CHAPTER IV

RESULTS AND DISCUSSION

Cloning and Analysis of nucleotide sequence of the Human chromogranin A

1. DNA amplification and DNA analysis

The wild-type plasmid pET21b(+)-hCgA was obtained from Mr. Arthid Tim-uam, Department of Biochemistry, Faculty of Medical Science, Naresuan University (Arthid Tim-uam, 2010) to be used as a template for gene amplification. The full-length and truncated hCgA were amplified by PCR using truncation primers. In this study the cloned genes do not contain its own stop codon, but utilize stop codon in the vector. This amplification requires optimization of several parameters containing annealing temperature, concentration of template, primer and $MgCl_2$. The size PCR products were shown as 1334 bp (full-length), 1248 bp, 1122 bp, 1026 bp, 936 bp, 825 bp, 738 bp, 633 bp, 576 bp, 483 bp, 369 bp in Figure 10.

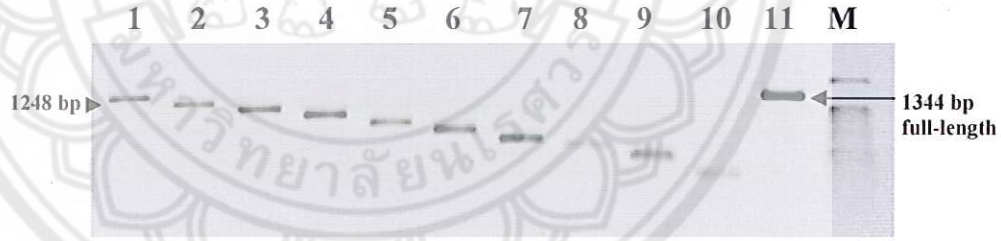

Figure 10 The 1% agarose gel electrophoresis of the full-length and truncated human chromogranin A gene

Lane1: 1248 bp Lane 2: 1122 bp Lane 3: 1026 bp Lane 4: 936 bp Lane 5: 825 bp Lane 6: 738 bp Lane 7: 633 bp Lane 8: 576 bp Lane 9: 483 bp Lane 10: 369 bp Lane 11: 1334 bp (full-length) Lane M: 200 bp DNA ladder

2. Determination of recombinant hCgA clones by colony PCR

The PCR products were purified and eluted from agarose gel electrophoresis. The various sizes of PCR products were cloned into the TA plasmid vector and then transformed into competent *E.coli* DH5α cells. The amplified full-length and the truncated hCgA cloned in the recombinant plasmid was confirmed by colony PCR using the M13 primer which provided the expected sizes approximately 1502, 1416, 1290, 1194, 1104, 993, 906, 801, 744, 651, 537 bp on agarose gel electrophoresis (Figure 11). For colony PCR, M13 were used so these sizes of colony PCR product would be higher than PCR product from the specific primers by 168 bp.

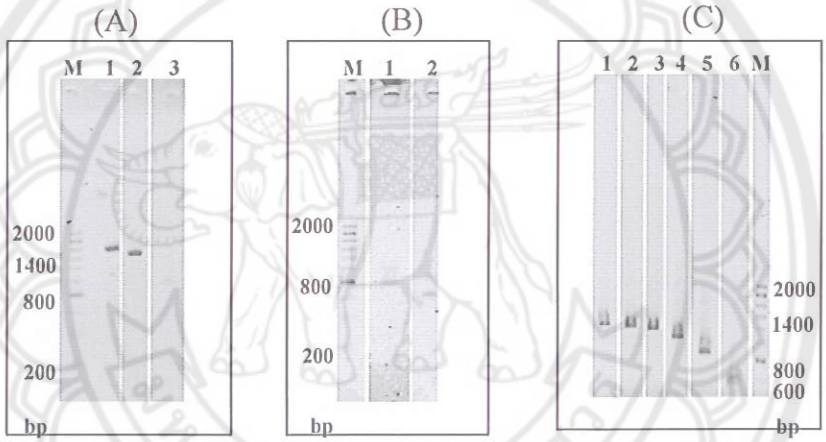

Figure 11 The 1% agarose gel electrophoresis of colony PCR of the TA-full-length and the truncated human chromogranin A recombinant clones

(A) Lane M: 200 bp DNA ladder
Lane 1: colony PCR TA-hCgA 1502 bp plasmid (full-length)
Lane 2: colony PCR TA-hCgA 1416 bp plasmid
Lane 3: colony PCR TA-hCgA 906 bp plasmid

(B) Lane M: 200 bp DNA ladder
Lane 1: colony PCR TA-hCgA 744 bp plasmid
Lane 2: colony PCR TA- hCgA 651 bp plasmid

(C) Lane 1: colony PCR TA-hCgA 1290 bp plasmid

Lane 2: colony PCR TA-hCgA 1194 bp plasmid

Lane 3: colony PCR TA- hCgA 1104 bp plasmid

Lane 4: colony PCR TA- hCgA 993 bp plasmid

Lane 5: colony PCR TA- hCgA 801 bp plasmid

Lane 6: colony PCR TA- hCgA 537 bp plasmid

Lane M: 200 bp DNA ladder

3. DNA digestion and Restriction analysis

The full-length and the truncated hCgA frangments were cloned into a TA plasmid, which was digested by *Nde* I and *Xho* I restriction enzymes to obtain the restriction sites. The pET21b(+) plasmid was digested as describe in method. The restriction mixture was determined gel electrophoresis to confirm correct insert sizes as shown in Figure 12.

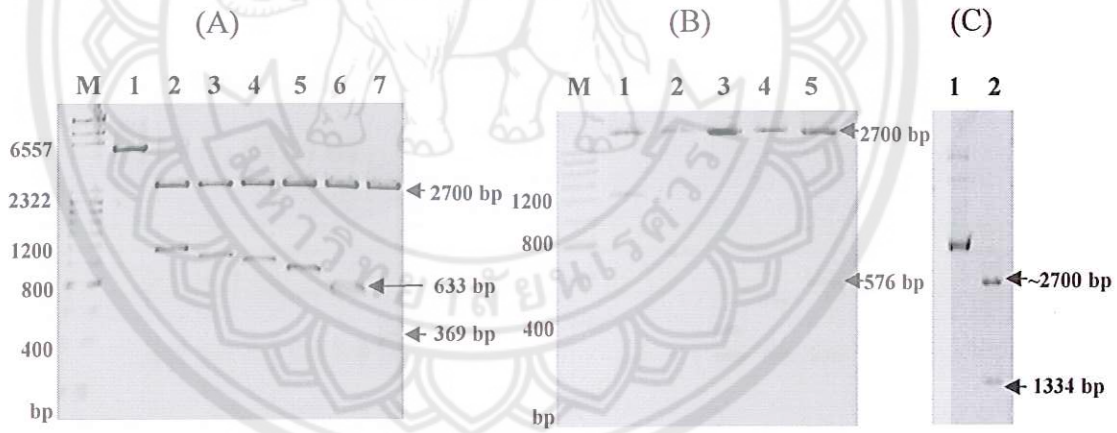

Figure 12 The 1% agarose gel electrophoresis of the full-length and the truncated human chromogranin A recombinant clones

(A) Lane M: 200 bp DNA ladder and λ HindIII ladder
Lane 1: digested pET21b(+)
Lane 2: digested TA- hCgA1122 bp plasmid
Lane 3: digested TA- hCgA1026 bp plasmid
Lane 4: digested TA- hCgA936 bp plasmid
Lane 5: digested TA- hCgA825 bp plasmid
Lane 6: digested TA- hCgA633 bp plasmid
Lane 7: digested TA- hCgA369 bp plasmid
(B) Lane M: 200 bp DNA ladder
Lane 1: digested TA- hCgA1248 bp plasmid
Lane 2: digested TA- hCgA738 bp plasmid
Lane 3: digested TA- hCgA369 bp plasmid
Lane 4: digested TA- hCgA483 bp plasmid
Lane 5: digested TA- hCgA576 bp plasmid
(C) Lane 1: TA- hCgA1334 bp plasmid (full-length)
Lane 2: digested TA- hCgA1334 bp plasmid

4. DNA sequencing and sequencing identification

DNA sequencing of TA-hCgA full-length recombinant plasmid showed 98.5 % identity to the known DNA sequence of hCgA gene (NCBI, Genebank accession NM_001275.3). The DNA sequence is shown in Figure 13. The mismatch was observed at nucleotide #1278, which does not affect the translated protein product.

Although a change in the DNA sequence occurs, this sequence does not change the protein that is to be produced. This is because multiple genetic codons can encode for the same amino acid. Amino acids are coded for by three nucleotide sets called codons (National Human Genome Research Institute). For this change, the amino acid glutamic acid is coded for by several DNA codons including GAA and GAG. The DNA sequence GAA is changed to GAG, the amino acid glutamic acid will still be produced. The Pairwise Sequence Alignment, EMBOSS Needle program was used to produce the sequencing results. The result shows sequence similar to CgA protein. The hCgA protein is shown in Figure 14.

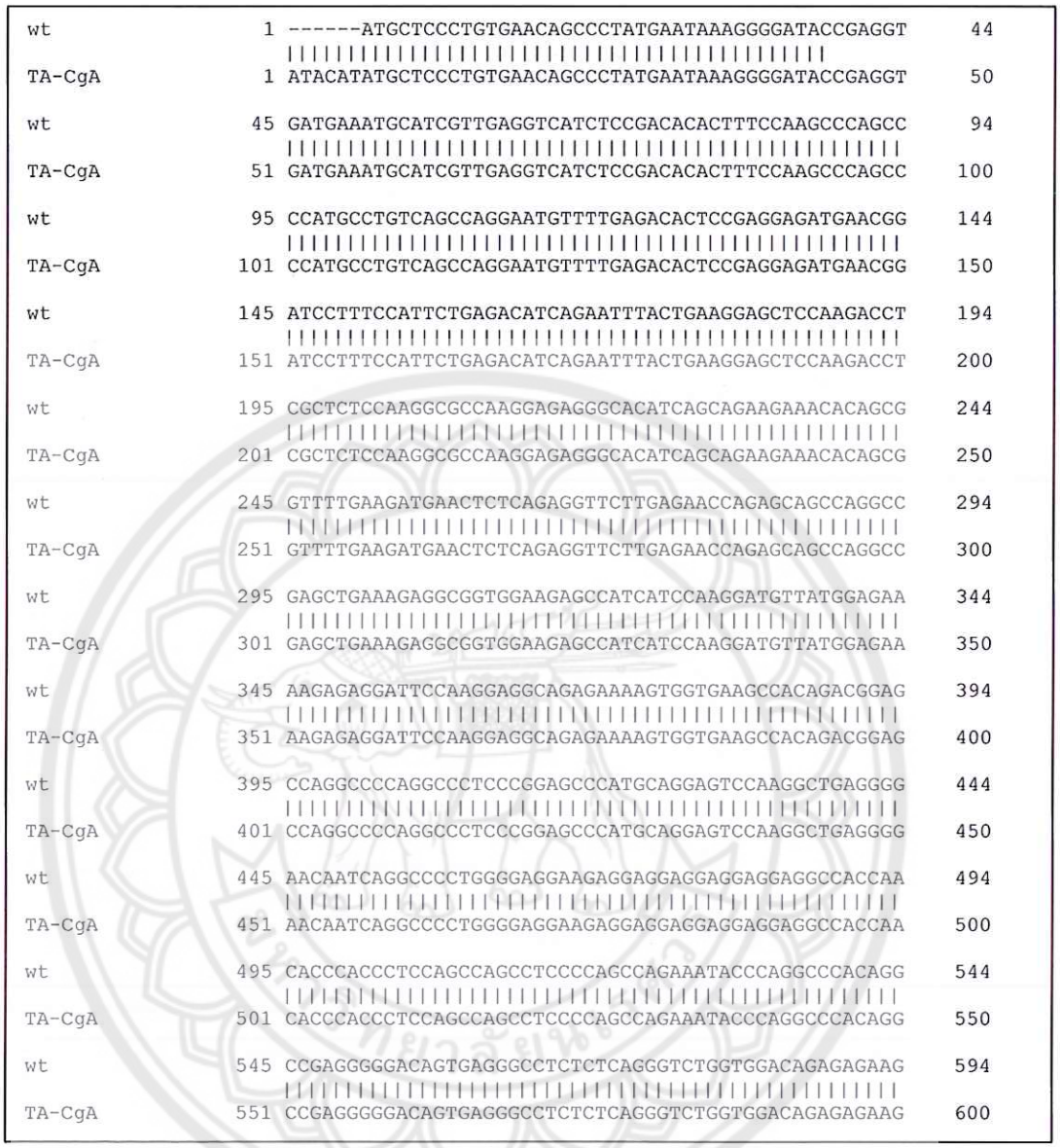

Figure 13 Alignment between the full-length *Homo sapiens* chromogranin A gene in TA plasmid without signal peptide and stop codon for in vitro expression

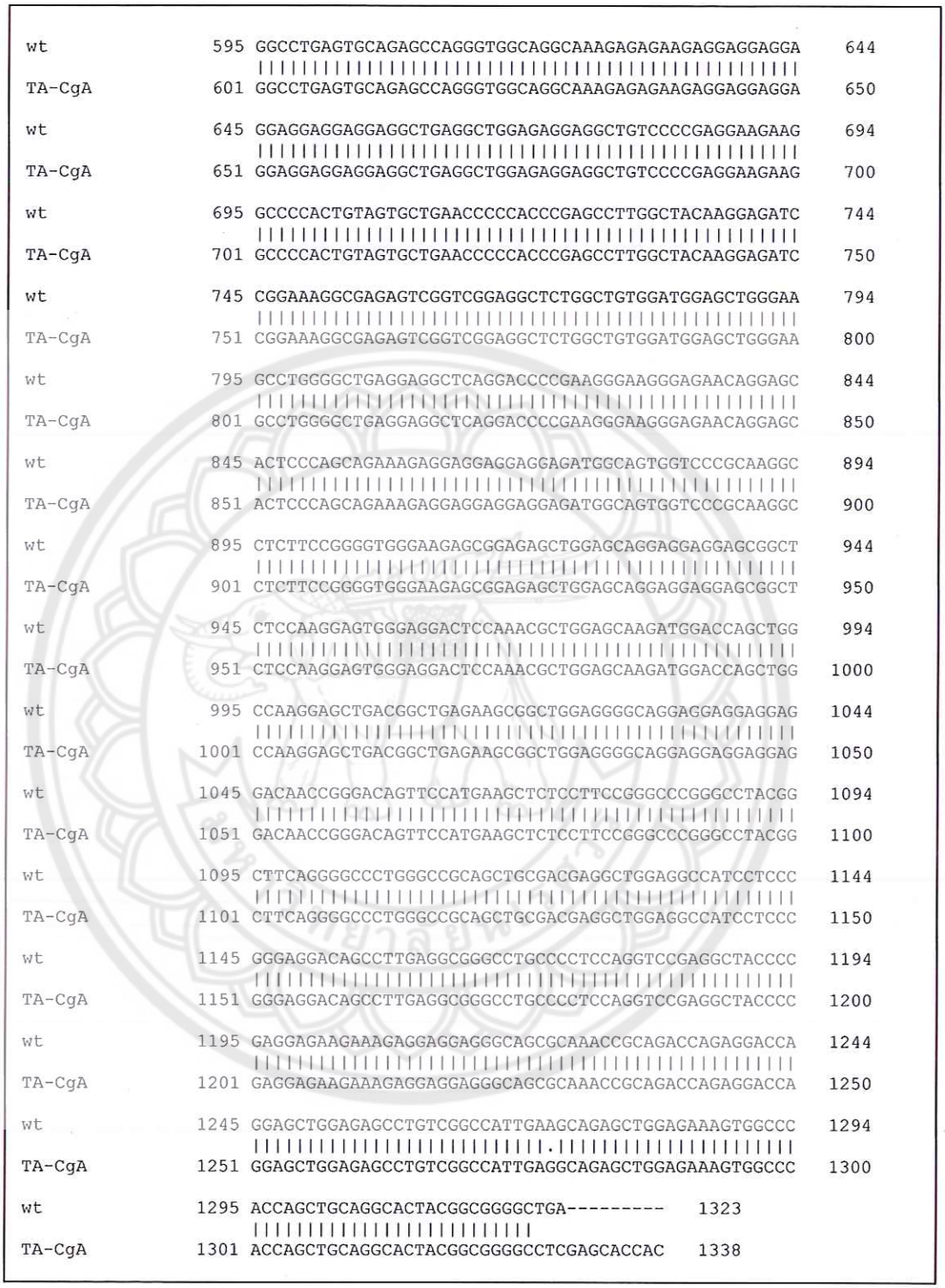

Figure 13 (Cont.)

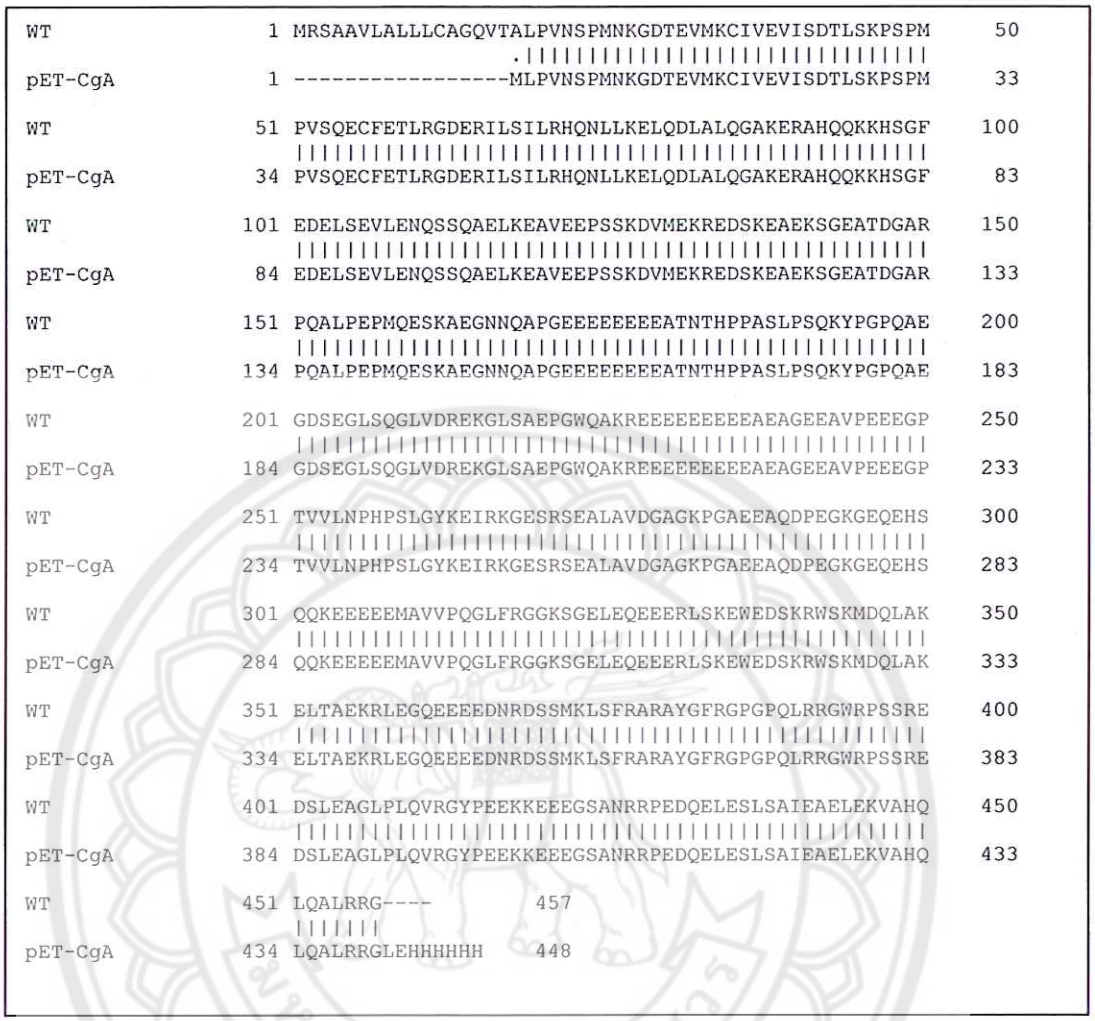

Figure 14 Alignment between the amino acid sequence of full-length human chromogranin A in pET21b(+) and wide type human chromogranin A without signal peptide and stop codon for in vitro expression

Expression of recombinant proteins in *Escherichia coli*

1. Construction of pET21b(+)-human chromogranin A gene and screening of recombinant clones by colony PCR.

The TA-full-length and the truncated hCgA recombinant plasmids and pET21b(+) plasmid were digested by The *Nde* I and *Xho* I restriction enzyme. The digested DNAs were purified and eluted from the gel. The purified DNAs were ligated between *Nde* I and *Xho* I site of the expression vector pET21b (+). The ligated product was transformed into *E.coli* strain BL21 (DE3)pLysS. The full-length and truncated hCgA genes were expressed by transcription of the T7 promoter from pET21b(+) plasmid. The recombinant clones were confirmed by colony PCR using specific primers shown in Table 6 providing the expected DNA sizes approximately 1334 bp (full-length), 1248 bp, 1122 bp, 1026 bp, 936 bp, 825 bp, 738 bp, 633 bp, 576 bp, 483 bp, 369 bp on agarose gel electrophoresis as shown in Figure 15. The full-length and truncated hCgA genes were successfully cloned into the pET21b(+) expression plasmid without its signal peptide.

Figure 15 The 1% agarose gel electrophoresis of colony PCR of the pET21b(+)-full-length and the truncated human chromogranin A recombinant clones

Lane M: 200 bp DNA ladder

Lane 1: PCR pET21b(+)-1338 bp (wild-type)

Lane 2: colony PCR pET21b(+)-hCgA 1334 bp (full-length)

Lane 3: colony PCR pET21b(+)-hCgA 1248 bp

Lane 4: colony PCR pET21b(+)-hCgA 1122 bp

Lane 5: colony PCR pET21b(+)-hCgA 1026 bp

Lane 6: colony PCR pET21b(+)-hCgA 936 bp

Lane 7: colony PCR pET21b(+)-hCgA 825 bp

Lane 8: colony PCR pET21b(+)-hCgA 738 bp

Lane 9: colony PCR pET21b(+)-hCgA 633 bp

Lane 10: colony PCR pET21b(+)-hCgA 576 bp

Lane 11: colony PCR pET21b(+)-hCgA 483 bp

Lane 12: colony PCR pET21b(+)-hCgA 369 bp

2. Expression of the full-length chromogranin A and its variants in *E. coli* BL21 (DE3)pLysS

Effect of induction time on the truncated recombinant hCgA protein expression is shown in Figure 16. The truncated recombinant hCgA proteins were expressed in *E. coli* strains BL21(DE3)pLysS and the highest expression was obtained after induction at 37 °C with addition of 1 mM IPTG for 3 h as shown that lane no. 4 of 10% SDS-PAGE.

The hCgA gene is coded by a unique gene coding for a protein of 457 amino acid residues; the first 18 residues represent the signal peptide giving rise to 439 amino acid of the mature protein (Mouland, et al., 1994). The truncated hCgA clones encode for proteins of 419, 377, 345, 315, 278, 249, 214, 195, 126 amino acid residues.

In the overproduction study of the full length and truncated hCgA proteins, pET21b(+) was used. The full length and truncated hCgA proteins were expressed as C-terminal 6His-tag fusion. The 6His-tag generally has no significant effect on the native protein structure and facilitates binding to a nickel affinity column (Carson, et al., 2007).

In this study hCgA proteins were expressed in *E. coli* strains BL21(DE3) pLysS and the highest expression was obtained after induction at 37 °C for 3 h after the addition of 1mM IPTG. The hCgA gene products may be toxic to the cells and lead to instability of *E.coli* host cells after 3 h post-induction, hence reduced the amount of the recombinant hCgA protein detected.

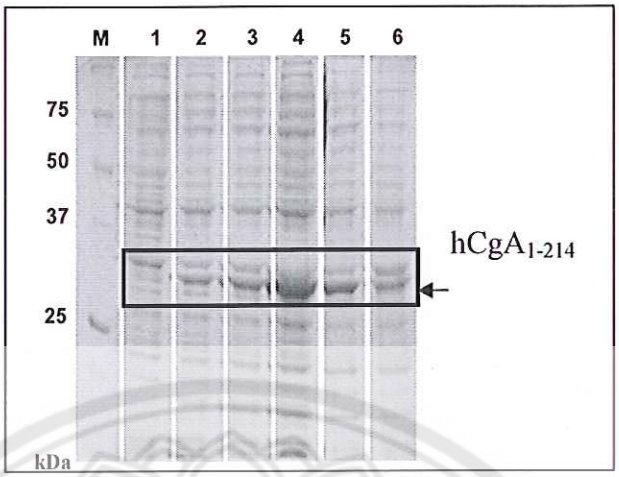

Figure 16 Effect of induction time on the truncated recombinant hCgA$_{1-214}$ proteins expression

Lane M: protein markers; Lane 1: pET21b(+)-hCgA clone, non IPTG induction ; Lane 2 to Lane 6 are pET21b(+)-hCgA clone after induction for 1 h, 2 h, 3 h, 4 h and 24 h consequently.

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

Effect of heating time on the truncated recombinant hCgA proteins was shown in Figure 17. The heat-stable CgA$_{1-419}$ of protein were analyzed by SDS-PAGE and characterized by western blotting. The blotting of heat-stable CgA$_{1-419}$ protein was recognized by monoclonal antibodies against 6 His-tagged protein. The heated full-length and the truncated hCgA recombinant were analyzed by 10% SDS-PAGE found that the expected sizes approximately 70, 67, 60, 55, 49, 44, 40, 34, 31, 26 and 20 kDa. (as shown in Figure 18)

Chromogranins constitute a family of highly acidic, heat stable glycoproteins originally from chromaffin granules of the adrenal medulla. Chromogranins A (CgA) is one of the most characterized members of this family and is present in endocrine tissue (Simon and Aunis, 1989). In order to evaluate heat-stable of the truncated recombinant hCgA proteins, the recombinant hCgA proteins were boiled at 100 ^{o}C in order to exclude other proteins. Therefore, the starting motived prior to the chosen affinity column chromatography can be performed with better

efficiency. The heated full-length and the truncated hCgA recombinant were analyzed by electrophoresis, and the results showed expected proteins of $hCgA_{1-448}$ (full-length), $hCgA_{1-419}$, $hCgA_{1-377}$, $hCgA_{1-345}$, $hCgA_{1-315}$, $hCgA_{1-278}$, $hCgA_{1-249}$, $hCgA_{1-214}$, $hCgA_{1-195}$, $hCgA_{1-164}$ and $hCgA_{1-126}$.

The mature hCgA is a single polypeptide chain, the protein is composed of 439 amino acid long protein with a molecular mass of 46-50 kDa for the unmodified protein (Bruno, et al., 2006; Mouland, et al., 1994). The difference between the apparent and real molecular mass of CgA possibly arises from large acidic amino acid composition of CgA which may inhibit SDS binding, resulting in decreased SDS-PAGE mobility and hence increased apparent molecular mass (Eskeland, et al., 1996).

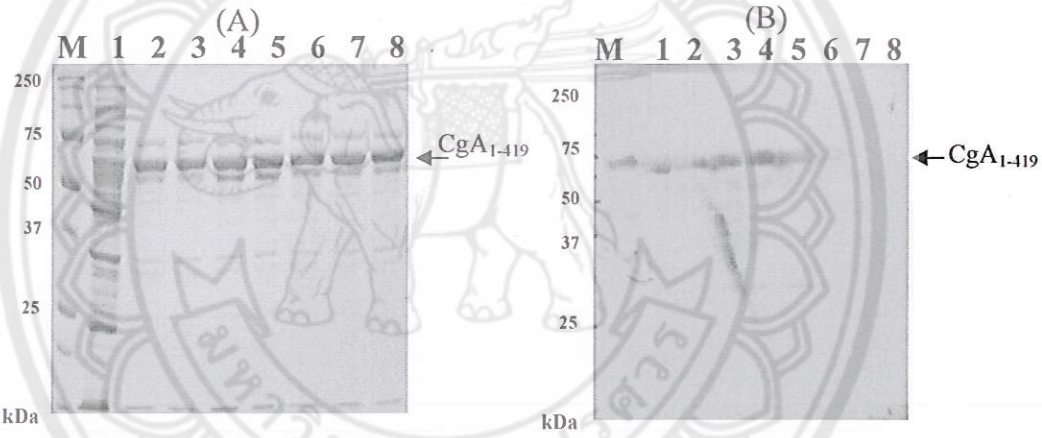

Figure 17 The heat-stable $hCgA_{1-419}$ of proteins were analyzed by 10% SDS-PAGE (A) and characterized by western blotting (B)

Lane M: protein markers; Lane 1: pET21b(+)-hCgA clone, non heating; Lane 2 to Lane 8 are pET21b(+)-hCgA clone after heating for 5, 10, 15, 20, 25, 30 and 35 min, consequently.

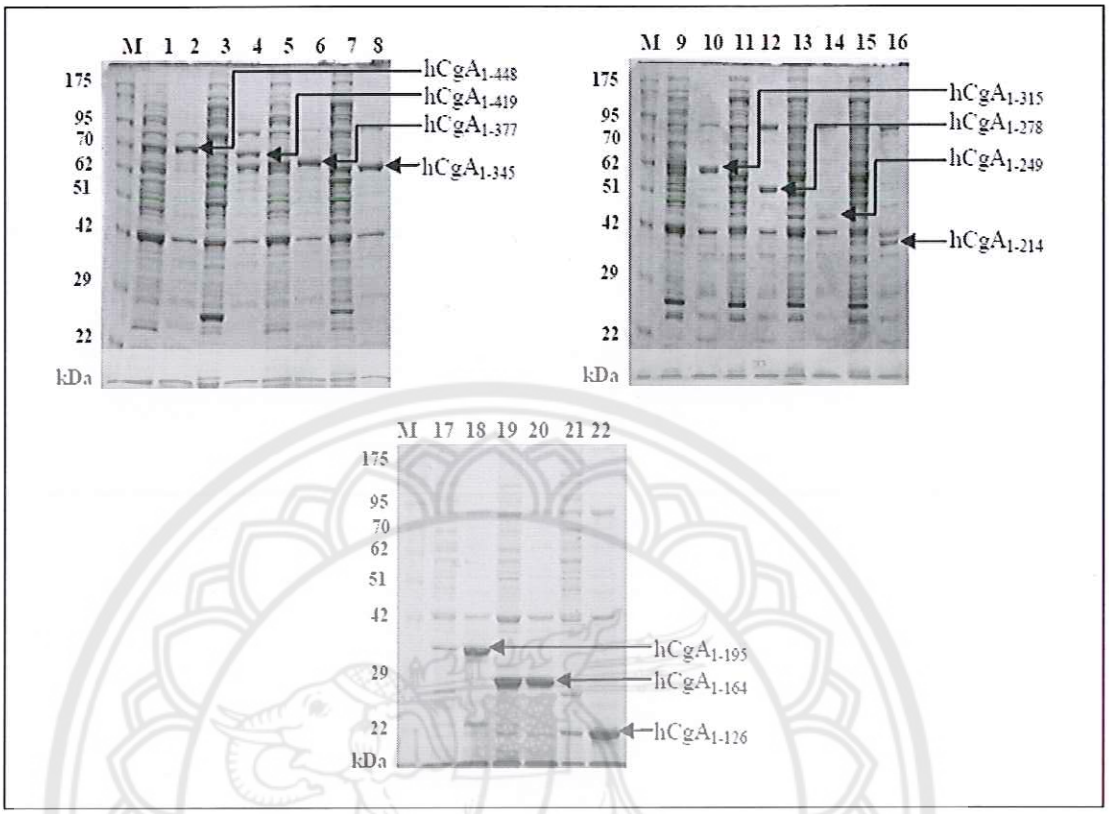

Figure 18 **Expression of human chromogranin A proteins lysates from *E. coli* BL21(DE3) pLysS cells containing pET21b(+)-hCgA recombinant plasmids. The heat-stable full-length and the truncated human chromogranin A recombinant protein were analyzed by 10% SDS-PAGE**

Lane M: protein markers

Lane 1: The $hCgA_{1-448}$ protein (full-length) non heating

Lane 2: The $hCgA_{1-448}$ protein (full-length) after heating for 10 min

Lane 3: The $hCgA_{1-419}$ protein non heating

Lane 4: The $hCgA_{1-419}$ protein after heating for 10 min

Lane 5: The $hCgA_{1-377}$ protein non heating

Lane 6: The $hCgA_{1-377}$ protein after heating for 10 min

Lane 7: The $hCgA_{1-345}$ protein non heating

Lane 8: The $hCgA_{1-345}$ protein after heating for 10 min

Lane 9: The $hCgA_{1-315}$ protein non heating

Lane 10: The $hCgA_{1-315}$ protein after heating for 10 min

Lane 11: The $hCgA_{1-278}$ protein non heating

Lane 12: The $hCgA_{1-278}$ protein after heating for 10 min

Lane 13: The $hCgA_{1-249}$ protein non heating

Lane 14: The $hCgA_{1-249}$ protein after heating for 10 min

Lane 15: The $hCgA_{1-214}$ protein non heating

Lane 16: The $hCgA_{1-214}$ protein after heating for 10 min

Lane 17: The $hCgA_{1-195}$ protein non heating

Lane 18: The $hCgA_{1-195}$ protein after heating for 10 min

Lane 19: The $hCgA_{1-164}$ protein non heating

Lane 20: The $hCgA_{1-164}$ protein after heating for 10 min

Lane 21: The $hCgA_{1-126}$ protein non heating

Lane 22: The $hCgA_{1-126}$ protein after heating for 10 min

4. Western blot analysis of the expression of recombinant pET-21b(+)-human chromogranin A protein and variants in *E.coli* BL21(DE3)pLysS

The corrected recombinant protein was confirmed by western blot analysis. The proteins were recognized by monoclonal antibodies against 6 His-tagged proteins. These results revealed that hCgA proteins were expressed as a fusion protein with the C-terminal (6 His) as shown in Figure 19. The protein sample were also characterized by western blotting using mouse Anti-chromogranin A monoclonal antibody that against hCgA. The full-length and truncated hCgA protein reacted with the antibodies, as shown in Figure 20.

The full-length and the truncated recombinant human chromogranin A were characterized by Western blotting using two different antibodies. All rechCgA fragments were recognized by mouse Anti-Histidine(6X) antibody, as expected confirming the integrity of 6 His-tagged recombinant proteins. However, truncated proteins smaller than $hCgA_{1-315}$ were not recognized by mouse anti-chromogranin A monoclonal antibody. This result suggested that the monoclonal antibody recognized the C-terminal region of the hCgA protein.

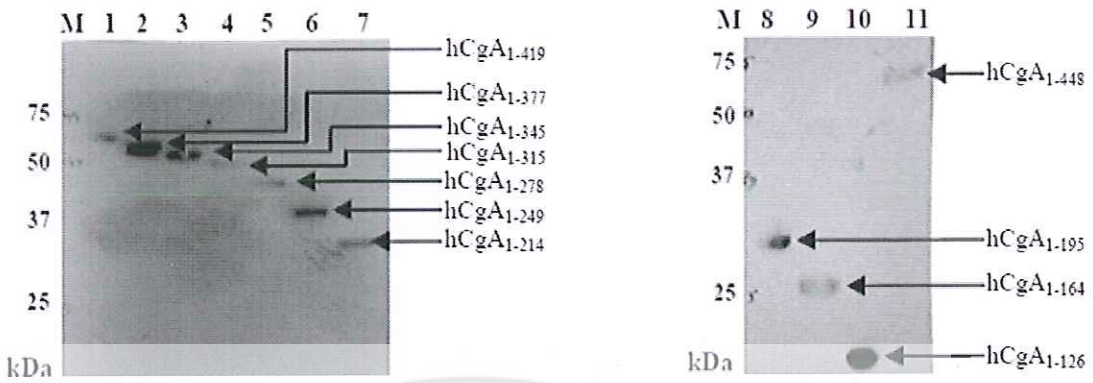

Figure 19 Western blot analysis of full-length and truncated recombinant human CgA proteins were characterized by mouse anti-Histidine (6X) antibody

Lane M: protein markers
Lane 1: The $hCgA_{1-419}$ protein
Lane 2: The $hCgA_{1-377}$ protein
Lane 3: The $hCgA_{1-345}$ protein
Lane 4: The $hCgA_{1-315}$ protein
Lane 5: The $hCgA_{1-278}$ protein
Lane 6: The $hCgA_{1-249}$ protein
Lane 7: The $hCgA_{1-214}$ protein
Lane 8: The $hCgA_{1-195}$ protein
Lane 9: The $hCgA_{1-164}$ protein
Lane 10: The $hCgA_{1-126}$ protein
Lane11: The $hCgA_{1-448}$ protein (full-length)

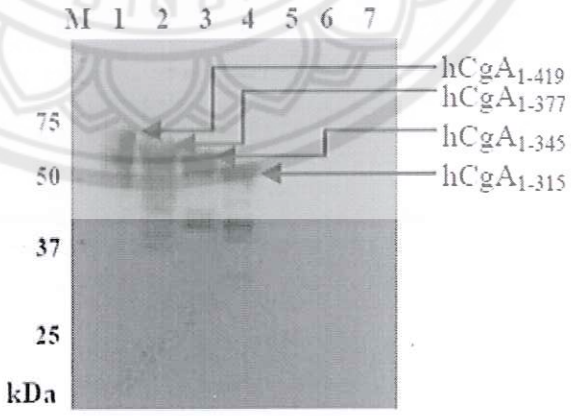

Figure 20 Western blot analysis of truncated recombinant human CgA proteins were characterized by mouse Anti-chromogranin A monoclonal antibody

Lane M: protein markers
Lane1: The $hCgA_{1-419}$ protein
Lane 2: The $hCgA_{1-377}$ protein
Lane 3: The $hCgA_{1-345}$ protein
Lane 4: The $hCgA_{1-315}$ protein
Lane 5: The $hCgA_{1-278}$ protein
Lane 6: The $hCgA_{1-249}$ protein
Lane 7: The $hCgA_{1-214}$ protein

Purification of recombinant human chromogranin A protein by affinity column chromatography

The purification method described by Ugendra Kumar (Ugendra, 1997) was modified to purify hCgA. Fraction containing hCgA were identified on polyacrylamide gel electrophoresis as shown in Figure 21-28. After the affinity column chromatography purification, proteins were purified by electroelution. The indeed purified full-length and truncated hCgA proteins were identified by polyacrylamide gel electrophoresis as shown in Figure 29.

The CgA is the major soluble and heat-stable protein that was secreted from the secretory granules of chromaffin cells of adrenal medulla (Huttner, et al., 1991). The purification of full-length and truncated hCgA proteins was performed by a three-step protocol. The recombinant proteins were first processed by a heat step. Most of the bacterial proteins were precipitated by centrifugation, and the supernatant contained hCgA and heat-stable proteins of the cell lysate. Then the recombinant full-length and truncated hCgA proteins (C-terminal contain 6His-tag fusion) can be purified by Ni^{2+} affinity column chromatography. The 6His-tag specifically binds to Ni^{2+} and the trapped proteins can be eluted by imidazole, which competitively bind to Ni^{2+}. The recombinant full-length and truncated hCgA proteins were eluted by 250 mM imidazole. In order to obtain improve the purity of purified recombinant proteins, full-length and truncated hCgA proteins were finally purified by electroelution. The purified recombinant proteins were used in assays in order to define the region responsible for the plasminogen activation property.

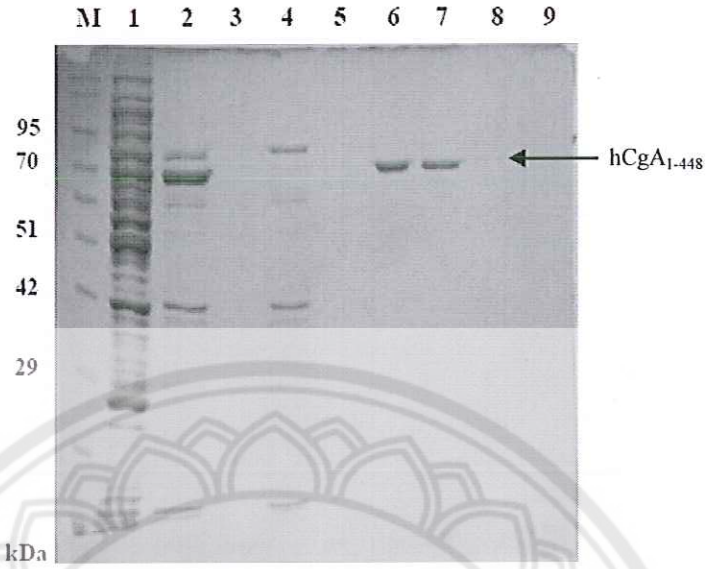

Figure 21 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-448}$ by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify $hCgA_{1-448}$ protein.

Lane M: protein markers

Lane 1: The $hCgA_{1-448}$ protein (full-length) non heating

Lane 2: The $hCgA_{1-448}$ protein (full-length) after heating for 10 min

Lane 3: Soluble protein before loading into column

Lane 4: Flow-through fraction (unbound protein)

Lane 5: Wash fraction

Lane 6: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 7: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 8: Elution fraction 3 (Elution buffer containing 350 mM imidazole)

Lane 9: Elution fraction 4 (Elution buffer containing 350 mM imidazole)

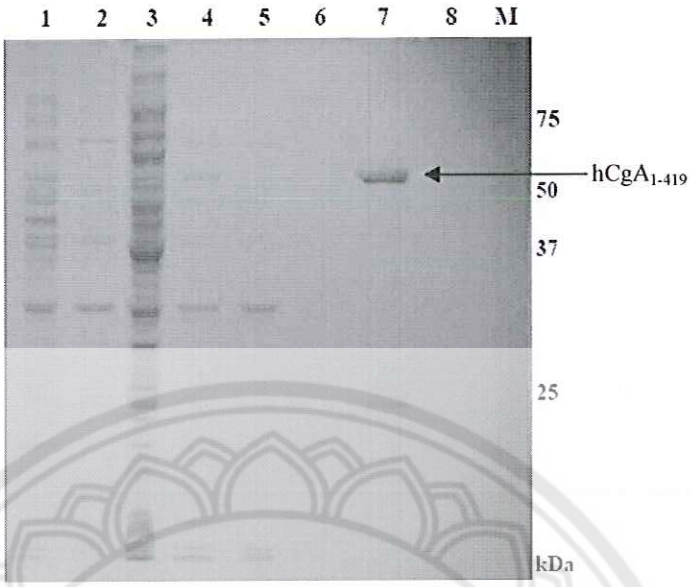

Figure 22 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-419}$ by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify $hCgA_{1-419}$ protein.

Lane 1: The CgA_{1-419} protein non induction

Lane 2: The $hCgA_{1-419}$ protein non heating

Lane 3: The $hCgA_{1-419}$ protein after heating for 10 min

Lane 4: Soluble protein before loading into column

Lane 5: Flow-through fraction (unbound protein)

Lane 6: Wash fraction

Lane 7: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 8: Elution fraction 2 (Elution buffer containing 350 mM imidazole)

Lane 9: Elution fraction 3 (Elution buffer containing 500 mM imidazole)

Lane M: protein markers

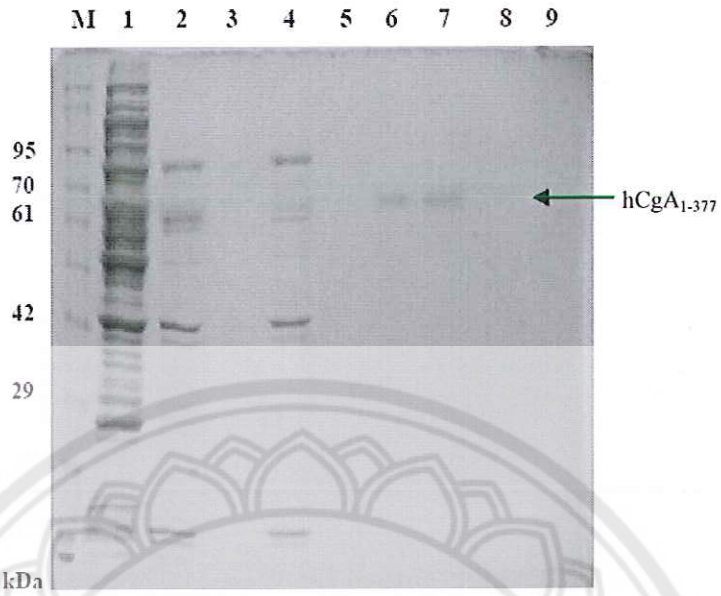

Figure 23 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-377}$ by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify $hCgA_{1-377}$ protein.

Lane M: protein markers

Lane 1: The $hCgA_{1-377}$ protein non heating

Lane 2: The $hCgA_{1-377}$ protein after heating for 10 min

Lane 3: Soluble protein before loading into column

Lane 4: Flow-through fraction (unbound protein)

Lane 5: Wash fraction

Lane 6: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 7: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 8: Elution fraction 3 (Elution buffer containing 350 mM imidazole)

Lane 9: Elution fraction 4 (Elution buffer containing 350 mM imidazole)

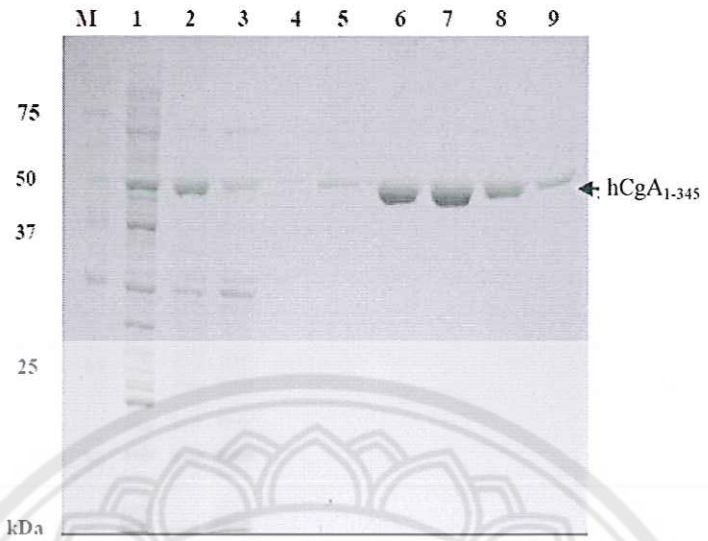

Figure 24 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-345}$ by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify CgA_{1-345} protein.

Lane M: protein markers

Lane 1: The $hCgA_{1-345}$ protein non heating

Lane 2: The $hCgA_{1-345}$ protein after heating for 10 min

Lane 3: Flow-through fraction (unbound protein)

Lane 4: Wash fraction

Lane 5: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 6: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 7: Elution fraction 3 (Elution buffer containing 250 mM imidazole)

Lane 8: Elution fraction 4 (Elution buffer containing 250 mM imidazole)

Lane 9: Elution fraction 5 (Elution buffer containing 250 mM imidazole)

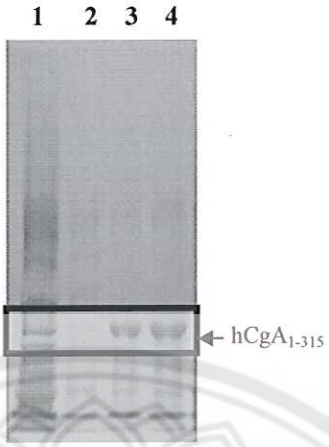

Figure 25 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-315}$ by affinity column chromatography.

The Ni^{2+} affinity chromatography utilizing imidazole was used to purify $hCgA_{1-315}$ protein.

Lane 1: Soluble protein before loading into column

Lane 2: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 3: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 4: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

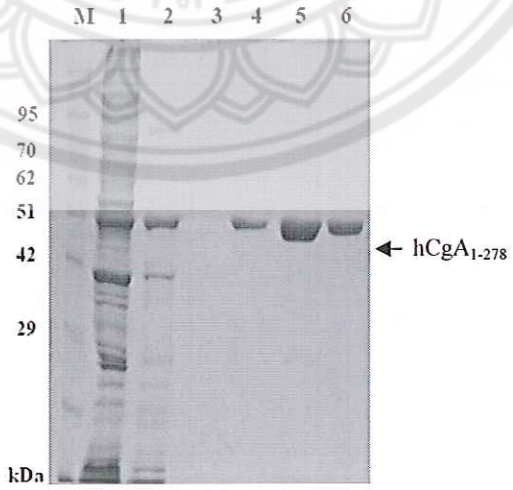

Figure 26 SDS-PAGE analysis of purified recombinant hCgA proteins of the CgA_{1-278} by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify $hCgA_{1-278}$ protein.

Lane M: protein markers

Lane 1: The $hCgA_{1-278}$ protein non heating

Lane 2: The $hCgA_{1-278}$ protein after heating for 10 min

Lane 3: Flow-through fraction (unbound protein)

Lane 4: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 5: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 6: Elution fraction 3 (Elution buffer containing 250 mM imidazole)

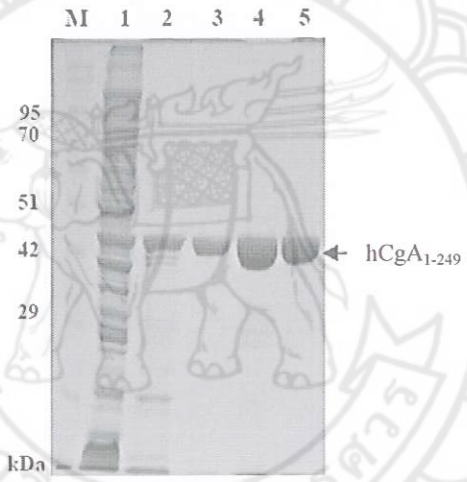

Figure 27 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-249}$ by affinity column chromatography.

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify $hCgA_{1-249}$ protein.

Lane M: protein markers

Lane 1: The $hCgA_{1-249}$ protein non heating

Lane 2: The $hCgA_{1-249}$ protein after heating for 10 min

Lane 3: Elution fraction 1 (Elution buffer containing 250 mM imidazole)

Lane 4: Elution fraction 2 (Elution buffer containing 250 mM imidazole)

Lane 5: Elution fraction 3 (Elution buffer containing 250 mM imidazole)

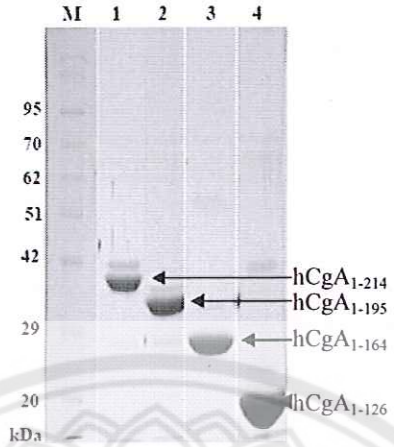

Figure 28 SDS-PAGE analysis of purified recombinant hCgA proteins of the $hCgA_{1-214}$, $hCgA_{1-195}$, $hCgA_{1-164}$, $hCgA_{1-126}$ by affinity column chromatography

The Ni^{2+}affinity chromatography utilizing imidazole was used to purify the $hCgA_{1-214}$, $hCgA_{1-195}$, $hCgA_{1-164}$, $hCgA_{1-126}$ protein.

Lane M: protein markers

Lane 1: Elution fraction 1 (250 mM imidazole) of $hCgA_{1-214}$ protein

Lane 2: Elution fraction 1 (250 mM imidazole) of $hCgA_{1-195}$ protein

Lane 3: Elution fraction 1 (250 mM imidazole) of $hCgA_{1-164}$ protein

Lane 4: Elution fraction 1 (250 mM imidazole) of $hCgA_{1-126}$ protein

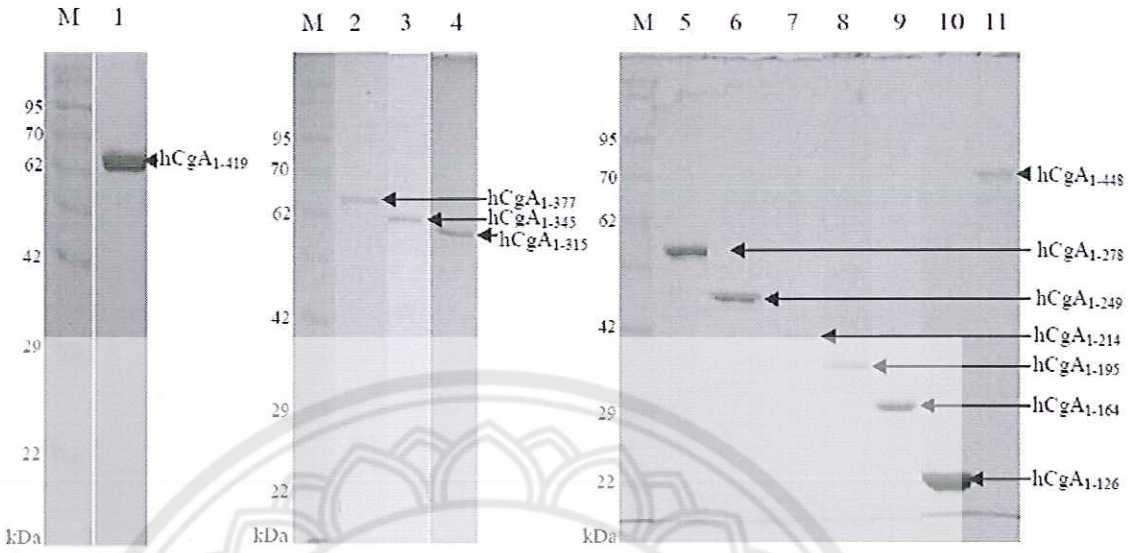

Figure 29 SDS-PAGE analysis of purified recombinant hCgA proteins by electroelution

Lane M: protein markers

Lane 1: Purified $hCgA_{1-419}$ protein after electroelution

Lane 2: Purified $hCgA_{1-377}$ protein after electroelution

Lane 3: Purified $hCgA_{1-345}$ protein after electroelution

Lane 4: Purified $hCgA_{1-315}$ protein after electroelution

Lane 5: Purified $hCgA_{1-278}$ protein after electroelution

Lane 6: Purified $hCgA_{1-249}$ protein after electroelution

Lane 7: Purified $hCgA_{1-214}$ protein after electroelution

Lane 8: Purified $hCgA_{1-195}$ protein after electroelution

Lane 9: Purified $hCgA_{1-164}$ protein after electroelution

Lane 10: Purified $hCgA_{1-126}$ protein after electroelution

Lane 11: Purified $hCgA_{1-448}$ protein (full-length) after electroelution

Plasminogen activation assays and kinetic study

The tPA activated plasminogen to plasmin which was assayed in a extracellular buffer. The rates of plasminogen activation were evaluated by the cleavage of fluorogenic substrate (Boc-Val-Leu-Lys-MCA) to release the free AMC fluoropore. K_m (Micharis-Menten constants) and K_{cat}/K_m (the rates of catalytic efficiency), were calculated. Addition of full-length and truncated recombinant hCgA increased the activation rate of plasminogen by tPA as shown in plasmin activity assay (Figure 30). The $hCgA_{1-249}$ is the best enhancer of the activation rate of conversion of plasminogen to plasmin. Summary of kinetic parameters were calculated as shown in Table 9. At the highest concentration of $hCgA_{1-249}$ (50 nM), the K_m values decreased from 0.53 μM to 0.04 μM, when the catalytic efficiency values (K_{cat}/K_m) were increased from 4,968.75 $\mu M^{-1}S^{-1}$ to 51,335.66 $\mu M^{-1}S^{-1}$.

The C-terminal part and the N-terminal part of CgA possess a disulphide bridge formed by two cysteine residues at amino acid positions 17 and 38 that appears important for several CgA-related biological activities (Konecki, et al., 1987; Lugardon, et al., 2000). Many proteins, such as some membrane and secreted proteins in both bacteria and eukaryotes, fold into their native structures requiring the formation of disulfide bonds. Disulfide bonds are covalent interactions between cysteine residues, and are also vital for the stability and activities of the proteins (Frand, et al., 2000; Qin, et al., 2006).

The first hypothesis is that plasminogen and tPA cannot bind to hCgA when hCgA folds, so plasminogen activation process cannot be enhanced by hCgA (Figure 31).

The second hypothesis is that CgA is a large single polypeptide (Benedum, et al., 1986), therefore, plasminogen and tPA may bind to hCgA on a different sites, consequently tPA cannot convert plasminogen to plasmin (Figure 32).

The presence of numerous paired basic amino acids in granins suggests that they function as prohormones, giving rise to bioactive peptides as a result of post-translational proteolytic processing (Taupenot, et al., 2003). Hence, hCgA may be cleaved by proteolytic enzymes at dibasic residues. Plasminogen and tPA may bind to small fragments of hCgA at the same site, therefore plasminogen activation can occur on hCgA fragment.

The specific binding site of plasminogen and tPA is not understood. This study is aimed at determination of structural region of the recombinant hCgA responsible for the enhancement of plasminogen activation. Therefore hCgA gene was reconstructed by PCR. The compositions of the polypeptide EEIIMD and fibrinolysis agents, which relates to methods of enhancing the fibrinolytic activity, reducing the side effects due to vasoactivity caused by the fibrinolytic agents (Rashida, 2006), contain EE, which is the same component of highly glutamic acid in fibrin. CgA is remarkably heat-stable, hydrophilic and acidic protein with large hydrodynamic volume, mostly in random coil (60–65%) and α helix (25–40%) conformations, due to high content of glutamic acid residues (Yoo, et al., 1990). The $hCgA_{1-249}$, the best enhancer of plasminogen activation in this study, also exhibits high content of glutamic acid.

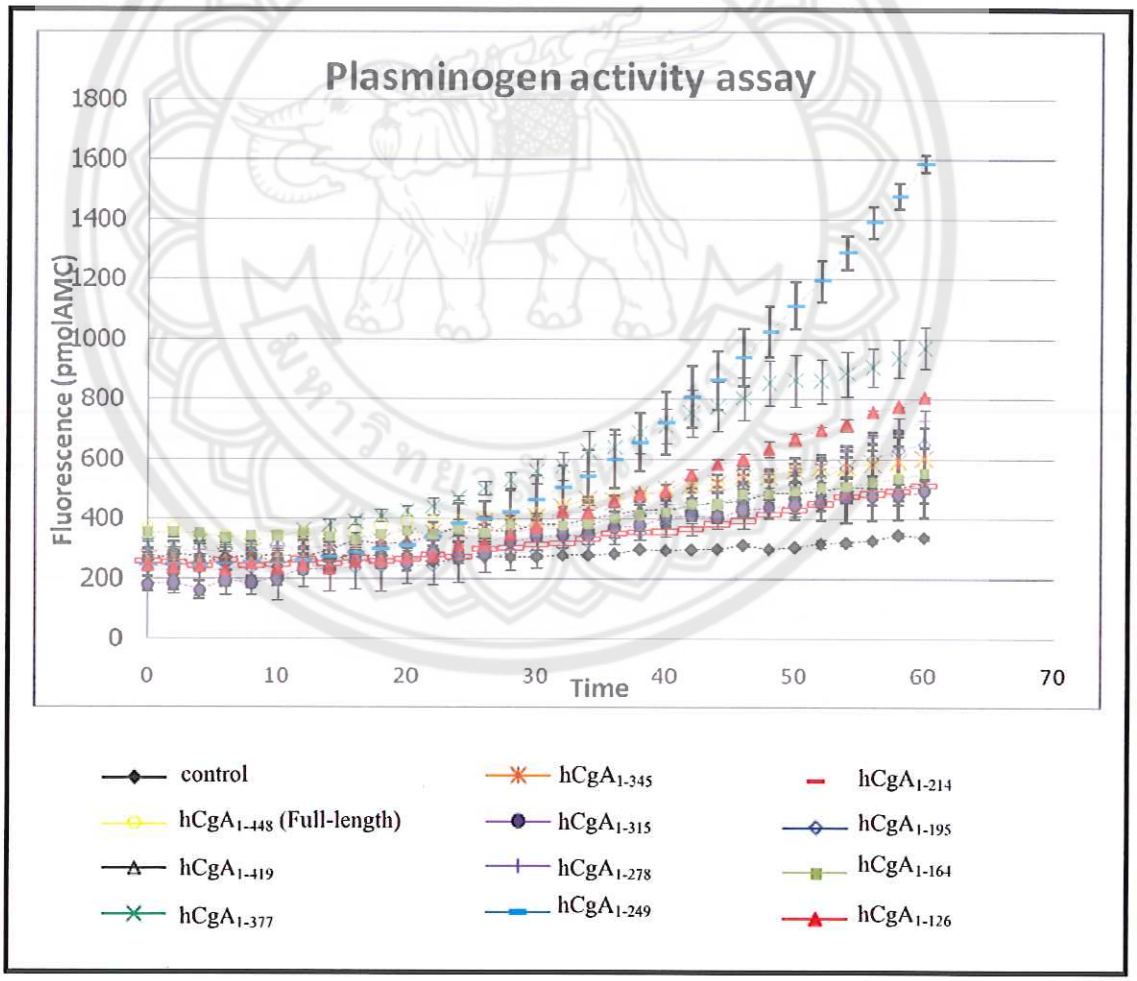

Figure 30 Effect of full-length and truncated recombinant hCgA on plasmin activity assays

Table 9 Summary of kinetic parameters containing K_m, K_{cat}, K_{cat}/K_m and %Efficiency of full-length and truncated recombinant hCgA in plasmin activity assays

rec hCgA (μM)		K_m @pH7.4 (μM)	K_{cat} (S^{-1})	K_{cat} / K_m ($\mu M^{-1}S^{-1}$)	%Efficiency
$hCgA_{1-448}$ (Full-length)	0	0.53	2633.53	4968.75	100.00
	0.01	0.09	360.70	3843.01	77.34
	0.02	0.07	438.43	5993.71	120.63
	0.03	0.06	348.85	5523.45	111.16
	0.04	0.04	546.87	13940.26	280.56
	0.05	0.02	494.76	23832.00	479.64
$hCgA_{1-419}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.33	4092.04	12389.15	249.34
	0.02	0.31	3377.05	10941.77	220.21
	0.03	0.27	3768.12	14047.51	282.72
	0.04	0.26	3747.17	14475.18	291.32
	0.05	0.25	4057.63	15993.94	321.89
$hCgA_{1-377}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.33	1437.85	4353.15	87.61
	0.02	0.28	1292.15	4682.73	94.24
	0.03	0.29	1283.58	4483.66	90.24
	0.04	0.26	3105.26	12025.93	242.03
	0.05	0.25	2845.34	11506.42	231.58
$hCgA_{1-345}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.21	1806.48	8712.58	175.35
	0.02	0.20	1720.02	8760.86	176.32
	0.03	0.16	1709.52	10504.71	211.42
	0.04	0.15	1544.99	9998.24	201.22
	0.05	0.15	1691.29	11356.42	228.56
$hCgA_{1-315}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.25	1985.65	8013.08	161.27
	0.02	0.17	2935.36	17581.44	353.84
	0.03	0.12	2762.79	23521.86	473.40
	0.04	N/A	N/A	N/A	N/A
	0.05	N/A	N/A	N/A	N/A

Table 9 (Cont.)

rec hCgA (μM)		K_m @pH7.4 (μM)	K_{cat} (S^{-1})	K_{cat} / K_m ($\mu M^{-1}S^{-1}$)	%Efficiency
$hCgA_{1-278}$	0	0.53	2633.53	4968.752	100.00
	0.01	0.45	4079.34	9100.92	183.16
	0.02	0.27	3503.95	13045.44	262.55
	0.03	0.26	4270.06	16607.40	334.24
	0.04	0.24	3930.20	16343.57	328.93
	0.05	0.23	3715.69	16287.26	327.79
$hCgA_{1-249}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.28	3696.54	13174.94	265.16
	0.02	0.24	4170.53	17436.87	350.93
	0.03	0.11	2357.61	20516.67	412.91
	0.04	0.09	2639.93	29607.48	595.87
	0.05	0.04	2184.53	51335.66	1033.17
$hCgA_{1-214}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.37	2768.60	7395.70	148.84
	0.02	0.26	2847.35	10931.15	220.00
	0.03	0.16	1979.60	12701.60	255.63
	0.04	0.12	1870.21	15319.55	308.32
	0.05	0.08	1566.45	19742.97	397.34
$hCgA_{1-195}$	0	0.53	2633.53	4968.752	100.00
	0.01	0.33	1016.36	3039.632	61.17
	0.02	0.05	559.70	10630.48	213.95
	0.03	0.03	400.98	12337.56	248.30
	0.04	0.02	474.96	20493.84	412.45
	0.05	0.02	419.07	20579.98	414.19
$hCgA_{1-164}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.23	1912.32	8330.77	167.66
	0.02	0.16	4312.77	26727.53	537.91
	0.03	0.15	4388.03	29579.35	595.31
	0.04	0.13	4057.35	30993.35	623.76
	0.05	0.11	3606.06	32448.79	653.06

Table 9 (Cont.)

rec hCgA (μM)		K_m @pH7.4 (μM)	K_{cat} (S^{-1})	K_{cat} / K_m ($\mu M^{-1}S^{-1}$)	%Efficiency
$hCgA_{1-126}$	0	0.53	2633.53	4968.75	100.00
	0.01	0.39	1491.16	3838.47	77.25
	0.02	0.34	2601.88	7530.31	151.55
	0.03	0.26	1822.52	6920.09	139.27
	0.04	0.25	1877.20	7537.53	151.70
	0.05	0.21	1929.01	9070.68	182.55

Note: This table shows normalization of K_m and K_{cat}.

N/A is not available

% Efficiencies were calculated by using the k_{cat}/K_m values of no hCgA as 100%.

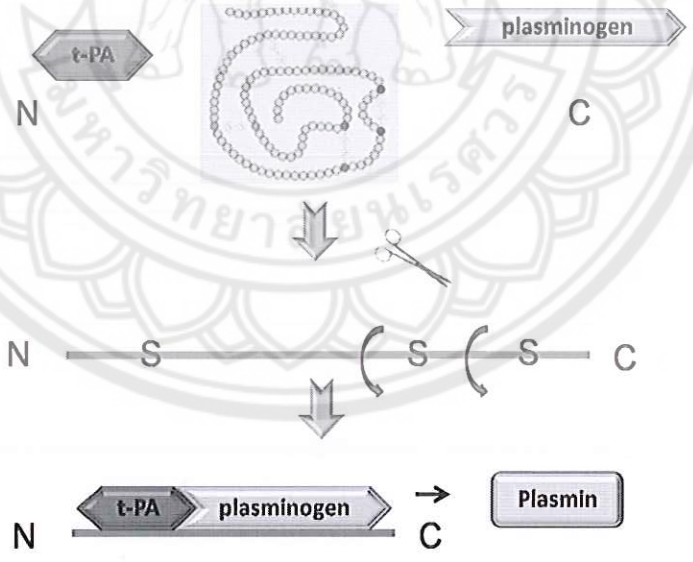

Figure 31 Proposed effect of protein folding of hCgA on plasminogen activation

Source: Department of Chemistry The University of Maine, 2002

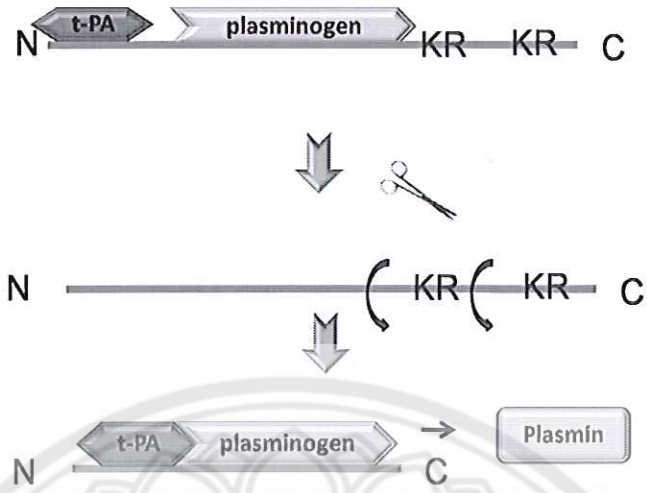

Figure 32 Proposed effects of paired dibasic residues of hCgA on plasminogen activation