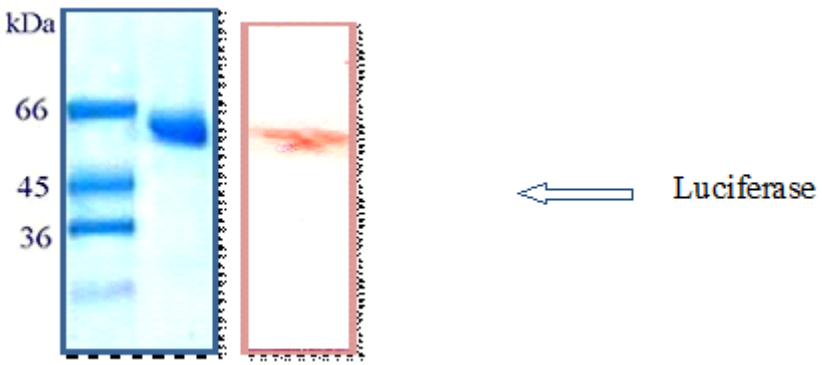


**Fig. 5:** The molecular weights (kDa) of standard marker are on the left. The arrow on the right shows the luciferase band at 62 kd .

*Discussion:*

Monoclonal antibodies are utilized for detection of proteins in various systems such as ELISA (Burg and Rossenen, 1985) and western blotting (towbin et. Al., 1979).

Many proteins are used as reporter proteins , for example chloramphenicol acetyltransferase (CAT) , ß galactosidase and luciferase. Firefly luciferases are abundantly utilized as gene reporter and catalyze a bioluminescense reaction which are directly detected. Emitted light is contingent upon measuring the activity of luciferase. Luciferase assay is very sensitive, rapid and precise and in contrast with CAT it does not need radioactive substrate.

Anti-luciferase is a stable detection system for luciferase . It requires neither luciferase activity ,nor measurement of rapid reaction kinetics besides, for its detection, luminometer is not essential. Moreover, antibodies can detect the luciferase expression in situ, and it provides a strategy to study the cellular localization of signaling cascades.

In this paper we produced monoclonal antibody for first time against firefly luciferase from Lampyris turkestanicus. The antibody was named $M_2G_{11}$. $M_2G_{11}$ hybridoma generated after four fusions. After of third cloning, this hybridoma was tested for its ability to interact with luciferase on ELISA. $M_2G_{11}$ was specific for luciferase and recognized it with a very high affinity . This antibody recognizes luciferase in its native and denatured forms by SDS. So that, its epitope can be a peptid segment on its surface . Our results indicate that this mAb is useful for detection of the luciferase. It interacts with luciferase in ELISA and western blotting. $M_2G_{11}$ also detects firefly luciferase from Lampyris turkestanicus. Furthermore, we produced the first reporter monoclonal antibody against Lampyris turkestanicus.

We can make use of this Ab in luciferase antibody staining kit which is an easy and rapid method for detection of luciferase in cells. In this method, antibody bonds to the cells which express luciferase and the antigen-antibody complex is detected by a secondary antibody. It does not need the luminometer for detection. Moreover, we are able to conjugate $M_2G_{11}$ with biotin-HRP and use this complex to detect cells which express luciferase without a secondary antibody. By applying this method, the sensitivity of detection increases several times.

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