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Full Length Research Paper

Preparation and partial characterization of monoclonal antibody against human phosphdiesterase 5 (PDE5)

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Phosphodiesterase 5 (PDE5) is a catalytic enzyme for degradation of cyclic guanosine monophosphate (cGMP) in human smooth muscle cells. Inhibition of this enzyme by certain chemicals including sildenafil, vardenafil and tadalafil, is considered a breakthrough treatment of erectile dysfunction. Unfortunately, these drugs are associated with several side effects. In this study, another strategy is adopted in the inhibition of the PDE5 enzyme by using a monoclonal antibody (Mab) against the enzyme. The Mab was prepared by immunizing a mouse, taking the spleen to make single cell suspension of lymphocytes. Hybridoma cells were obtained by fusion of BALB/c myeloma cells with the immunized lymphocytes using the polyethylene glycol method. Several clones were produced that have been separated by limiting dilution method. The antibodies were tested by enzyme-linked immunosorbent assay (ELISA) and western blot. The obtained Mab has titer of 625, immunoglobulin G1 (IgG1) isotype and 3.9×10^4 mol^{-1} affinity constant.

Key words: Phosphodiesterase (PDE), chemical inhibition, monoclonal antibody (Mab).

INTRODUCTION

Phosphodiesterase (PDE) (EC 3.1.4.-) enzymes catalyze the degradation of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) to the corresponding AMP or GMP. To date, at least 11 different families of PDE isozymes have been identified (Gupta et al., 2005; Lugnier, 2006; Matsumoto et al., 2003). The PDE isozymes described as being expressed in distinct types of vascular smooth muscle are PDE1, PDE3, PDE4 and PDE5 (Maurice et al., 2003; Noguera et al., 2001; Rybalkin et al., 2003). In humans, three PDE5 isoforms (PDE5A1, A2, and A3) have been identified. These isoforms are the products of alternatively spliced messenger RNAs and are identical in all of the known regulatory and catalytic domains (Sussman, 2004). PDE5 inhibitors were firstly implicated in vasorelaxation, since the specific inhibition of PDE5 by zaprinast was shown to induce an increase in cGMP associated with a vasore-laxing effect (Kameni et al., 2006). In that way, new PDE5 inhibitors derived from zaprinast were designed as antihypertensive compounds or coronary vasodilators. Unexpectedly, during clinical studies, sildenafil amelio-rated erectile dysfunction pointed out PDE5 inhibition as a new target for treatment of erectile dysfunction and increasing the development of PDE5 inhibitors (Rapoport and Murad, 1983). PDE5 encompass from a catalytic domain encompassing a region of ~270 amino acids; a regulatory domain between the amino terminus (Figure 1).

Nowadays, three different PDE5 inhibitors are used to

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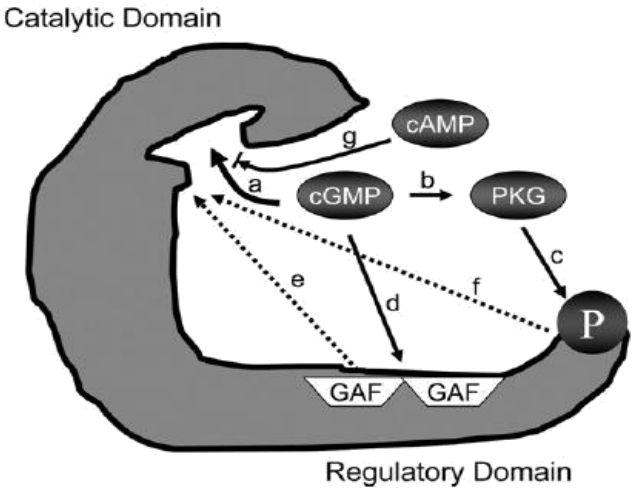

Figure 1. Schematic of mechanisms by which cGMP/PKG interacts with a PDE to influence its catalytic activity. Diagram shows PDE with GAF binding domains, such as PDE2 or PDE5. (a), cGMP can interact with the PDE in the catalytic domain to undergo hydrolysis to GMP; (b), cGMP can activate PKG which in turn can phosphorylate; (c), the N-terminus to (f) enhance catalytic activity; (d), cGMP can bind to regulatory GAF domains that facilitate phosphorylation by PKG and (e) also enhance PDE catalytic activity; and (g) cGMP binding at the catalytic site can impact PDE hydrolysis for cAMP (as the case for PDE3).

treat erectile dysfunction. Unfortunately; these drugs have a lot of serious side effects. Due to these side effects, another strategy to inhibit PDE5 enzyme was adopted using a monoclonal antibody (Mab).

The objective of this study was to use another strategy for the inhibition of the PDE5 enzyme by using a Mab. So preparation, purification and partial characterization of Mab against human PDE5 are the main objectives of this study.

MATERIALS AND METHODS

Extraction of platelets by ultrasound sonicator

The method described by Asru et al. (1976) was adapted. Ten units of human male platelets were brought from the blood bank of King Abdullah hospital. The units were aliquoted into 50 ml tubes, then centrifuged at 900 × g at room temperature for 15 min. After removing the supernatant, the pellet was washed three times with 10 ml of phosphate buffer saline (PBS) and centrifuged at 700 × g for 12 min at room temperature. After that, the pellet was re-suspended in 5 ml of PBS, and sonicated by ultrasound sonicator for 10 min. The samples were centrifuged at 700 × g for 10 min and the supernatants were pooled. Protein concentration in the supernatants was determined by method of Bradford (1976).

PDE5 partial purification using ion exchange chromatography

All chromatography experiments were performed at room temperature. One (1) ml column was packed with Q-sepharose A-25 resin (GE Healthcare) using the ethanol slurry packing technique and equilibrated with buffer A (20 mM Tris-HCl, pH = 8.2) at a flow rate of 6 ml/min. 2 M NaCl solution was used to remove impurities absorbed inside the columns. Salt and absorbed impurities were removed by flushing the columns with buffer A. The dead volume of the columns was measured by injecting 50 µl of 20% acetone solution via the injection loop. One (1) ml of platelet sonicated sample was diluted with 4 ml of binding buffer and was applied to the column. The column was washed with buffer A and a linear gradient elution was generated by changing from 100% buffer A to 0% buffer B (20 mM Tris-Cl, plus 0.2 M NaCl pH 8.20) at a flow rate of 1 ml/min. Fractions were collected and assayed for protein at 280 nm and for PDE5 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and PDE assay kit (Sun and Palmer, 2008). After each run, the column was regenerated by flushing it with 2 M NaCl and equilibrating it with buffer A.

PDE5 assay

PDE5 activity was assayed according to the company instructions (BPS Bioscience).

Mice immunization and Mab production

Four 6 weeks old BALB/c female mice were inoculated intra-peritoneally with 100 µg of partially purified PDE5 mixed (1:1) with Complete Freund's adjuvant (Sigma, USA). Two (2) and 3 weeks later, booster immunizations were given to each mouse with 100 µg of partially purified PDE5 in incomplete Freund's adjuvant. A final boost of 100 µg PDE5 in PBS buffer was given to each mouse after 4 weeks. Six (6) weeks after the first immunization, test-bleed sera were assayed by indirect enzyme-linked immunosorbent assay (ELISA). Microtiter plate (Nunc MaxiSorp, Denmark) were coated with PDE5 obtained from a native gel using a preparative electrophoresis by adding 100 µg/ml in coating buffer and incubation overnight at +4°C. The plates were blocked for 30 min at room temperature with 2% bovine serum albumin (BSA) in PBS buffer and then incubated with primary antibody for 1 h at room temperature. After washing, the plates were incubated with goat-anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate diluted 1:2000 in PBS containing 1% BSA for 1 h at room temperature. After each step, wells were washed with PBS containing 0.05% Tween 20 for three times. The enzymatic reaction was developed with developing solution and stopped by adding 1 M H_2SO_4. The optical density was measured at 450 nm using Bio-Rad ELISA reader (Zvirbliene et al., 2010). The mouse producing the highest titer of antibody was boosted at 8 weeks. The mouse spleen cells were fused with sp2/0 myeloma cells using standard procedures. Resulting hybridoma colonies were maintained on RPMI-1640 medium supplemented with 20% fetal calf serum (FCS). The hybridoma culture supernatants were screened for antibody reactivity by indirect ELISA. Selected hybridoma colonies were then cloned for three cycles by limiting dilution, and expanded into 75 cm^2 tissue culture flasks. The hybridoma culture supernatants which had specific reaction with SGIV were collected, and Ig was precipitated and concentrated by ammonium sulfate.

Mab isotype identification

To screen and determine classes and subclasses for mouse Mabs in the supernatants of hybridoma, ELISA assay was done. Instead of the HRP-labelled goat anti-mouse IgG secondary antibody five antibody HRP conjugates were applied, including anti IgG1, IgG2a, IgG2b, IgG3 and IgM.

Mab purification using affinity chromatography

The column which was packed with protein G was fixed and the ultraviolet (UV) lamp was sat on zero. The device was started running with 1 ml/min flow rate of elution buffer for 10 min, then with binding buffer for 10 min. E8 Mab diluted 1:2 with binding buffer was run. After the UV readings were raised, the fractions were collected. Elution fractions were neutralized immediately by addition of a small amount of neutralizing buffer and dialyzed (0.15 M PBS, pH 7.4). The eluted E8 Mab samples were analyzed by a discontinuous 12.5% SDS-PAGE. The column was regenerated with 20% ethanol and store at 2 to 8°C (Farbridge et al., 1999).

SDS-PAGE and western blot

SDS-PAGE was carried out as described by Laemmli (1970) with 12.5% separating. After around 1 h electrophoresis at 150 V, fractionated proteins were transferred onto a 0.45 mm pore nitrocellulose membrane (Bio-Rad, Hercules) at 70 V for 1 h and subsequently 30 V overnight in a Bio-Rad mini Trans- Blot electrophoretic transfer cell. The blotted membrane was rinsed with distilled water and then blocked with Tris buffered saline (TBS) containing 2% (w/v) BSA for 1 h. The membrane was then cut into several strips according to the sample lane and these strips were put into supernatants of Mab or into mouse polyclonal antiserum raised against PDE5 diluted by TBS containing 1% (w/v) BSA, and incubated for 1 h at room temperature, followed by washing for 3 to 5 min in TBS containing 0.05% Tween 20. These strips were then incubated with goat-anti-mouse IgG HRP conjugate for 1 h at room temperature, followed by 3 to 5 min washing in TBS- Tween 20. After the final wash, membrane strips were put into substrate solution for 30 min. The developed reaction was stopped by rinsing strips with distilled water.

Immunodot blot

Two microgram (2 ∝g) of aliquots of extracted platelet proteins diluted as 1:10 in TBS was dotted onto four 0.45 mm pore nitrocellulose membrane strips (Bio-Rad). Antigen-blotted membranes were blocked with 2% BSA in TBS for 1 h at room temperature. The strips were placed in supernatants of Mab and mouse polyclonal antiserum raised against PDE5 diluted by TBS containing 1% (w/v) BSA, and incubated for 2 h at room temperature. The strips were then washed for 3 to 5 min in TBS containing 0.05% Tween 20, followed by incubation with goat-anti-mouse IgG HRP conjugate for 1 h at room temperature. After another 3 to 5 min of washing in TBS-T, the membrane strips were put into substrate solution for 20 min. The development reaction was stopped by rinsing strips with distilled water (Shi et al., 2003).

Determination of Mab titer

Twelve wells in ELISA plate were coated with 100 µl of PDE5 (5000 ng/ml). After 1 h of incubation at room temperature, the wells were blocked with blocking solution (2% BSA in 1 × PBS buffer). Then 100 µl of Mab with serial dilutions (80000, 40000, 20000, 10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.1 and 39 ng/ml) were added to the wells. After incubation for 1 h at room temperature with continuous shaking, 100 µl of the total antimouse Ig-HRP secondary antibody diluted in diluent buffer were added to each well. After each step, wells were washed with PBS-Tween 20 (0.05%) for three times. Lastly, 100 µl of substrate solution was added to each well. The absorbance was measured at 450 nm using Bio-Rad ELISA reader (Ya et al., 2006).

Measurement of Mab affinity constant by an ELISA based method

Two rows of ELISA plates were pre-coated for 1 h with two different concentrations of PDE5 ([Ag: 5000 ng/ml] and [Ag′: 2500 ng/ml]). Wells were blocked with blocking solution (2% BSA in 1 · PBS buffer) for 1 h. Then they were incubated with serial concentrations of Mab (80000, 40000, 20000, 10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.1 and 39 ng/ml) for 1 h. 100 µl of the total antimouse Ig-HRP secondary antibody diluted in diluent buffer were added to each well. After each step, wells were washed with PBS-Tween 20 (0.05%) for three times. Lastly, 100 µl of substrate solution was added to each well. Sigmoid curves were constructed using the absorbance values obtained for different concentrations of Mab. Two non-overlapping curves were selected for Mab to calculate the affinity constant. The half maximum optical density (OD-50) was assigned for the selected curves from which the corresponding antibody concentrations (Ab, Ab′) were extrapolated. Accordingly, Ab and Ab′ are the measurable total Ab concentrations at OD-50 and OD′-50 for plates coated with Ag and Ag′, respectively. The affinity constant was determined using the following equation (Beatty et al., 1987):

$$K_{aff} = (n - 1)/2(n[Ab'] - [Ab])$$

Where n = [Ag]/ [Ag′]

RESULTS

The concentration of protein in sonicated platelet sample was 1.32 mg/ml. SDS-PAGE analysis for the extracted platelet proteins revealed the presence of several proteins with molecular weights ranging from about 15 KD to more than 225 KD (Figure 2). Two bands with molecular weight (MW) of 97 and 105 KD could be seen which correspond to the known two bands for PDE5.

The elution profile of the platelet proteins from Q-sepharose gel chromatography system shows two peaks of eluted proteins with the fractions 8, 9, 10, 11, 15, 16 and 17 (Figure 3). PDE5 assay for the mentioned fractions revealed that fractions 8 and 9 showed high fluorescence peaks which indicate high enzyme activity (Figure 4). SDS PAGE for the collected fractions shows two bands in fractions 8 and 9 with MW of 105 and 97KD which correspond to PDE5 (Figure 5).

Hyper immunized mouse 1 was sacrificed and its spleen cells were fused with myeloma cells in 24-well plate. After 15 days of fusion, the supernatant from wells with healthy cells were tested using ELISA. All tested supernatants showed Ab reactivity against PDE5 enzyme, but the highest reactivity was obtained in B2, B6 and C4 wells.

The medium in A1, B2, B6 and C4 wells were collected to be used as a positive control in the next ELISA experiment. The cells in B6 well were chosen for cloning using the limiting dilution in 96 well plates which already coated with peritoneal macrophages as a feeder layer to support the growth of hybridoma cells. After 7 days of cloning, each well was checked. E8 and F3 wells contained just a

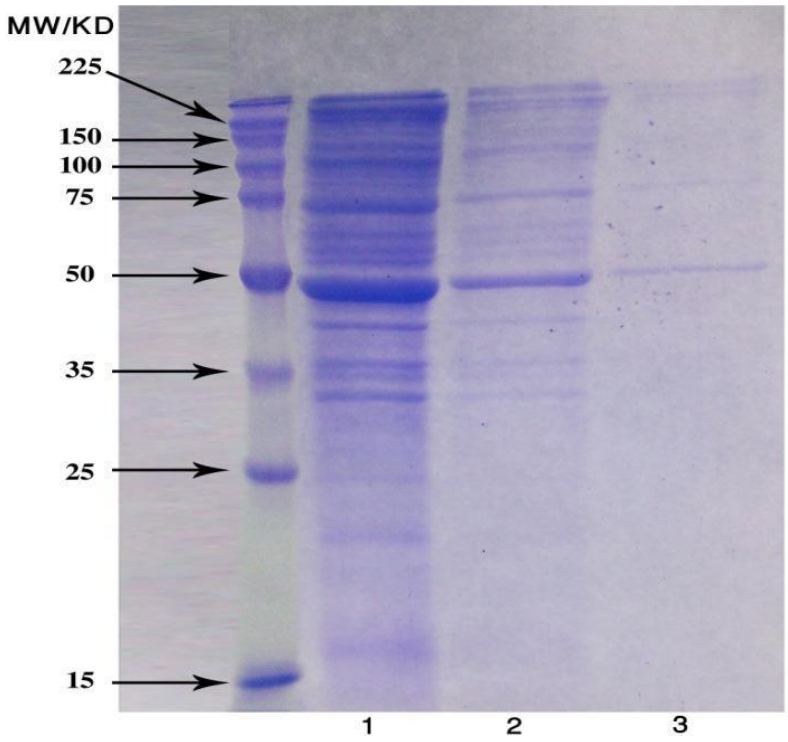

Figure 2. SDS-PAGE analysis (12.5% acrylamide) of the extracted platelets proteins profile in three different dilutions 1:1, 1:2 and 1:3 that correspond to lanes 1, 2 and 3, respectively.

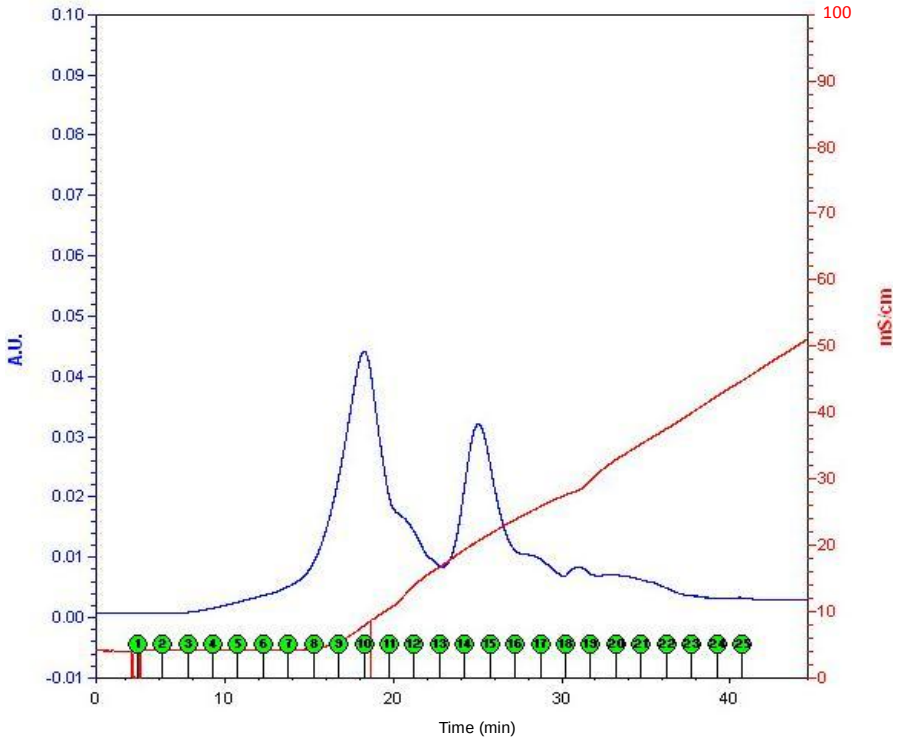

Figure 3. Linear gradient elution curve of PDE5 using Q-Sepharose gel column (1 ml packed per volume). The gel was washed with binding buffer and the unbound proteins were eluted with gradient salt concentration (0.02 to 0.5 M).

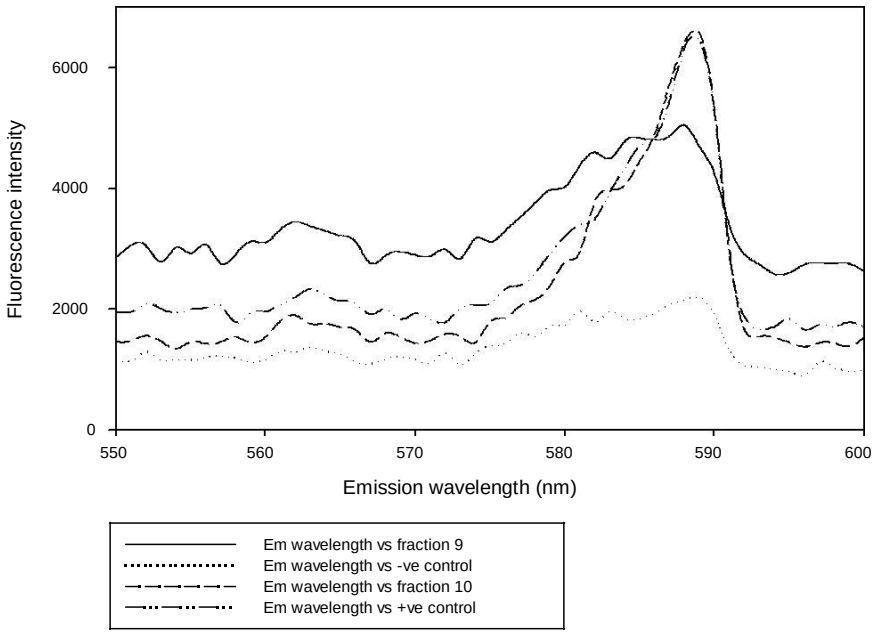

Figure 4. PDE5 assay for fractions 8 and 9 collected from Q-Sepharose column.

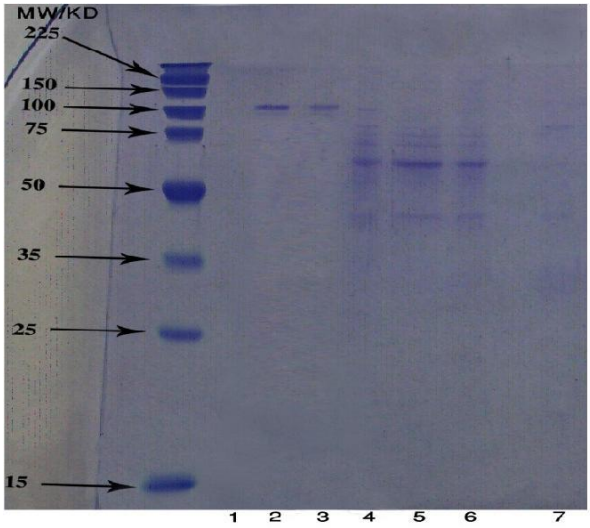

Figure 5. SDS-PAGE analysis (12.5% acrylamide) for seven fractions of IEC. The eluted fractions (8, 9, 10, 11, 15, 16 and 17) that correspond to lanes (1 to 7, respectively) from the IEC were applied.

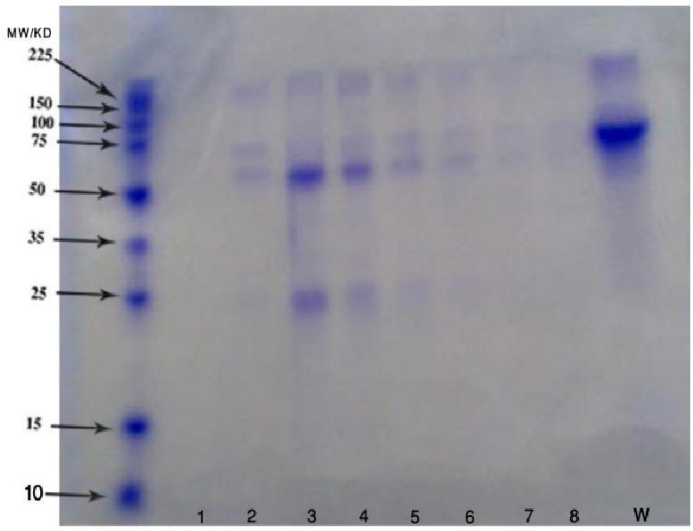

Figure 6. SDS-PAGE analysis (12.5% acrylamide) of the fractions obtained from protein G affinity chromatography. Lanes 1 to 8 correspond to the eluted fractions. W, Fraction of washed column.

single colony originating from single cell. The wells which presented healthy cells were tested by ELISA.

The supernatants from E8 and F3 wells with high reactivity against PDE5 were isotyped using ELISA with different secondary antibodies against IgG1, IgG2a, IgG2b, IgG3 and IgM. The class of Mab produced from E8 was IgG1, and F3 clone produced IgG1 and IgM which means it still needs further recloning. Therefore, E8 clone was further characterized.

The fractions which were collected from protein G column and represented IgG against PDE5 was subjected to SDS-PAGE. Two bands of 50 and 25 KD were obtained (Figure 6).

The dot blot assay was carried out to investigate the reaction between E8 Mab and PDE5 which was adsorbed to the nitrocellulose membrane paper. Figure 7 shows a

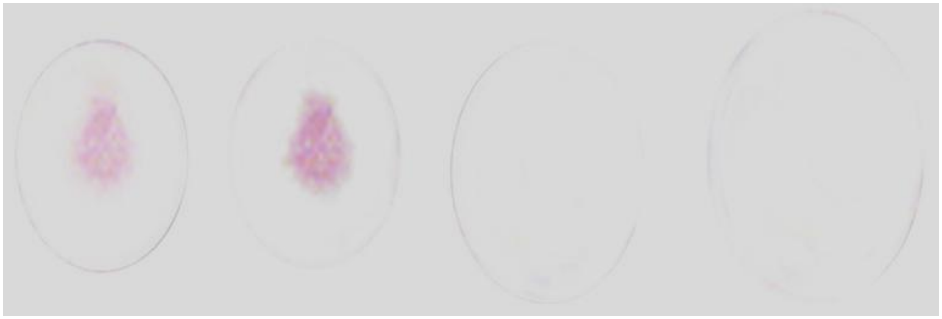

Figure 7. Dot blot assay for PDE5 enzyme with the purified E8 Mab.

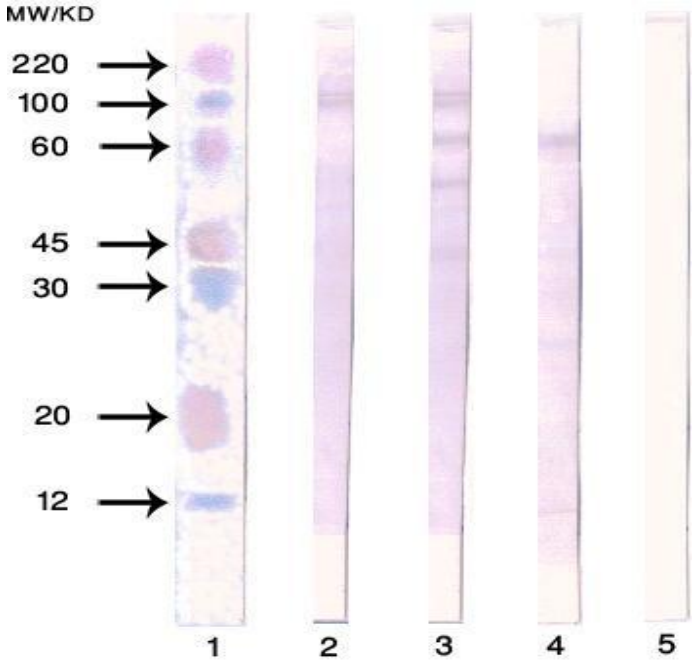

Figure 8. Immunoblot for SDS-PAGE analysis (12.5% acrylamide) of PDE5 with E8 Mab (2), positive polyclonal anti serum (3), negative serum (4) and negative media (5), MW marker (1).

strong binding between PDE5 and E8 Mab.

To detect the specificity of E8 Mab, an immune blot assay was carried out. E8 Mab displayed a specific binding with 105 and 97 KD of PDE5. In positive polyclonal anti serum, the previous two bands appeared in addition to other bands (Figure 8).

The titer of E8 Mab is (625), because at this dilution the Mab gives a half-maximal binding with PDE5 (Figure 9)

A sigmoid curve was constructed to determine the OD-50 for a given concentration of PDE5 and the corresponding Mab [Ab] (Figure 10). The Ab concentration [Ab] which gave a (OD-50) for a [PDE5 = 5000 ng/ml] was 625 ng/ml. On the other hand, the Ab concentration [Ab′] which gave a OD'-50 for a [PDE5′= 2500 ng/ml] was 951ng/ml. According to the equation previously given in the work, the affinity constant for E8 Mab is 3.9×10^{4} Mol^{-1}.

DISCUSSION

Pharmacological inhibition of PDE5 exhibits differential effects in different organs according to its expression in various tissues (Wallis et al., 1999). Unfortunately, chemical inhibition of PDE 5 by sildenafil or vardenafil is associated with several side effects such as blindness and heart problems (Boolell et al., 1996).

The PDE5 enzyme contains a regulatory site and a

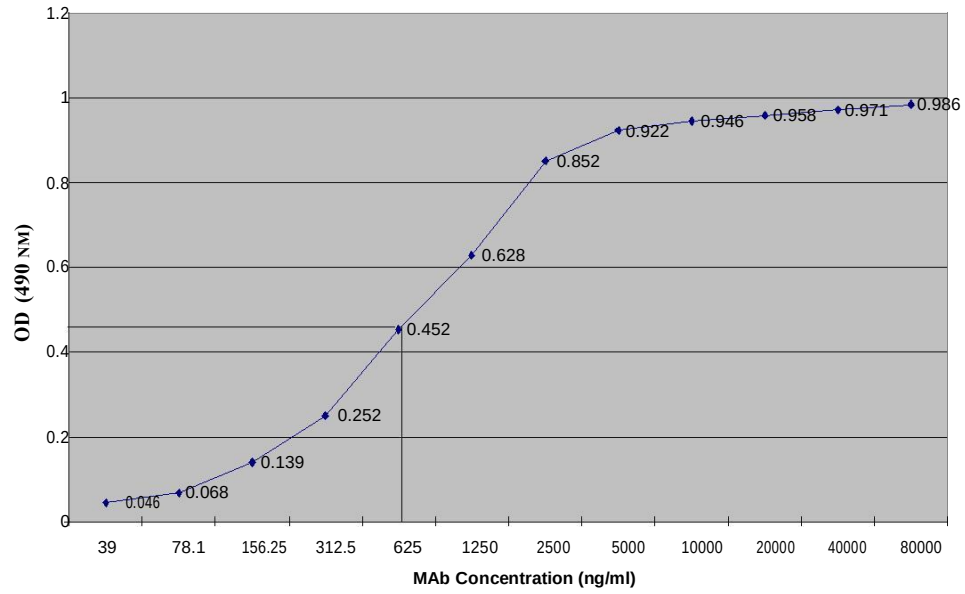

Figure 9. MAb titer determination. The reactivity of serial dilution of Mab against a constant concentration of PDE5.

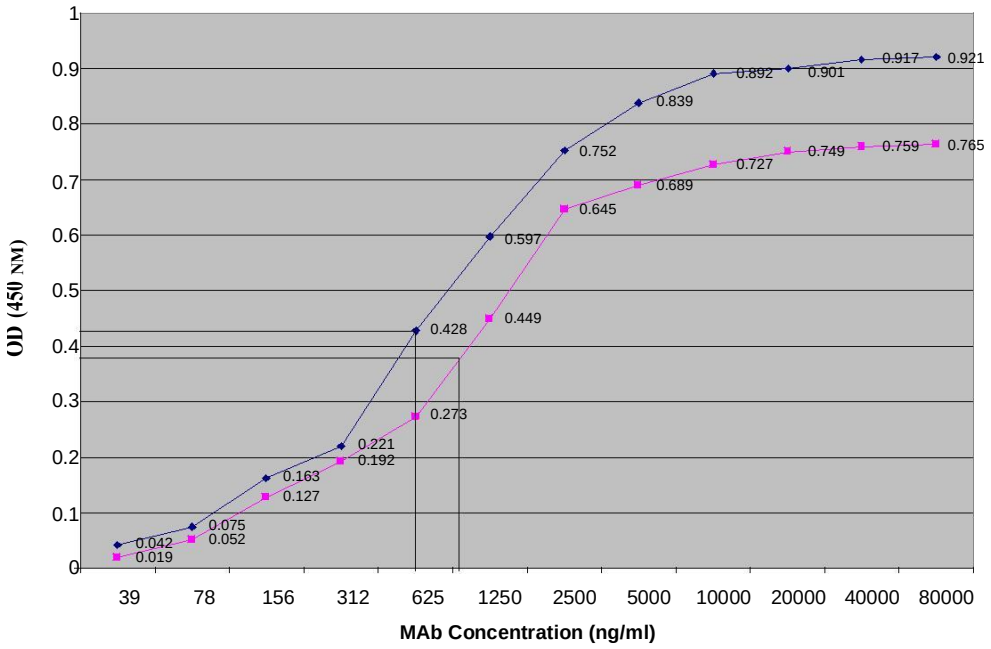

Figure 10. Representative binding curves employed for extrapolation of affinity constant of E8 Mab.

catalytic site. A Mab directed to either the catalytic site or to the regulatory site of PDE has no or less side effects when compared with other PDE inhibitors now in use (Kulkarni and Patil, 2004).

For inhibition of the enzyme, the Mab should bind either to the catalytic site or to the regulatory site. It would be better if the E8 Mab binds the regulatory site because of the structural homology between PDE5 and PDE6. If the Mab binds to the catalytic site, both PDE5 and PDE6 will be inhibited. This contributes to the side effects resulted from the chemical inhibition specifically the impairment of the vision. Binding to the regulatory site results in inhibition

of the PDE5 only (Zoraghi et al., 2004).

E8 Mab may have no or less side effects, but it should be administered by injection and not as tablets or capsules like the chemical treatment. This disadvantage should be considered and weighed to the serious side effects of the chemical treatment.

Purification of the PDE5 was not necessary for the immunization of mice. Partial purification using extraction and anion exchange chromatography was performed. For the identification of the enzyme, the two bands of MW of 97 and 105 KD in SDS-PAGE and one band of 200 KD in the native PAGE were followed. In addition, the enzymatic assay for PDE5 was used.

The E8 Mab is highly specific; it detects only the PDE5 enzyme as it appears in SDS-PAGE. It is expected that the E8 Mab binds both bands of 97 and 105 KD because of the structural resemblance between both enzyme subunits.

The titer of E8 Mab was very low (625), but as the case in all Mabs, the concentration can be increased easily and the titer will be also increased.

The low affinity of the E8 Mab (3.9×10^4 Mol^{-1}) could be useful in treating the erectile dysfunction patients. This needs further investigation.

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