CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Wild-type plasmid

pET21b(+)- hCgA wild-type plasmid from Mr. Arthid Tim-uam, Department of Biochemistry, Faculty of Medical Science, Naresuan University (Arthid Tim-uam, 2010)

2. Bacterial strains

The host cell for amplification of recombinant plasmids is *Escherichia coli* DH5α (Madison, WI, USA). Expression host *Escherichia coli* strain BL21 (DE3)plysS was from Novagen (Madison, WI, USA)

3. Cloning and expression vectors, and host cells

The TA plasmid vector systems were supplied by RBC bioscience (Taipei, Taiwan). Plasmid expression vector pET21b was from Novagen (Madison, WI, USA). The PCR products were cloned into this vector according to the method recommended by the manufacture protocol.

4. Enzymes

The *Taq* DNA polymerase used for PCR amplification of hCgA gene from pET21b(+)- hCgA wild-type plasmid was purchased from Invitrogen (USA). The *NdeI* and *Xho*I restriction enzyme from Invitrogen were used for digestion of plasmid vector and PCR product. The ligation reaction of the digested plasmid vector and PCR product was performed by T4-DNA ligase supplied by Invitrogen (USA).

5. Antibodies

Mouse anti hCgA monoclonal antibody was purchased form GeneTex, Inc. (USA), Goat Anti-Mouse IgG polyclonal antibody, HRP conjugate were purchased from Millipore (USA).

6. Reconstruction Primer

All reconstruction primers for truncating of CgA gene were designed and ordered from Biodesign (Pathumthani, Thailand) as shown in Table 5.

Equipments

Sterilization of reagents and equipments for cloning system was performed by Autoclave (Sanyo electric Co., LTD., Japan) and Hot air oven (MMM Medcenter Einrich-tungen GmbH Co., LTD., Germany). All bacteria cells were lysed by sonicator (Sonics VCx-130, USA). All PCR amplifications were performed in the Thermo Scientific Hybaid PX2 thermal cycler Thermo Scientific (USA). The centrifugation of lysis cell was performed by Refrigerated microcentrifuge MX301 Tomy (Tokyo, Japan). SDS-PAGE and agarose gel were photographed with Gel Document SYDR2/2002 (Syngene bioimage). Heating block (Labnet International, Inc., USA) was used for protein boiling. All Protein determinations were performed by SDS-Polyacrylamide Gel Electrophoresis invitrogen (CA, USA). Bacteria cells were incubated in incubator shaker Shel Lab (Oregon USA). The pH is measured using pH meter Seven easy Mettler Toledo (Ohio, USA). Enzyme kinetic is measured using Synergy HT Multi-Mode Microplate Reader Biotek (Washington, USA). Concentrations of protein, DNA and bacteria cells were detected by UV/Vis Spectrophotometer (Beckman Coulter Co., LTD., USA). Western blotting was performed by AE-6675 Semi Dry Blotting ATTO (Japan).

Molecular reagents and chemicals

Sodium chloride and magnesium chloride were purchased from Fluka (Steinheim, Switzerland). Sodium dodecyl sulphate (SDS) was purchased from Amersham (USA). Methanol was purchased from LAB-SCAN (Ireland). 100 bp Plus DNA ladder was purchased from Fermentas (USA). DNA ladder (200 bp) was purchased from Bio basic (Canada). Boric acid, glacial acetic acid, Glycerol and isopropyl-*b*-D-thiogaltopyranoside (IPTG) were purchased from Merck (Damsladt, Germany). Bovine serum albumin (BSA), Precision Plus ProteinTM All Blue Standards, Precision Plus ProteinTM Unstained Standards, Ethidium bromide, Ethylenediaminetetraacetic acid (EDTA), Mercaptoethanol, Ammonium persulfate, Acrylamide/Bis Solution 19:1, Glycine and Coomassie Brilliant Blue G-250 were purchased from BIO-RAD (California, USA). Chromatein Prestained Protein Ladder was purchased from was purchased from Vivantis (California, USA). Ampicillin, Ponceau S Solution, Tween 20 and chloroform were purchased from Sigma (St. Louis,

USA). Agarose, T4 DNA ligase and X-Gal from Promega (Madison, USA). *Taq* DNA polymerase, 10X PCR buffer, 50 mM $MgCl_2$, restriction endonuclease, *Nde I,* and *Xho I* and deoxyribonucleotide (dATP, dCTP, dGTP, dTTP) from Invitrogen (California, USA). GI/PCR DNA Fragment Extraction kit was purchased from RBC bioscience (Taipei, Taiwan). LB Broth and LENNOX were purchased from DIFCO (Grayson, USA). *N,N',N'',N'''* tetramethylethylenediamine (TEMED), bromophenol blue, chloroform, sodium hydroxide, ethanol, sodium bicarbonate, sodium dihydrogen phosphate, and hydrochloric acid were purchased from Carlo ERBA (Rodano, Milano, Italy). Tris base, Boric acid and Agarose were purchased from USB (USA). Agar powder was purchased form Himedia (Himedia Laboratories Pvt. Ltd., India). Calcium chloride was purchased from Ajax Finechem (New Zealand). Other chemicals and solvents used but not listed here were purchased from various suppliers. Tissue plasminogen activator and glu-plasminogen were purchased from American diagnostic (USA). Boc-Val-Leu-Lys-MCA was purchased from Bachem (California, USA).

Methods of Experiment

1. Cloning and sequencing of the Human chromogranin A

1.1 Primer design and DNA amplification

In order to obtain the truncated hCgA fragment, 11 primers were used for the reconstruction of hCgA (Table 6). Series of recombinant hCgA clones were generated by PCR with truncated C-terminus (Figure 9). DNA amplification by polymerase chain reaction (PCR) was performed using 10-100 ng of the pET21b(+)-hCgA wild-type plasmid as a template with the same components, but with different primers. The reaction mixture was composed of 0.1 µl plasmid, 0.3 µl 10 mM forward primer, 0.3 µl 10 mM reverse primer and 1 µl PCR Master Mix (0.02 units/µl *Taq* DNA Polymerase in reaction buffer, 3 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, and 0.2 mM dTTP) and distilled water to make the volume of up to 10 µl. The PCR program is shown in the following Table 7.

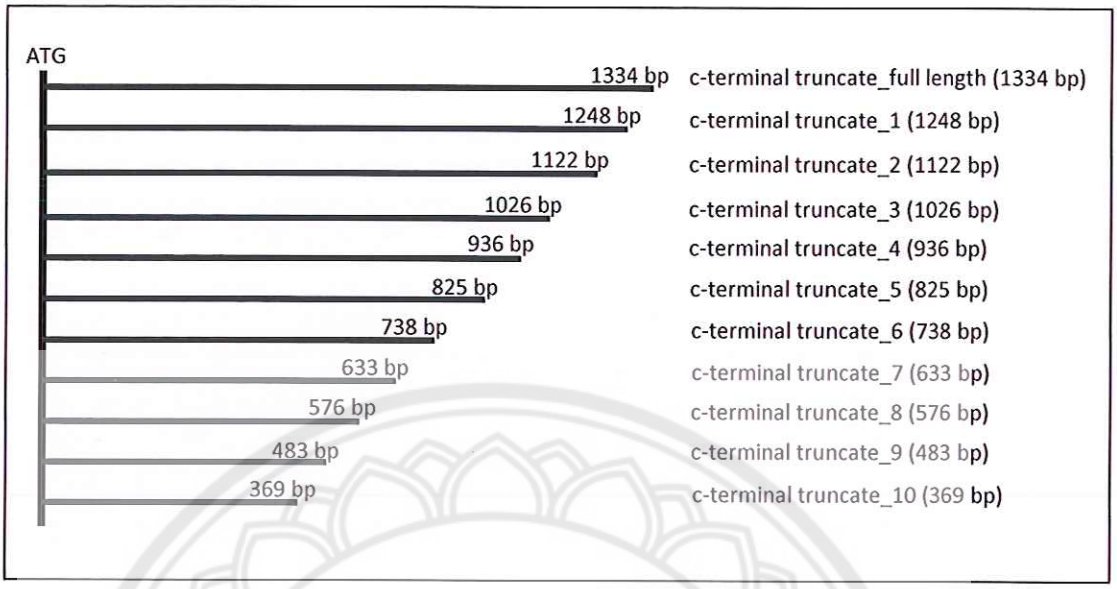

Figure 9 Series of recombinant hCgA clones generated by PCR with truncated C-terminus

Table 6 Oligonucleotide primers used for DNA amplification of human chromogranin A

Primer name		Sequence
c-terminal truncate	Forward	5'-ATACATATGCTCCCTGTGAACAGCCC- 3'
	Reverse	5'-TGGTGGTGCTCGAGGCCCCGCCGTAGT- 3'
c-terminal truncate_1	Reverse	5'-TATCTCGAGTCTGCGGTTTGCGCTGCC- 3'
c-terminal truncate_2	Reverse	5'-TATCTCGAGAGGGCCCCTGAAGCCGTA-3'
c-terminal truncate_3	Reverse	5'-TATCTCGAGAGCCGTCAGCTCCTTGGCCA-3'
c-terminal truncate_4	Reverse	5'-TATCTCGAGCTCTCCGCTCTTCCCACC-3'
c-terminal truncate_5	Reverse	5'-TATCTCGAGCTCCTCAGCCCCAGGCTTCC-3'
c-terminal truncate_6	Reverse	5'-TATCTCGAGCGGGTGGGGGTTCAGCACTA -3'
c-terminal truncate_7	Reverse	5'-TATCTCGAGCCACCCTGGCTCTGCACTC-3'
c-terminal truncate_8	Reverse	5'-TATCTCGAGTTCCTCCCCAGGGGCCTGAT-3'
c-terminal truncate_9	Reverse	5'-TATCTCGAGCTCACTGTCCCCCTCGGCCT-3'
c-terminal truncate_10	Reverse	5'-TATCTCGAGATCCTCTCTTTTCTCCATAA-3'

Table 7 PCR conditions for truncate CgA

Primer	PCR condition
c-terminal	94 °C 2 min
Full- length	94 °C 30 sec, 65 °C 30 sec,72 °C 1.30 min; 30 cycles
	72 °C 10 min
c-terminal truncate1-10	94 °C 5 min
	94 °C 30 sec, 64°C 30 sec,72 °C 1 min; 30 cycles
	72 °C 10 min

1.2 Agarose gel electrophoresis and DNA analysis

For a 1% Agarose gel, 1 gram of Agarose and 100 ml of 0.5X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) were mixed together. Agarose was completely melted by heat and cooled down to 50-60 °C before poured the molten Agarose on the tray. The comb was placed into the tray. After the agarose gel has solidified, add 0.5x TBE buffer covering the gel. The comb was removed. Suitable volumes of nucleic acid samples were mixed with 6X loading dye and loaded into the gel wells. The amplified PCR products were analyzed by electrophoresis on 1% agarose gel in 0.5X TBE buffer. PCR sample were mixed with 6X loading dye. Electrophoresis was performed at a constant 70 V for 90 min using the 0.5X TBE buffer. After electrophoresis, the gel was stained with 10 µg/ml of ethidium bromide solution for 15 min and destained with distilled water. The band in the gel was visualized by Gel documentation system (Syngene bioimage).

1.3 Purification of PCR product from agarose gels

PCR products in agarose gel were extracted using the Gel/PCR DNA Fragments Extraction Kit (RBC bioscience, (Taipei, Taiwan). PCR products on the agarose gel visualized under UV light. PCR products were cut out of the gel and transferred into 1.5 ml sterile microcentrifuge tube. The gel block was dissolved by adding 500 µl of DF buffer and incubated at 55 °C for 10-15 minutes until the gel slice was completely dissolved. During incubation, invert the tube every 2–3 mins, then cool the dissolved sample mixture to room temperature, before transferring it to the

DF column and centrifuge at 10,000 x g for 30 seconds. The flow-through was discarded and placed the DF Column back in the collection tube, then 600 µl of wash buffer was added into the DF column and left standing for 1 min. After centrifugation for 30 sec the flow-through was discarded and centrifuged again for 2 min to dry the column matrix. Finally, the bound DNA was eluted with 20 µl of sterile distilled water and then centrifuged at 10,000 x g for 2 min. The supernatant containing the eluted DNA was transferred to a new centrifuge tube. The purified DNA solution was determined by measuring the absorbance at 260 nm.

1.4 Preparation of competent cells

The $CaCl_2$ method using to prepare competent *E. coli* DH5 α cells (Sambrook, et al., 1989). A single colony of *E. coli* was transferred to 3 ml of LB broth and incubated at 37 °C in incubator shaker machine for 16-18 h. A 1% overnight culture of starter culture was inoculated in 100 ml of LB broth and incubated for 3 h in incubator shaker machine at 37 °C until the OD at 600 nm was approximately 0.3-0.4. The cells were transferred into a sterile ice-cold 50 ml polypropylene tube, the cells were recovered by centrifugation at 6000 x g for 10 min at 4 °C. The culture medium was decanted from the cell pellet, and pellet was resuspended in 20 ml of ice cold 50 mM $CaCl_2$ and stored on ice for 20 min. The cells were collected by centrifugation at 6000 x g for 10 min at 4 °C. The supernatant was decanted from the pellet, and pellet was resuspended in 10 ml of ice cold 50 mM $CaCl_2$ and 15% glycerol and stored on ice for 2 h. The aliquots of 50 µl suspension of competent cells was transferred to sterile microcentrifuge tubes and stored at -80 °C.

1.5 Ligation of purified DNA fragment into vector

Purified DNA fragment was ligated to TA plasmid vector according to the plasmid supplier's recommendation (RBC Bioscience Cloning System T&A Cloning Vector Kit Protocol Book). The reaction mixture was composed of 2 µl of TA plasmid vector (50 ng/µl), 3 µl of purified DNA fragment (10-50 ng), 1 µl of Ligation buffer A, 1 µl of Ligation buffer B, 1 µl of T4 DNA ligase and 2 µl of sterile water. The reaction mixture solution was incubated at 4 °C overnight.

1.6 Transformation and selection

Frozen competent cells were thawed on ice. The ligation mixture was transferred to the thawed competent cells, mixed gently and stored on ice for 30 min. The cells were heat shocked at 42 °C for 90 sec and rapidly transferred to ice box and left on ice for 2 min. LB medium 250 µl was added to the ligation mixture and incubated at 37 °C for 1 h. This culture was spreaded on an LB medium agar plate containing 50 µg/ml ampicillin along with 40 µl of 100 mM IPTG and 40 µl of 20 mg/ml X-Gal and incubated at 37°C overnight. The recombinant clones were identified as white colonies. The cells contained non-recombinant plasmid gave blue colonies. The recombinant clones were confirmed by colony PCR amplification using M13 forward and M13 reverse primers, which present in plasmid vector or confirmed by restriction enzyme digestion.

1.7 Determination of recombinant hCgA clones by colony PCR

The lacZ system was used to select recombinant clones following standard protocols (Sambrook, et al., 1989). Only white colonies containing the recombinant plasmids were selected. Colony PCR was performed to specify the insert sizes of positive clones. Colony PCR was provided in 10 µl reactions containing 10X PCR buffer, 10 µM of each specific primer. Selected colonies were individually picked by inoculation loop and to spin in the 10 µl sterile water. The colony suspension of 2 µl was added to reaction mixer of colony PCR containing 1 µl of PCR Master Mix (0.02 units/µl *Taq* DNA Polymerase in reaction buffer), 10 µM of M13 forward primer, 10 µM of M13 reveres primer and dNTP. Condition of PCR program for each recombinant hCgA clones was shown in Table 8. The colony PCR products were electrophoresed through 1% agarose gels. The gel was stained with 0.5 µg/ml of ethidium bromide and visualized by UV light transillumination of Gel documentation system (Syngene bioimage).

Table 8 Primer and colony PCR condition for determine recombinant CgA clones

Primer M13	Forward: 5'- GTTTTCCCAGTCACGAC- 3'
	Reveres: 5'- TCACACAGGAAACAGCTATGAC-3'
PCR condition	94 °C 5 min
	94 °C 30 sec, 64°C 30 sec, 72 °C 1 min; 30 cycles
	72 °C 10 min

1.8 Restriction analysis

The recombinant CgA plasmid was digested using restriction enzyme according to the manufacture's recommendation (Invitrogen). The reaction mixture contained 1 µg of purified recombinant CgA plasmid, 3 µl of 10X buffer, 10 U/µl of restriction enzyme (*Nde*I and *Xho*I) and adjusted volume to 11 µl by sterile water. After incubation at 37 °C for overnight, the reaction mixtures of restriction enzyme digestion were analyzed by agarose gel electrophoresis.

1.9 Plasmid isolation using GF-1 plasmid DNA extraction Kit

GF-1 Plasmid DNA Extraction Kit protocol was used to purify plasmid for sequencing using the recommended protocol (Vivantis nucleic acid extraction kit handbook). A white colony containing recombinant plasmid was grown in 5 ml LB medium with 100 mg/ml ampicillin at 37 °C for 12-16 h with agitation. The bacteria cultures were pelleted by centrifugation at 6000 x g for 2 min. The supernatant were decanted then 250 µl S1 was added to the pellets and resuspended the cells by vortexing. The suspension was transferred to a clean 1.5 ml microcentrifuge tube. Then 250 µl of S2 was added and mixed gently by inverting tube 4-6 times to obtain a clear lysate and incubated on ice or at room temperature for no longer than 5 min. Afterward, 400 µl of NB buffer was added and slowly mixed by inverting the tube 6-10 times. The plasmid solution was divided by centrifugation at 14,000 x g for 10 min at room temperature. The GF-1 column was placed in a 1.5 ml collection tube. The plasmid solution supernatant was transferred into the column and centrifuged at 10,000 x g for 1 min. The plasmid solution was packed into the column and was washed with 700 µl of Wash buffer. After that it was centrifuged at 10,000 x g for 1 min, discarding the flow through. Plasmid was eluted from column by adding

50 µl of sterile distilled water to the center of column, leaving it to stand for 1 min and centrifuging at 10,000 x g for 1 min. The concentration of plasmid solution was determined by measuring the absorbance at 260 nm. Automated DNA sequencing was used to confirm the correct assembly of the recombinant plasmid.

1.10 DNA sequencing and sequencing identification

Recombinant hCgA plasmid clones were unidirectional sequenced using M13 forward primer and M13 reverse primer on an automatic sequencer at the DNA sequencing service. Assembled nucleotide sequences from the sequencing results were blasted against Genbank database using Blast*N* (nucleotide similarity) and Blast*X* (translated protein similarity). The Pairwise Sequence Alignment, EMBOSS Needle program was used to blast the sequencing results.

2. Expression of recombinant proteins in *Escherichia coli*

2.1 Construction of pET 21b(+)-human chromogranin A gene.

The *Nde I* and *Xho I* restriction enzymes were used to digest pET21b (+) plasmids (Novagen, Madison, WI). The restriction mixture contained 1 µl 10X buffer, 1 µl pET21b (+) vector, 1 µl *Nde I* (10 unit/ml), 1 µl *Xho I* (10 unit/ml) and 6 µl of distilled water. The reaction mixture was incubated at 37 °C for 16-18 h and the expected DNA size was purified by 1% agarose gel electrophoresis. The purified DNA sample obtained previously was ligated between *Nde I* and *Xho I* site of the expression vector pET21b (+). The plasmid was transformed into *E. coli* strain BL21 (DE3)pLysS. The pET21b (+) - hCgA plasmid was added to 100 µl thawed Origami (DE3) competent cells. The microcentrifuge tube was stored on ice for 30 min. The cells were heat shocked at 42 °C for 90 sec and the tube was immediately transferred to ice for 2 min. The transformed cells were grown by adding 250 µl of LB medium and incubated at 37 °C for 1 h, then plated directly on LB plate containing 100 µg/ml ampicillin, which was then incubated overnight at 37 °C. The recombinant clones were confirmed by colony PCR using expression primer and specific primer (Table 6). Automated DNA sequencing was used to confirm the correct assembly of the pET21b (+) - hCgA plasmid.

2.2 Expression of the wild-type human chromogranin A and variants in *Escherichia coli* BL21 (DE3)plysS

E. coli BL21(DE3)pLysS containing pET21b (+) - hCgA plasmid was grown in LB medium with 50 mg/ml of ampicillin at 37 °C overnight. The 1% bacterial cultures were grown in LB medium for 2-3 h, at 37 °C until OD at 600 nm reach 0.4-0.6. Then, the culture was induced by adding 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) and grown for 0, 1, 2, 3, 4 and 24 h at 37°C. Pre-cooled bacterial cells were collected by centrifugation at 5,000 x g for 5 min, and then kept at -80 °C.

2.3 Extraction of recombinant human chromogranin A from *E. coli* strain BL21 (DE3)PlysS

After growth and induction as described above, the cells were resuspended in 1X binding buffer (pH 7.4) and lysed by sonication on ice at the highest output (130 W) for 10 sec, 20 times using probe sonicator (Sonics VCx-130, USA). Soluble and insoluble proteins were separated by centrifugation at 15,000 x *g* for 30 min. The soluble proteins were boiled for 5, 10, 15, 20, 25, 30, 35 min and were separated by centrifugation at 15,000 x *g* for 30 min. Protein concentrations were determined by Bradford assay (Bradford, 1976). Samples were analyzed by SDS-PAGE.

3. Purification of recombinant human chromogranin A protein by chromatography

The extracted proteins were heated for 10 min in boiling water bath (Ugendra Kumar, 1997) and centrifuged at15,000 x *g* for 30 min. The proteins were purified on a Histrap FF crude column that is Ni^{2+} affinity chromatography (GE Healthcare, USA), with Binding buffer (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4), then with Wash buffer (20 mM sodium phosphate, 500 mM NaCl, 60 mM imidazole, pH 7.4) and Elution buffer (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 7.4). The purification was monitored by AKTAprime plus (GE Healthcare, USA) at 280 nm. Fractions containing hCgA were identified on polyacrylamide gel electrophoresis. Proteins were run on 10% Native-PAGE, cut expected size of hCgA protein. Proteins were purified

by electroelution, pooled, dialyzed at 4 °C overnight and concentrated using Vivaspin (GE Healthcare, USA).

4. Protein determination

4.1 Denaturing Gel Electrophoresis (SDS-PAGE)

The full-length and truncated recombinant hCgA proteins were determined by SDS-PAGE. Protein samples (~10 µg) were mixed with 4 volumes of 5X sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, and 14.4 mM 2-mercaptoethanol), and boiled for 5 min to denature the protein. The samples were analyzed on 4% stacking gel and 10% separating gel for 2 h at 20 mA. Protein bands were visualized by staining in a staining solution containing 0.1% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h, followed by several washes with a destaining solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

4.2 Western blot analysis

The corrected recombinant protein was confirmed by western blot analysis, Separated proteins were transferred from polyacrylamide gel to polyvinylidene fluoride (PVDF) membrane (Amersham, USA) with a AE-6675 Western blotting by AE-6675 Semi Dry Blotting ATTO (Japan) for 90 min at 400 mA. After western blotting, while the gel was stained with Coomassie Brilliant blue, the membrane was stained with Ponceau S to confirm complete transferring of protein from gel to membrane. The stained gel was destained with Coomassie blue destaining solution. The stained membrane was washed 3 times for 5 min with 1X Tris Buffer Saline Tween20 (TBST). The membrane was incubated with protein blocking solution containing 5% (w/v) non-fat dry milk in TBST at 4 °C overnight. Blocking buffer was discarded. The membrane was incubated with the primary antibody (Mouse anti human chromogranin A monoclonal antibody GeneTex, Inc USA) for 1 h at room temperature. The primary antibody was used at a dilution of 1:100 in blocking solution. The membrane was washed 3 times for 5 min with 1X TBST and then membrane was incubated with secondary antibody (goat anti-mouse antibody Millipore, USA) at a dilution of 1:5000 in blocking solution for 1 h at room temperature. Following 3 washes for 5 min with 1X TBST and 2 washes for 5 min with 1X PBS, the membrane was detected with chemiluminescence (GE Healthcare, UK). For 6 histidine at C-terminus determination

of all truncated hCgA and full-length, the membrane was incubated with primary antibody (Monoclonal Antibody Anti-His Tag Millipore, USA) at a dilution of 1:1500 in blocking solution for 2 h at room temperature. After this step the secondary antibody was incubated following by described above.

5. Plasminogen activation assays and kinetic study

5.1 Plasmin Activity Assay

In a 96-well microtiter plate containing 0.3 ug of plasminogen, 0.1 mM Boc-Val-Leu-Lys-MCA (BACHEM, USA), and 0.12 nM tPA were added in 1x Extracellular buffer (10 mM HEPES, pH 7.4/ 2 mM $CaCl_2$), in a total volume of 100 µL. The plate was placed in a preheated at 37 °C fluorometer and the reaction was monitored at excitation and emission wavelengths of 365 and 450 nm, respectively. At different time intervals (0-60 min), plasmin activity was measured by the release of free AMC from the synthetic peptide substrate. To observe kinetics of plasminogen activation 0.2 µM full-length or truncated recombinant hCgA was added into the reaction mixtures prior to the addition of tPA. Kinetics of the reactions (controlled (without enhancer), with recombinant hCgA) were observed and graphed as amounts of AMC released with times.

5.2 Kinetics of Plasminogen Activation in the Presence of Enhancers

Different amounts of recombinant hCgA (final concentrations of 10, 20, 30, 40, and 50 nM) were added to the standard mixtures of plasmin activity assays prior to addition of tPA (as described above). The reactions were monitored as free AMC fluorophore was released in a fluorometer at excitation and emission wavelengths of 365 and 450 nm, respectively. Plasmin activity of the control reactions (without enhancer) was observed and compared to recombinant hCgA as an enhancer. Also, different amounts of plasminogen (final concentration of 0.1-0.4 µM) were used at any fixed concentrations of the enhancers. Rates of the reactions at each condition were calculated as the amounts (pmoles) of AMC released/ min. Hofstee plots of the plasminogen activation gave K_m values of the control reaction (without enhancer). Kinetics of plasminogen activation was also observed in the comparison of the control plasmin activity (without enhancer), and the reactions containing 30 nM recombinant hCgA. The reactions were measured in the fluorometer as amount (pmol) of AMC released with times (0-60 min).