CHAPTER IV

RESULTS

Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from various tissues of human, buccal mucosal cell, white blood cell and neuroblastoma cell line (SH-SY5Y) by using TRI REAGENT. The quality of total RNA was electrophoretically determined by using 2% agarose gel. Predominant discrete bands were observed along with smeared high molecular weight RNA (Figure 11). The ratio of OD_{260}/OD_{280} of purified RNA were 1.9-2.0. cDNA synthesized from total RNA cover the large sized products indicating the acceptable quality of the synthesized first strand cDNA.

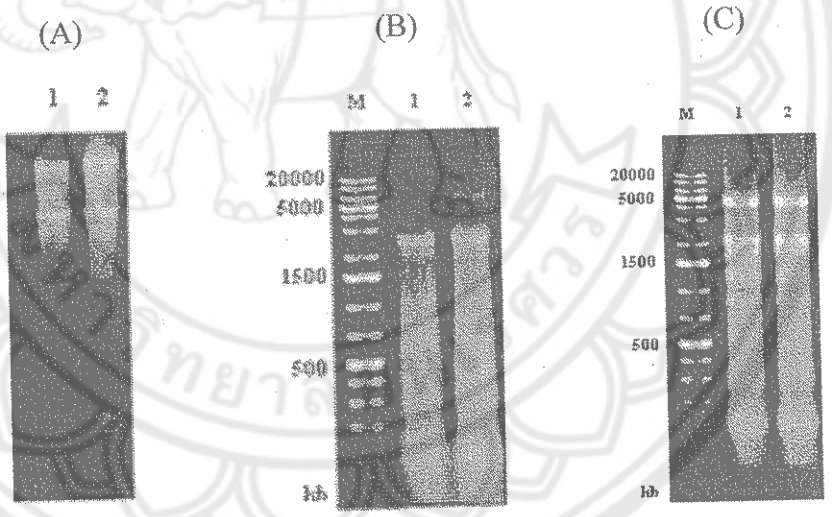

Figure 11 A 2 % ethidium bromide-stained agarose gel showing the quality of total RNA extracted from buccal mucosal cell (A) Lane 1 and 2: total RNA from human mucosa buccul cells (B) Lane M: 1 kb plus DNA ladder, Lane 1 and 2: total RNA from human white blood cells. (C) Lane M: 1 kb plus DNA ladder, Lane 1 and 2: total RNA from neuroblastoma cells

Cloning and sequencing of human chromogranin A

To determine the gene expression of human chromogranin A, total RNA were extracted from buccal mucosa cells and white blood cells of *Homo sapiens*. The result showed that both buccal mucosa cell and white blood cell were not expression of chromogranin A gene. Human neuroblastoma cell line was chosen for amplify the chromogranin A gene.

The first stand cDNA of human chromogranin A in neuroblastoma cell line was subjected to RT-PCR. To obtain the amplified gene, this technique requires optimization of several parameters including annealing temperature, concentration of primer and $MgCl_2$ and the type of DNA Taq polymerase.

RT-PCR was initially carried out with fixed components except primer concentration. Lower concentration may result in non-quantitative amplification whereas higher concentration of primer may leave a large amount of unused primer which could give rise to non-specific amplification products. The concentration of $MgCl_2$ that gave the highest yield and specificity for each PCR product was chosen.

1. Amplified gene by Nested-PCR

The two primers were designed and used in Nested- PCR. A sigle product was obtained from the outer primer set of approximately 1360 bp (Figure 12A) using High fidelity Taq DNA polymerase (Invitrogen, USA) and a single product of approximately 1317 bp was produced by re-amplification of this product with the inner primer set (Figure 12B). The products were purified and extracted from 1 % agarose gel electrophoresis. The PCR product was cloned into the plasmid pTZ57R/T and then was transformed into competent DH5α.

The amplified fragment cloned in the recombinant plasmid was confirmed by colony PCR using the specific primer which gave the expected 1317 bp band on an agarose gel electrophoresis (Figure 13). DNA sequencing of the recombinant plasmid showed 99 % identity to the known DNA sequence of human chromogranin A gene (Genbank Accession No. NM_001275). The sequenced is shown in Figure 14.

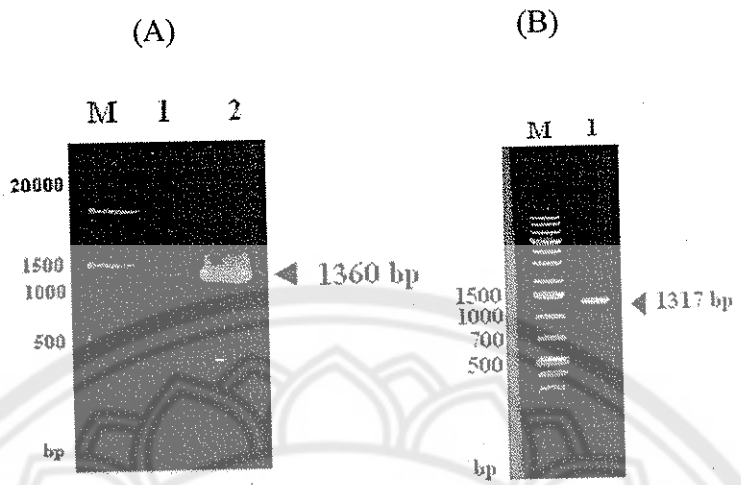

Figure 12 PCR products generated by the use of different primers.

Lane M: 1 kb plus DNA ladder; Lane 2: PCR product using outer primer;(B): Nested PCR using PCR product from lane 2 in Figure (A) as template and using inner primer.

Figure 13 A 1% agarose gel electrophoresis of colony PCR of recombinant plasmids containing human chromogranin A fragment.

Lane M: 1 kb plus DNA ladder. Lane 1-16: colony PCR of recombinant plasmids.

CATATGCTCCCTGTGAACAGCCCTATGAATAAAGGGGATACCGAGGTGATGAAATGCATCGTTGAGGTCATCTCCGACA
CACTTTCCAAGCCCAGCCCCATGCCTGTCAGCCAGGAATGTTTTGAGACACTCCGAGGAGATGAACGGATCCTTTCCAT
TCTGAGACATCAGAATTTACTGAAGGAGCTCCAAGACCTCGCTCTCCAAGGCGCCAAGGAGAGGGCACATCAGCAGAAG
AAACACAGCGGTTTTGAAGATGAACTCTCAGAGGTTCTTGAGAACCAGAGCAGCCAGGCCGAGCTGAAAGAGGCGGTGG
AAGAGCCATCATCCAAGGATGTTATGGAGAAAAGAGAGGATTCCAAGGAGGCAGAGAAAAGTGGTGAAGCCACAGACGG
AGCCAGGCCCCAGGCCCTCCCGGAGCCCATGCAGGAGTCCAAGGCTGAGGGGAACAATCAGGCCCCTGGGGAGGAAGAG
GAGGAGGAGGAGGAGGCCACCAACACCCACCCTCCAGCCAGCCTCCCCAGCCAGAAATACCCAGGCCCACAGGCCGAGG
GGGACAGTGAGGGCCTCTCTCAGGGTCTGGTGGACAGAGAGAAGGGCCTGAGTGCAGAGCCAGGGTGGCAGGCAAAGAG
AGAAGAGGAGGAGGAGGAGGAGGAGGAGGCTGAGGCTGGAGAGGAGGCTGTCCCCGAGGAAGAAGGCCCCACTGTAGTG
CTGAACCCCCACCCGAGCCTTGGCTACAAGGAGATCCGGAAAGGCGAGAGTCGGTCGGAGGCTCTGGCTGTGGATGGAG
CTGGGAAGCCTGGGGCTGAGGAGGCTCAGGACCCCGAAGGGAAGGGAGAACAGGAGCACTCCCAGCAGAAAGAGGAGGA
GGAGGAGATGGCAGTGGTCCCGCAAGGCCTCTTCCGGGGTGGGAAGAGCGGAGAGCTGGAGCAGGAGGAGGAGCGGCTC
TCCAAGGAGTGGGAGGACTCCAAACGCTGGAGCAGGATGGACCAGCTGGCCAAGGAGCTGACGGCTGAGAAGCGGCTGG
AGGGGCAGGAGGAGGAGGAGGACAACCGGGACAGTTCCATGAAGCTCTCCTTCCGGGCCCGGGCCTACGGCTTCAGGGG
CCCTGGGCCGCAGCTGCGACGAGGCTGGAGGCCATCCTCCCGGGAGGACAGCCTTGAGGCGGGCCTGCCCCTCCAGGTC
CGAGGCTACCCCGAGGAGAAGAAAGAGGAGGAGGGCAGCGCAAACCGCAGACCAGAGGACCAGGAGCTGGAGAGCCTGT
CGGCCATTGAGGCAGAGCTGGAGAAAGTGGCCCACCAGCTGCAGGCACTACGGCGGGGCTGACTCGAG

Figure 14 Nucleotide sequence of the cloned that were significantly matched human chromogranin A.

Primer were designed for the putative start (ATG) and stop (TGA) codons are boldfaced and underlined.

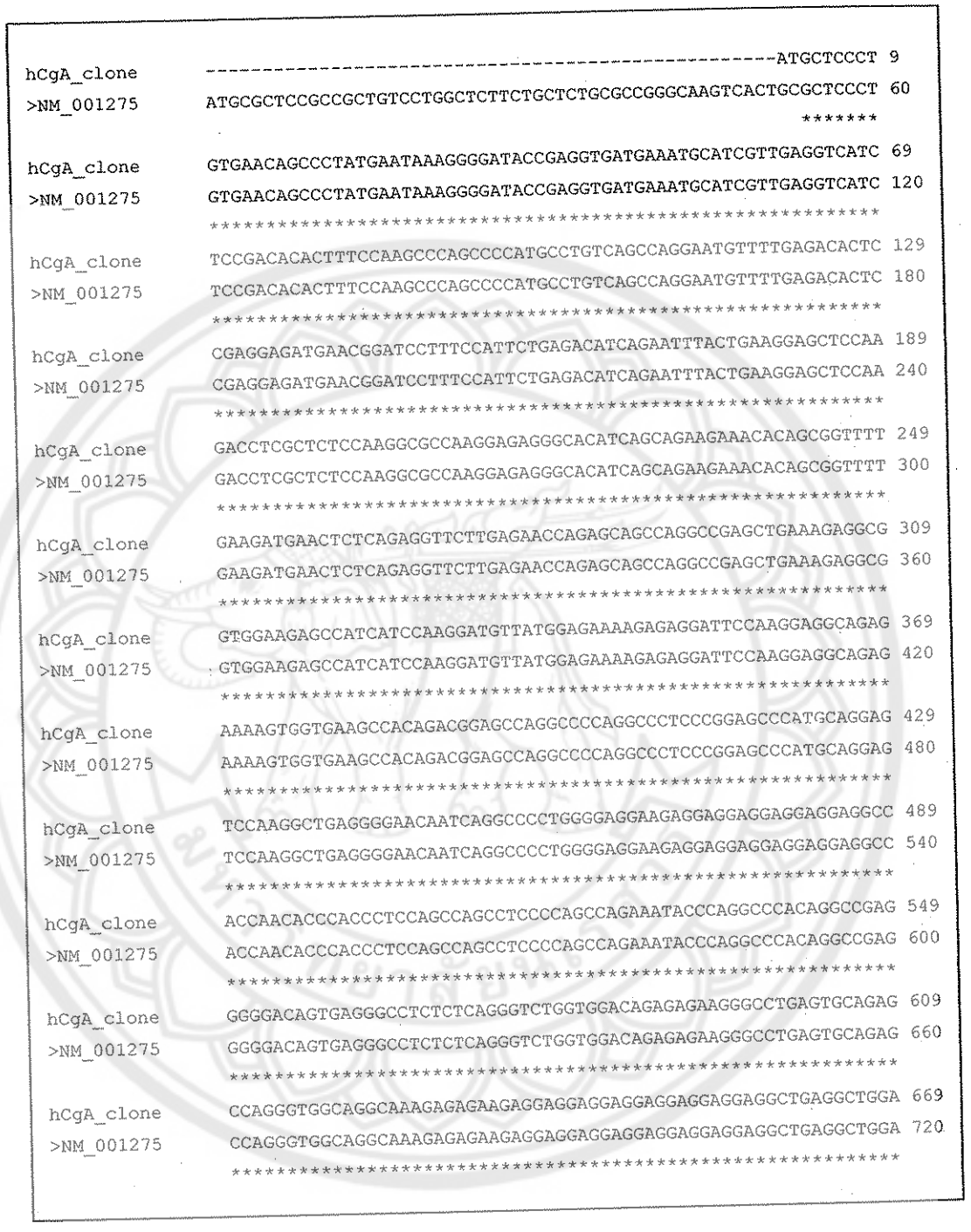

Figure 15 Alignment between the full lengh Homo sapiens chromogranin A (parathyroid secretory protein 1) (ChgA), mRNA from Genbank database Accession No. NM_001275 and mature proteins amplification without signal peptide (hCgA_clone) for *in vitro* expression.

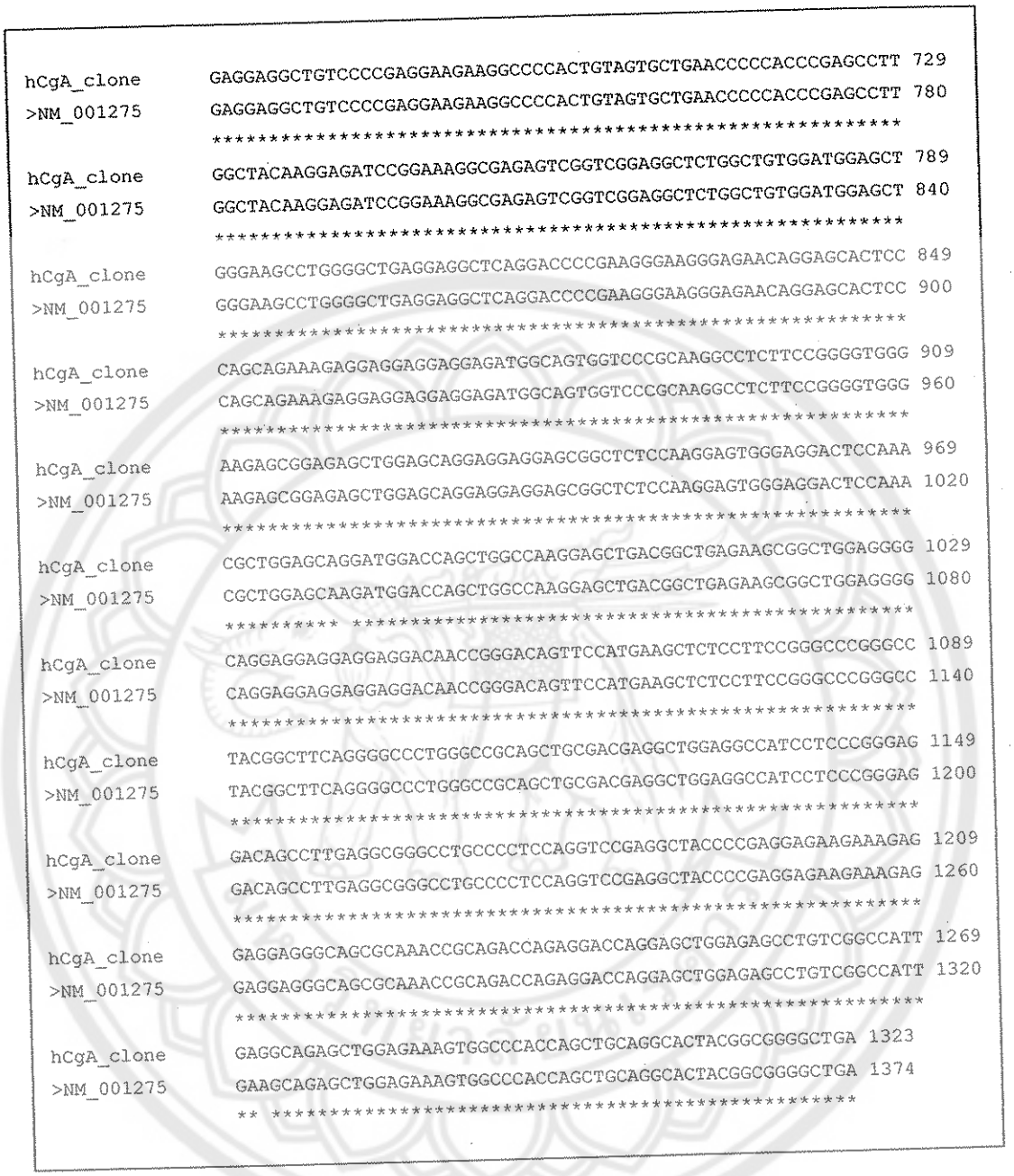

Figure 15 (Cont.)

```
hCgA_clone      ----------------MLPVNSPMNKGDTEVMKCIVEVISDTLSKPSPMPVSQECFETL 43
>NP_001266      MRSAAVLALLLCAGQVTALPVNSPMNKGDTEVMKCIVEVISDTLSKPSPMPVSQECFETL 60
                ****************************************

hCgA_clone      RGDERILSILRHQNLLKELQDLALQGAKERAHQQKKHSGFEDELSEVLENQSSQAELKEA 103
>NP_001266      RGDERILSILRHQNLLKELQDLALQGAKERAHQQKKHSGFEDELSEVLENQSSQAELKEA 120
                ************************************************************

hCgA_clone      VEEPSSKDVMEKREDSKEAEKSGEATDGARPQALPEPMQESKAEGNNQAPGEEEEEEEEA 163
>NP_001266      VEEPSSKDVMEKREDSKEAEKSGEATDGARPQALPEPMQESKAEGNNQAPGEEEEEEEEA 180
                ************************************************************

hCgA_clone      TNTHPPASLPSQKYPGPQAEGDSEGLSQGLVDREKGLSAEPGWQAKREEEEEEEEEAEAG 223
>NP_001266      TNTHPPASLPSQKYPGPQAEGDSEGLSQGLVDREKGLSAEPGWQAKREEEEEEEEEAEAG 240
                ************************************************************

hCgA_clone      EEAVPEEEGPTVVLNPHPSLGYKEIRKGESRSEALAVDGAGKPGAEEAQDPEGKGEQEHS 283
>NP_001266      EEAVPEEEGPTVVLNPHPSLGYKEIRKGESRSEALAVDGAGKPGAEEAQDPEGKGEQEHS 300
                ************************************************************

hCgA_clone      QQKEEEEEEMAVVPQGLFRGGKSGELEQEEERLSKEWEDSKRWSKMDQLAKELTAEKRLEG 343
>NP_001266      QQKEEEEEEMAVVPQGLFRGGKSGELEQEEERLSKEWEDSKRWSKMDQLAKELTAEKRLEG 360
                ************************************************************

hCgA_clone      QEEEEDNRDSSMKLSFRARAYGFRGPGPQLRRGWRPSSREDSLEAGLPLQVRGYPEEKKE 403
>NP_001266      QEEEEDNRDSSMKLSFRARAYGFRGPGPQLRRGWRPSSREDSLEAGLPLQVRGYPEEKKE 420
                ************************************************************

hCgA_clone      EEGSANRRPEDQELESLSAIEAELEKVAHQLQALRRG 440
>NP_001266      EEGSANRRPEDQELESLSAIEAELEKVAHQLQALRRG 457
                ***********************************
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Figure 16 Alignment of deduced amino acid sequences between of chromogranin A precursor from Genbank database and mature proteins without signal peptide (hCgA_clone) for recombinant protein expression.

Construction of pET21b(+)-human chromogranin A gene

Before construction of recombinant plasmid in expression vector, the amplified primer of human chromogranin A gene lacking the signal peptide sequence that covered from start to stop codon were designed. The amplified fragment cloned in the recombinant plasmid and pET21b(+) expression vector (Novagen, Madison, WI) were digested by restriction enzyme with *Nde I* and *Xho I* (Figure 17). The interested gene and expression vector were eluted from agarose gel and ligated. After that the ligated expression vector was transformed into *E. coli* BL21 (DE3)plysS. The recombinant plasmid of the positive clone was confirmed by colony PCR (Figure 18) and re-sequenced to confirm the nucleotide sequence of human chromogranin A.

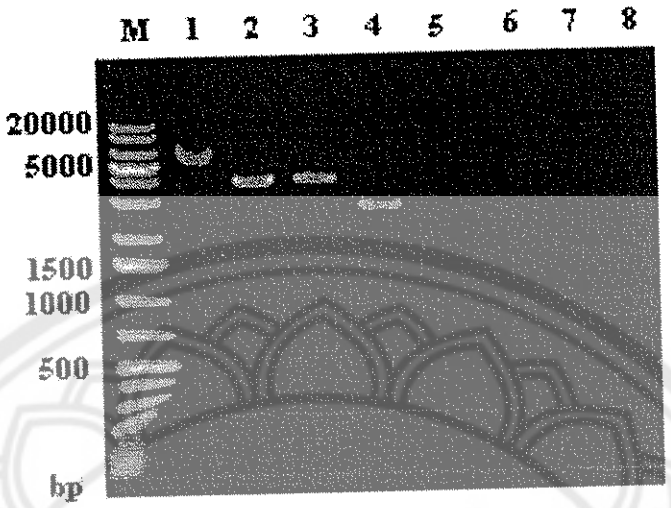


Figure 17 A1 % agarose gel electrophoresis of restriction analysis of recombinant plasmids containing human chromogranin A fragment.

Lane M 1 kb plus DNA ladder

Lane 1 Plasmid pTZ57R/T undigested

Lane 2 Plasmid pTZ57R/T digested with *Nde* I

Lane 3 Plasmid pTZ57R/T digested with *Xho* I

Lane 4 Plasmid pTZ57R/T digested with *Nde* I and *Xho* I

Lane 5 Plasmid pET21b(+) undigested

Lane 6 Plasmid pET21b(+) digested with *Nde* I

Lane 7 Plasmid pET21b(+) digested with *Xho* I

Lane 8 Plasmid pET21b(+) digested with *Nde* I and *Xho* I

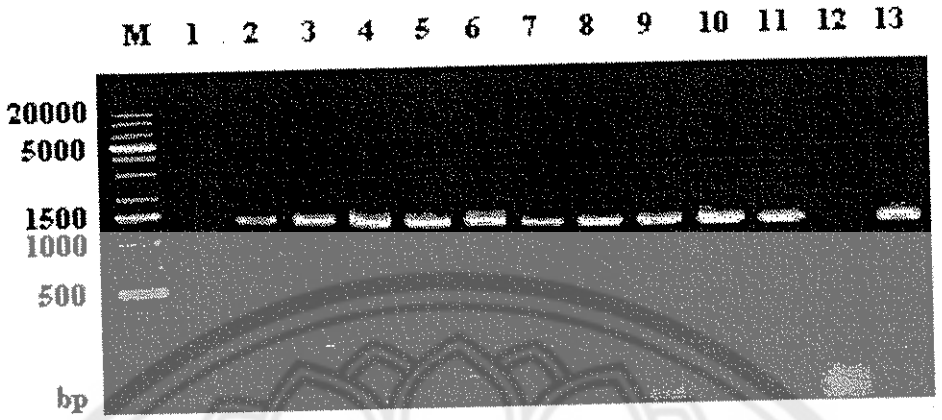


Figure 18 A1 % agarose gel electrophoresis of colony PCR of recombinant plasmids containing human chromogranin A fragment.

Lane M: 1 kb plus DNA ladder. Lane 1-13: colony PCR of recombinant plasmids.

Mutagenesis

Variant forms of human chromogranin A, according to the naturally occurring polymorphism, $Arg_{253}Trp$, $Arg_{374}Gln$ and $Arg_{381}Trp$, will be generated by site-directed mutagenesis. The pET21b (+)- human chromogranin A construct with the wild-type human chromogranin A will be used a template. The overlapping oligonucleotide primers containing the desired mutation were used (Figure 19) for construct the mutants. All of mutants were transformed into *E. coli* DH5α (Figure 20) and re-sequenced to confirm the nucleotide sequence (Figure 28). The last, plasmid containing the mutants were extracted and re-transformed into *E. coli* BL21 (DE3) plysS for *in vitro* proteins expression.

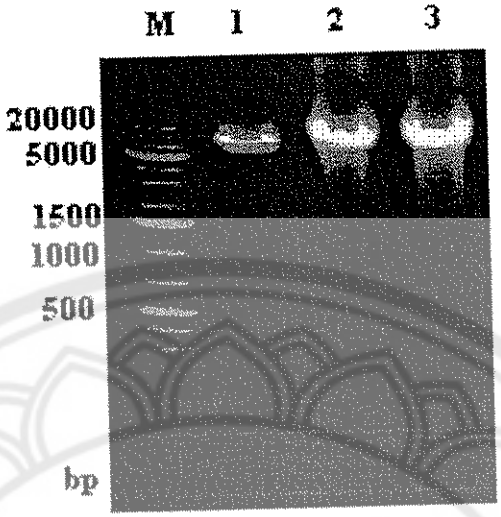


Figure 19 A1 % agarose gel electrophoresis of plasmid containing the mutant human chromogranin A gene.

Lane M : 1 kb plus DNA Marker; Lane 1 : CgA with mutation at $Arg_{253}Trp$;Lane 2 : CgA with mutation at $Arg_{374}Gln$ and Lane 3: CgA with mutation at $Arg_{381}Trp$.

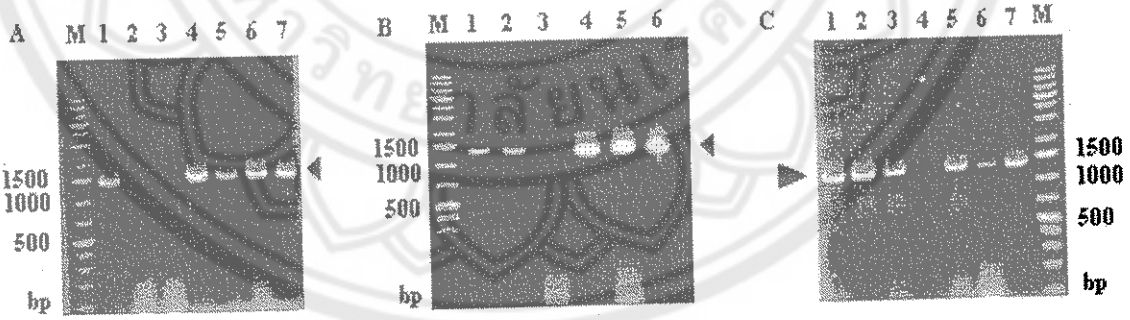


Figure 20 Colony PCR for determine of positive clone of the mutants.

The mutant at position 253 (A), 374 (B) and 381 (C) caused $Arg_{253}Trp$, $Arg_{374}Gln$ and $Arg_{381}Trp$ were checked by colony PCR using specific primer which gave the expected 1317 bp band on an agarose gel electrophoresis.

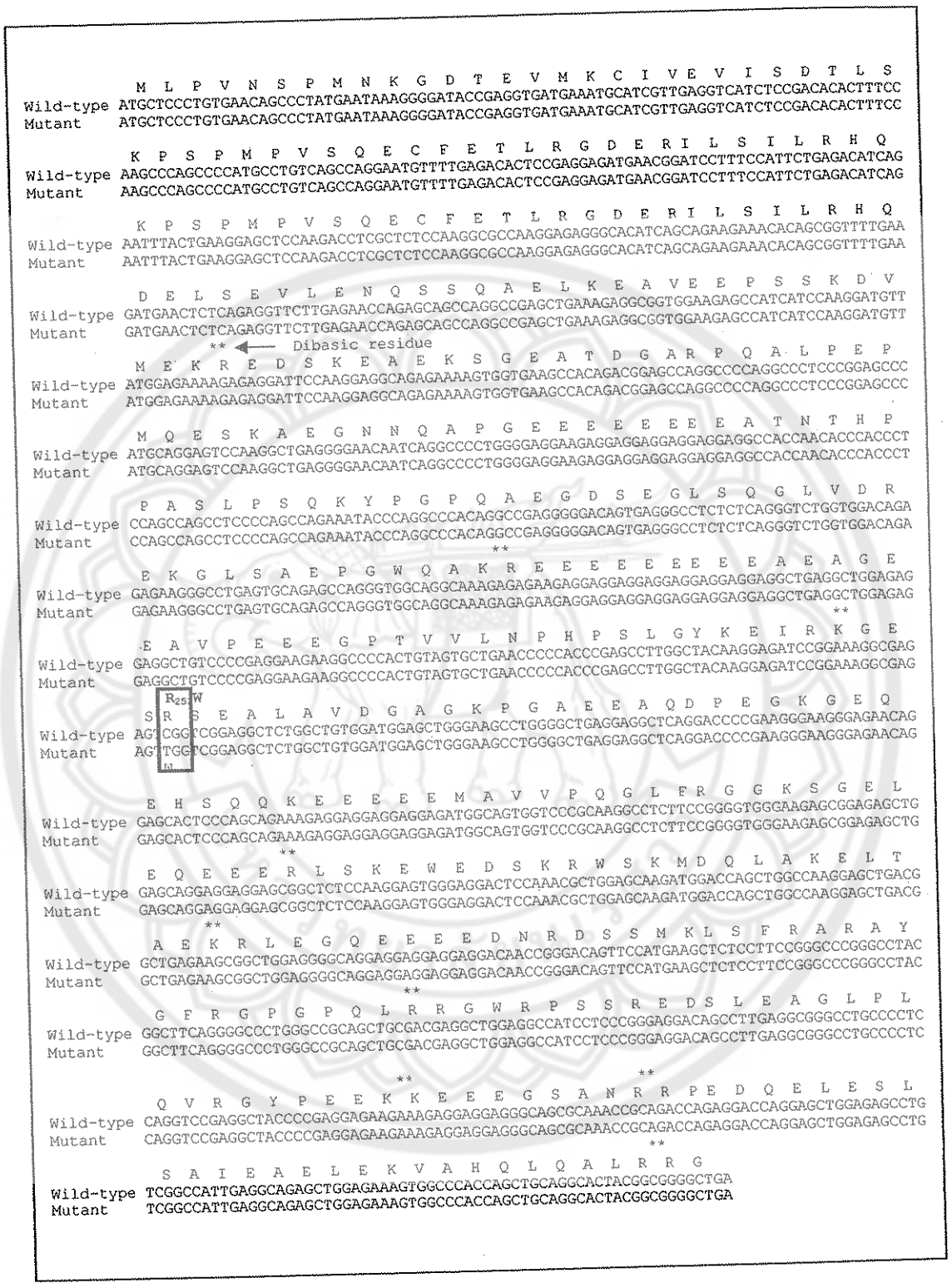


Figure 21 DNA sequence and 439 duduced amino acid sequence of full length human chromogranin A and its mutant at position caused Arg_{253}Trp(C to T).

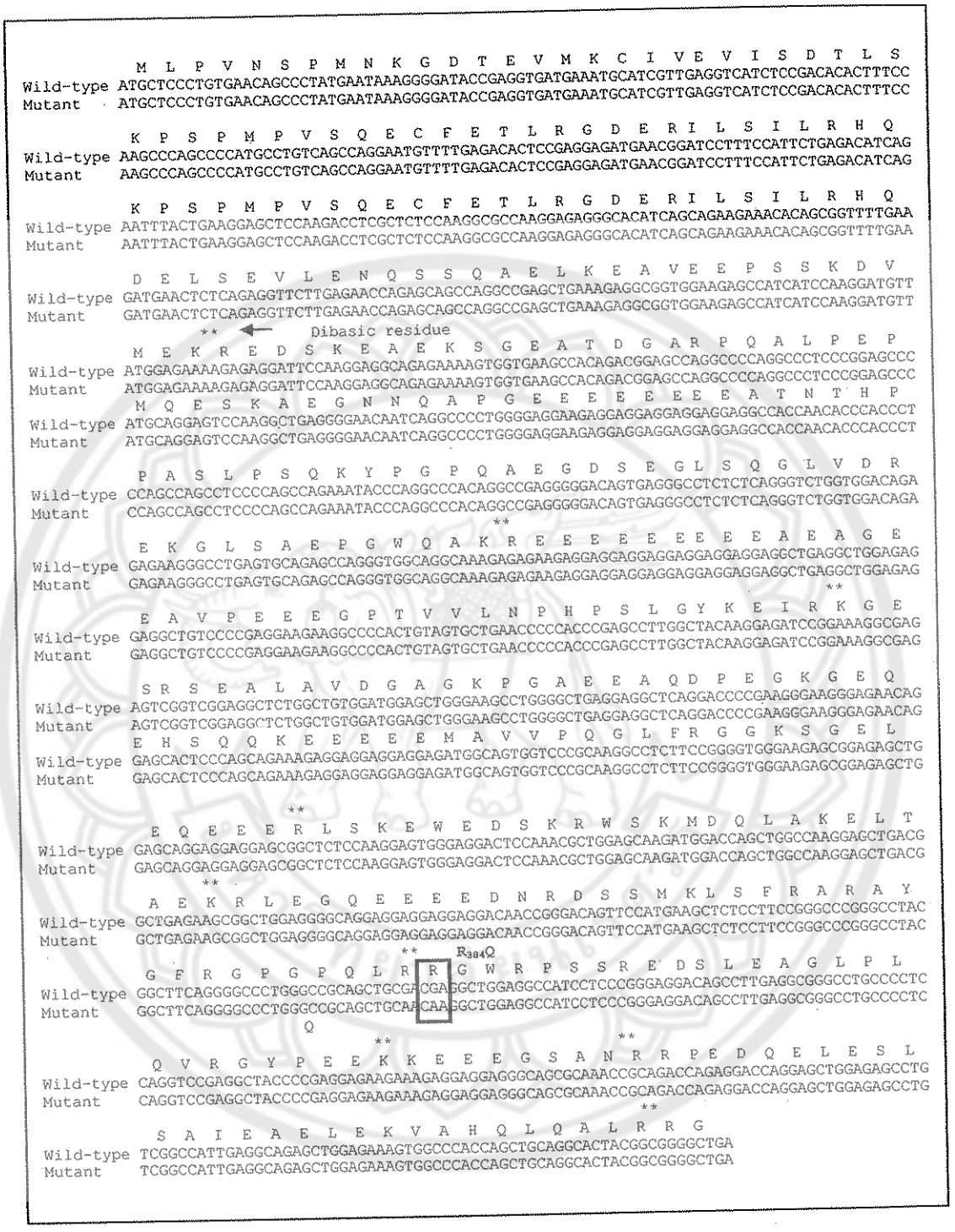


Figure 22 DNA sequence and 439 duduced amino acid sequence of full length human chromogranin A and its mutant at position caused $Arg_{374}Gln$ (G to A).

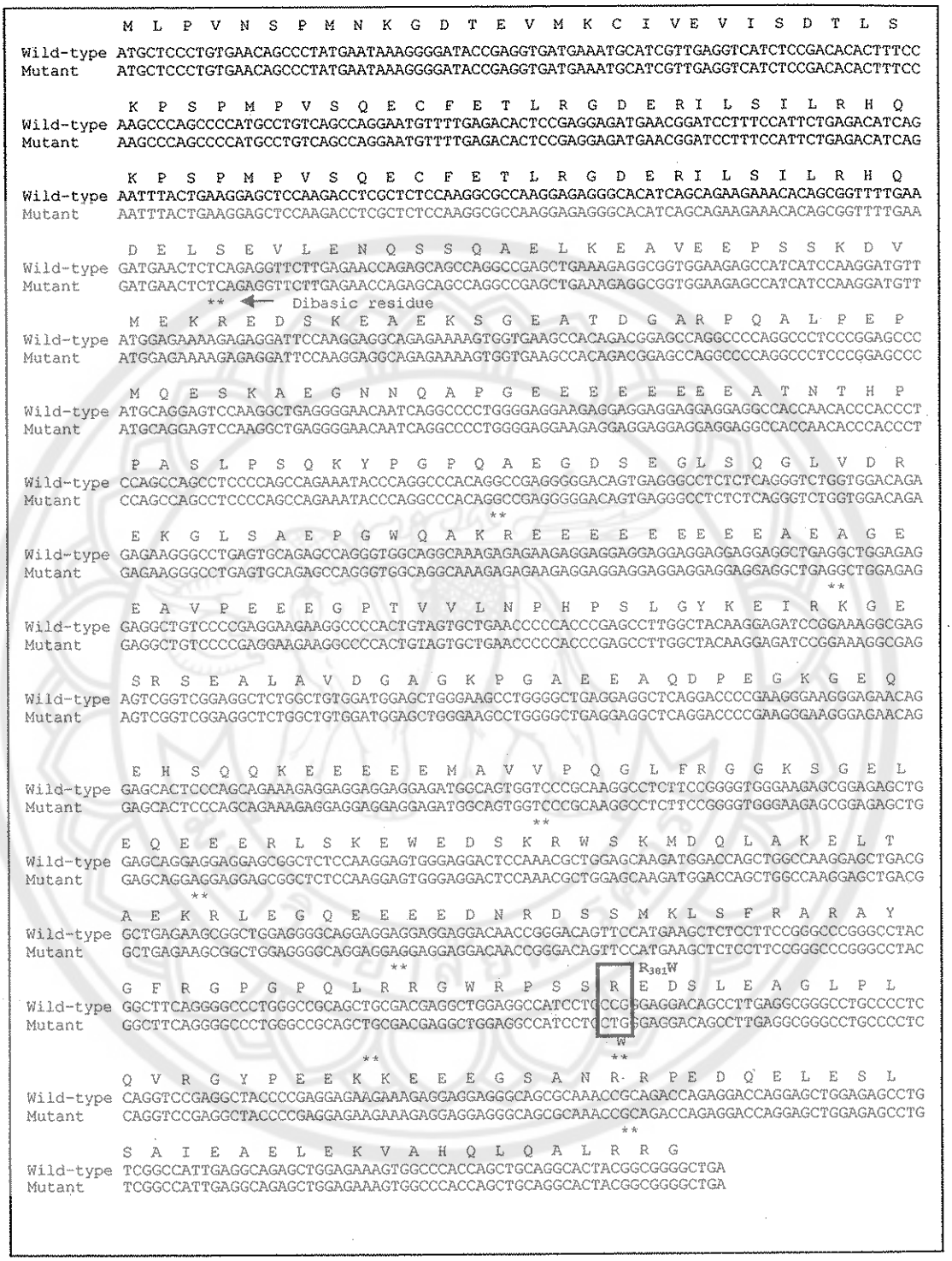


Figure 23 DNA sequence and 439 duduced amino acid sequence of full length human chromogranin A and its mutant at position caused Arg_{381}Trp(C to T).

Production of recombinant proteins

1. Selection of the clone

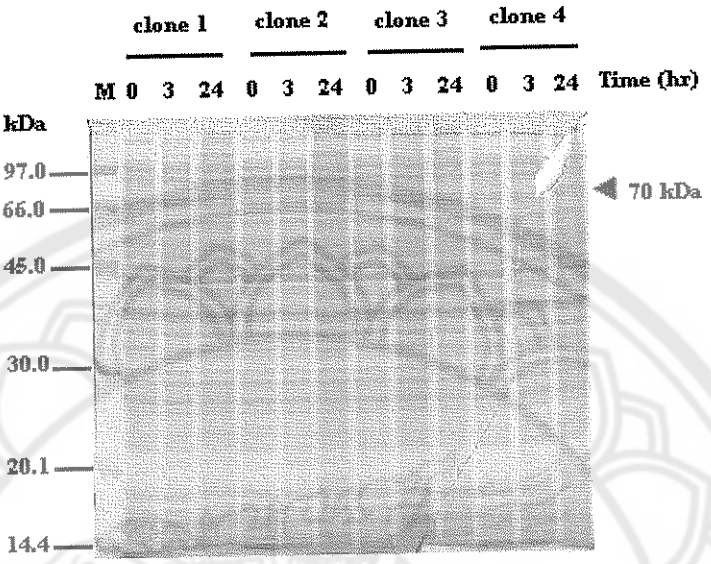


Figure 24 Proteins lysates from *E. coli* BL21 (DE3) plysS cells harbouring pET21b(+)-hCgA recombinant plasmid of clone 1, 2, 3 and 4 that had been induced by IPTG for 0, 3 and 24 hours.

Cell were then harvested and disrupted. Loaded samples were whole harvested cells taken from a grown culture after the specific induction times. Lanes were specified according to the induction times in hours and analyzed by 12.5 % SDS-PAGE. Lane M: standard protein marker.

2. Peptide fingerprints of the recombinant proteins by mass-spectrometry

ESI-LC-MS/MS was performed to confirm the expression. The results of clone 1 showed that human chromogranin A clone was significantly matched with Homo sapiens chromogranin A precursor (Figure 25). The highest match was a peptide similar to chromogranin A precursor of Homo sapiens (Score: 417; Queries matched = 9). For clone 2, the protein was significantly matched with with Homo sapiens chromogranin A precursor. The highest match was a peptide similar to chromogranin A precursor of Homo sapiens (Score: 374; Queries matched = 8).

The most significantly hit of clone 3 was that from Homo sapiens chromogranin A precursor protein (Score: 437; Queries: 8). The last, protein of clone 4 was significantly matched with Homo sapiens chromogranin A precursor. The highest match was a peptide similar to chromogranin A precursor of Homo sapiens (Score: 326; Queries matched = 5). In contrast, proteins of harvested cell in 24 hr after induced by IPTG from clone 1, 2, 3 and 4 were not significantly matched with any deduced peptide in the Homo sapiens library.

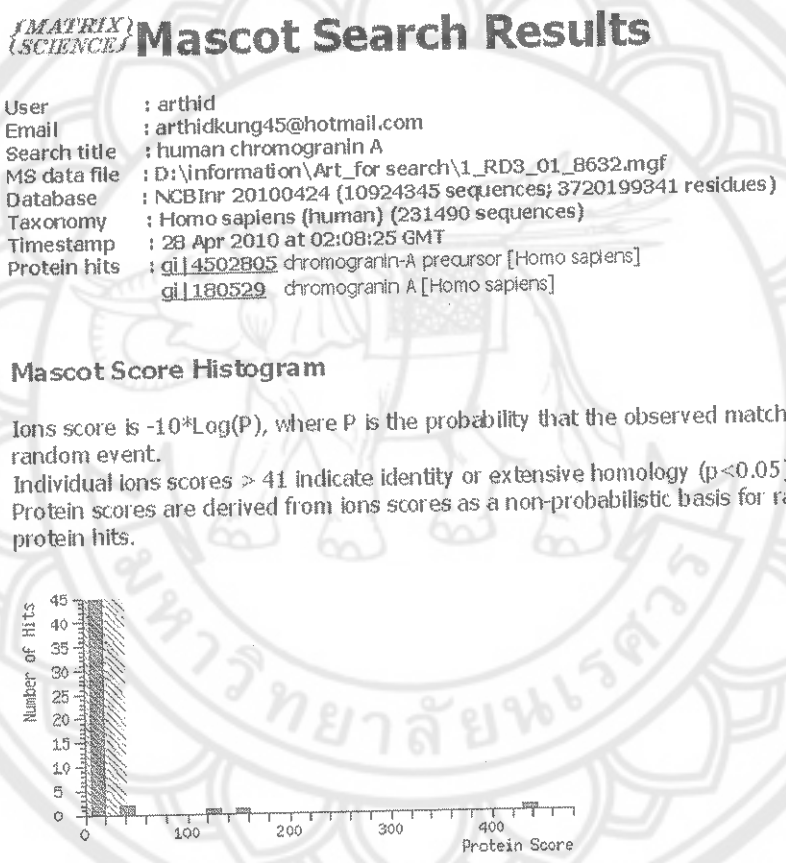


Figure 25 Identification of peptide mass fingerprints of human chromogranin A precursor protein clone 1 by ESI-LC-MS/MS.

The Mowse score is –10log(P), where P is the probability that the observed match is a random event. The height of each bar represents the number of protein(s) matched within a score range. The matches falling into the shaded area, where the probability of the random event is more than 5 % are considered to be insignificant.

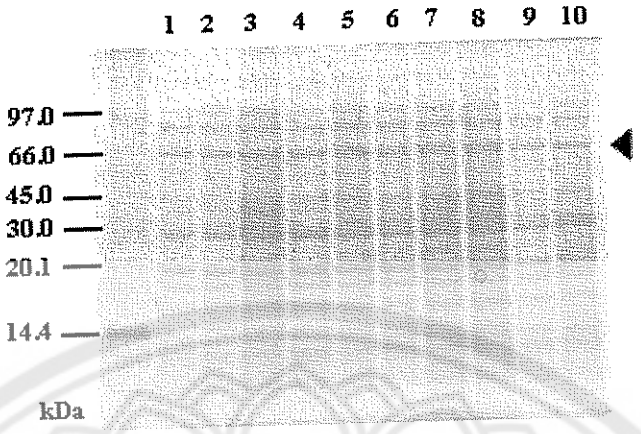


Figure 26 Small-scale selection of the colony for expression of recombinant chromogranin A protein in *E. coli* strain BL21(DE3)plysS analyzed by SDS-PAGE.

Loaded samples were whole harvested cells taken from a grown culture after 3 hr of IPTG induction. Lane were specified according to number of colony.

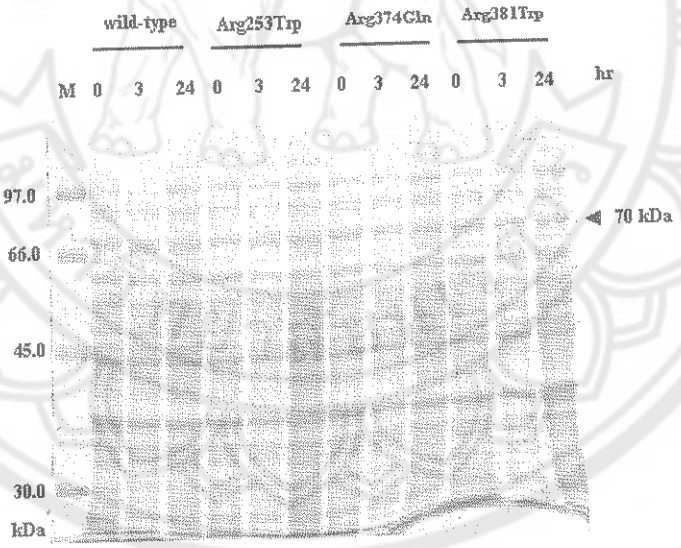


Figure 27 Small-scale expression of mutants chromogranin A protein in *E. coli* strain BL21(DE3)plysS analyzed by SDS-PAGE.

Loaded samples were whole harvested cells taken from a grown culture after 3 hr of IPTG induction. Lane were specified according to mutants at position $Arg_{253}Trp$, $Arg_{374}Gln$ and $Arg_{381}Trp$ and wild-type as a positive control for expressed proteins.

3. Time course study of the protein expression levels

Expression levels of the four recombinant proteins, i.e. wild-type, $Arg_{253}Trp$, $Arg_{374}Gln$ and $Arg_{381}Trp$ of human chromogranin A protein, were followed in time course to find out the optimal induction time yielding maximum amounts of the expressed proteins.

As shown in figures 4.18A-D, all of the recombinant proteins were expressed within the first hour. Their expression levels increased in line with the induction time. They are maximized at the hours of 3-4 and the proteins seemed to be affected by proteolysis even when the induction time was extended to 24 hr that showed in the previously study.

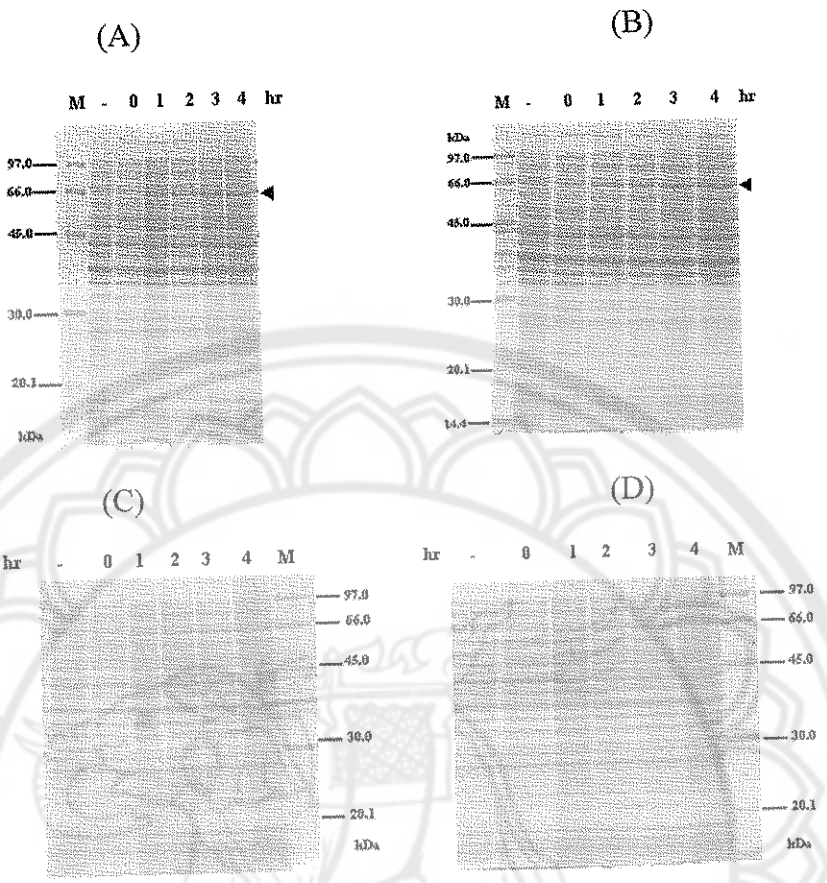


Figure 28 Time course study of recombinant expression of mature chromogranin A proteins in *E. coli* BL21 (DE3) plysS induced for 0, 1, 2, 3 and 4 hr analyzed by SDS-PAGE.

Wild-type (A), $Arg_{253}Trp$(B), $Arg_{374}Gln$(C) and $Arg_{381}Trp$(D). Loaded samples were whole harvested cells taken from a grown culture after the specified induction times. Lane were specified according to the induction times in hours. Lane "-" is a negative control derived from the host cell containing pET21(b) vector without insert gene.

Purification of the recombinant proteins

1. Purification of the recombinant proteins by heat treatment

A modification to the purification technique described by Ujendra Kumar, was employed to purify chromogranin A. The whole cells extracted were heated in boiling water, followed by centrifugation and supernatant was collected as shown in figure 29A-D.

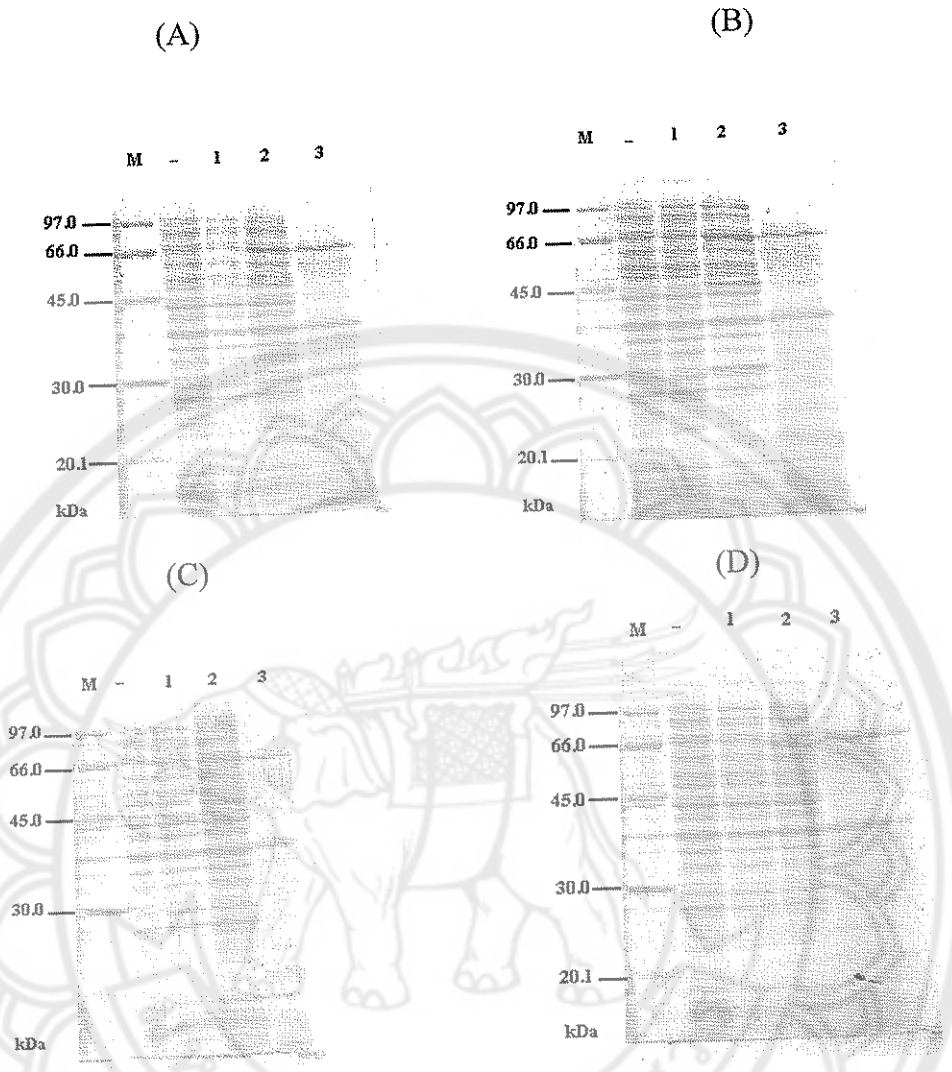


Figure 29 Small-scale purification of recombinant chromogranin A protein in *E. coli* strain BL21(DE3)plysS analyzed by SDS-PAGE.

Wild-type (A), $Arg_{253}Trp$(B), $Arg_{374}Gln$(C) and $Arg_{381}Trp$(D). All of them, lane 2 is whole harvested cells taken from a grown culture after 4 hr of IPTG induction. Lane "-" is a negative control derived from the host cell containing pET21b(+) vector without insert gene.

2. Protein purification using OFF GEL

To purify the recombinant proteins, isoelectric focusing in polyacrylamide gel was performed in the pH range 4.0-7.0 using Agilent 3100 OFFGEL Fractionator. The electrophoretic patterns obtained in Figure 39-42 showed a band of human chromogranin A (lane 3, Figure 30-32). The human chromogranin A protein from recombinant expression had pI of approximately 4.5-5 respectively.

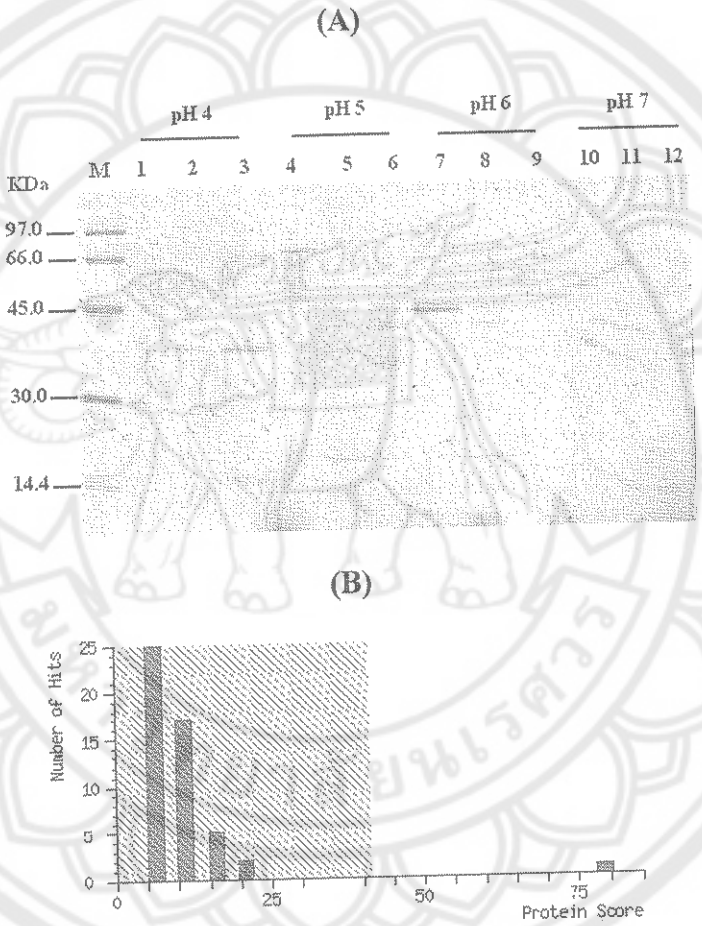
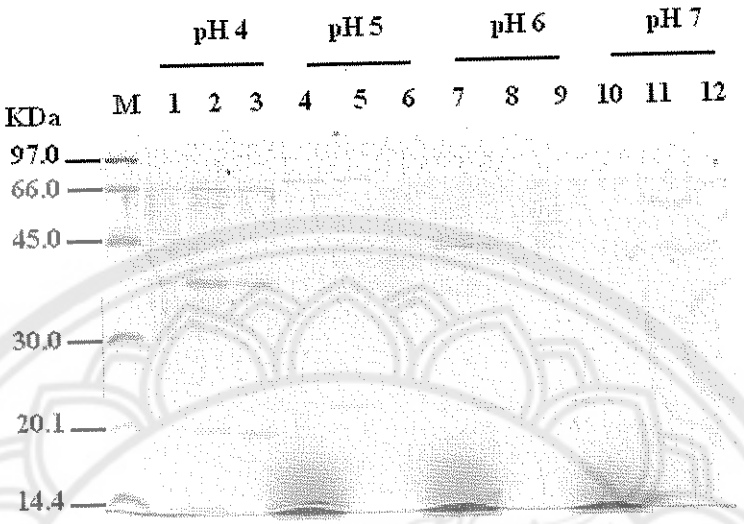


Figure 30 Isoelectric focusing of wild-type of recombinant human chromogranin A in *E. coli* strain BL21 (DE3) plysS analyzed by SDS-PAGE.

(A)The proteins were separated by OFFGEL Fractionator pI 4-7. Lane M: Protein makers (kDa), lane 1-3: pI 4, lane 4-6: pI 5, lane 7-9: pI 6 and lane 10-12: pI 7. (B) Identification of peptide mass fingerprints of human chromogranin A precursor protein.

(A)



(B)

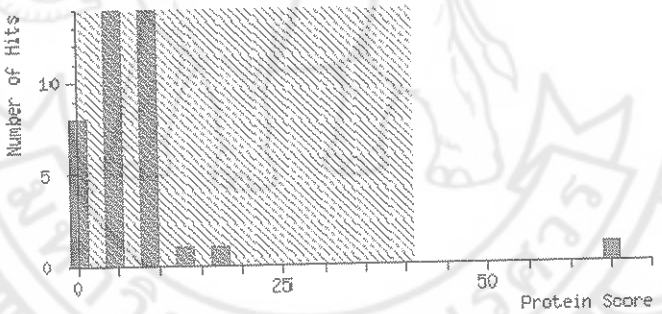


Figure 31 Isoelectric focusing of mutants at position $Arg_{253}Trp$ of recombinant human chromogranin A in *E. coli* strain BL21 (DE3) plysS analyzed by SDS-PAGE.

(A)The proteins were separated by OFFGEL Fractionator pI 4-7. Lane M: Protein makers (kDa), lane 1-3: pI 4, lane 4-6: pI 5, lane 7-9: pI 6 and lane 10-12: pI 7. (B) Identification of peptide mass fingerprints of human chromogranin A precursor protein.

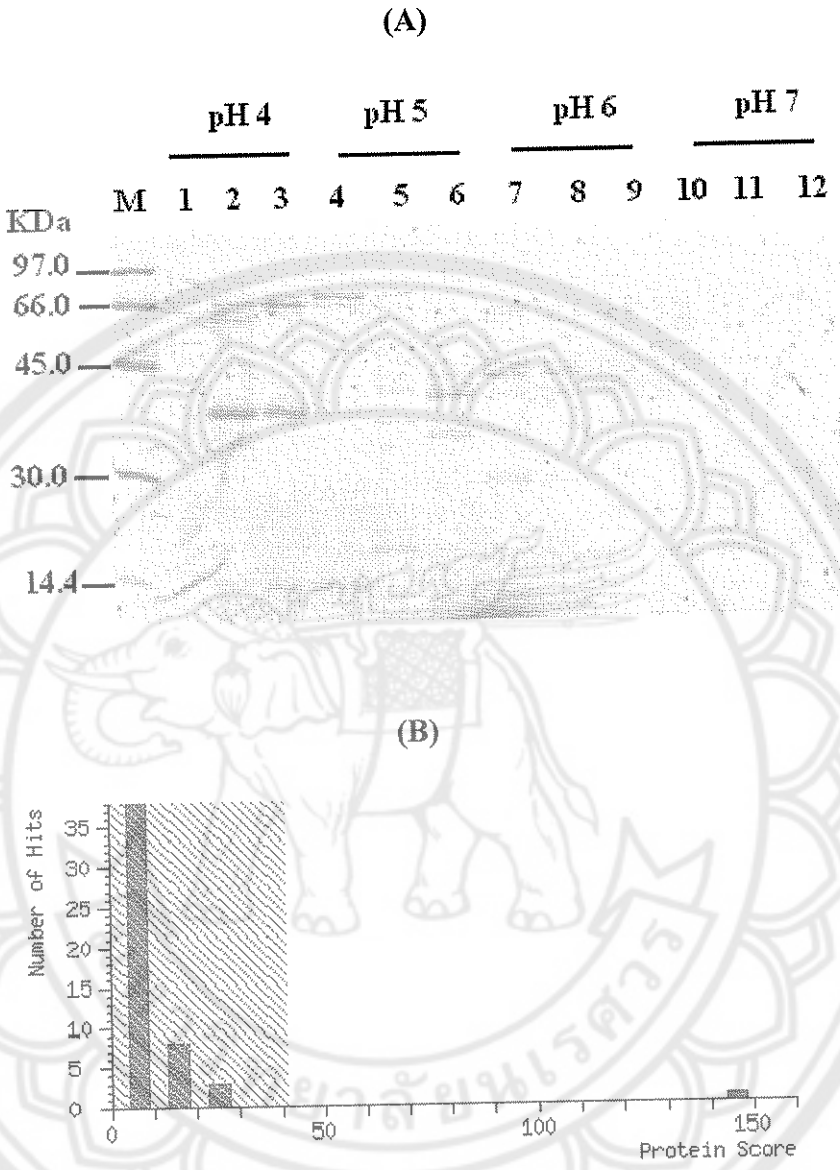
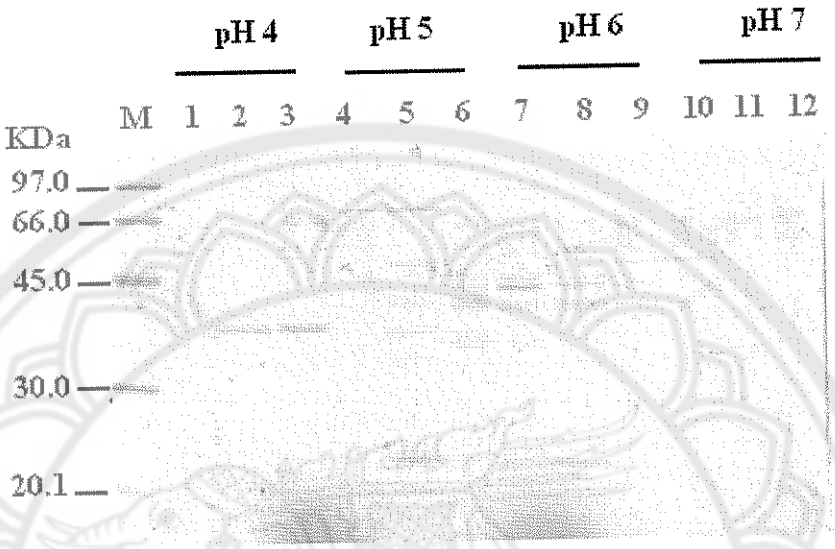


Figure 32 Isoelectric focusing of mutants at position $Arg_{374}Gln$ of recombinant human chromogranin A in *E. coli* strain BL21 (DE3) plysS analyzed by SDS-PAGE.

(A)The proteins were separated by OFFGEL Fractionator pI 4-7. Lane M: Protein makers (kDa), lane 1-3: pI 4, lane 4-6: pI 5, lane 7-9: pI 6 and lane 10-12: pI 7. (B) Identification of peptide mass fingerprints of human chromogranin A precursor protein.

(A)



(B)

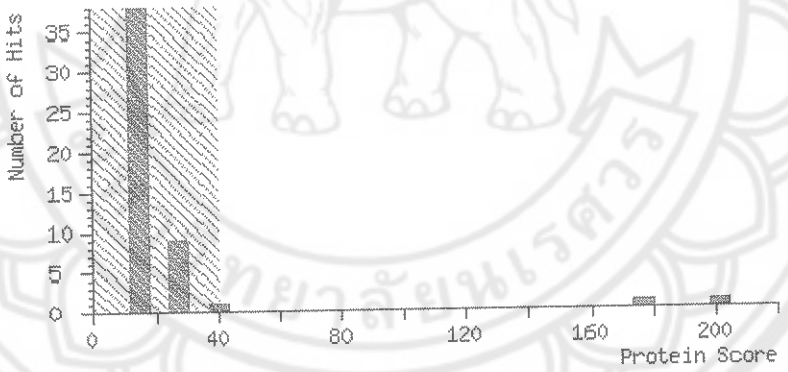


Figure 33 Isoelectric focusing of mutants at position Arg_{381}Trp of recombinant human chromogranin A in *E. coli* strain BL21 (DE3) plysS analyzed by SDS-PAGE.

(A)The proteins were separated by OFFGEL Fractionator pI 4-7. Lane M: Protein makers (kDa), lane 1-3: pI 4, lane 4-6: pI 5, lane 7-9: pI 6 and lane 10-12: pI 7. (B) Identification of peptide mass fingerprints of human chromogranin A precursor protein.

3. Purification by Microcon centrigugal filter device

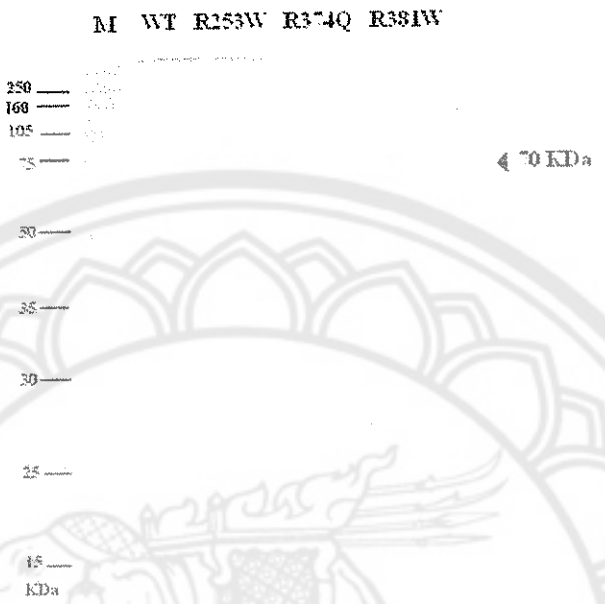


Figure 34 Purity of the recombinant human chromogranin A after purification by OFFGEL Fractionator and followed by microcon centrigugal filter device assessed by 12.5% SDS-PAGE.

Lane M: Board-range protein molecular weight marker. Lanes 2-5: Purified protein of wild-type, $Arg_{253}Trp$, Arg_{274Gln} and $Arg_{381}Trp$.

Mass spectrometry analyses of cathepsin L-cleaved human chromogranin A fragments

Purified recombinant CgA protein (10 μg) was incubated with 1 ng of CTSL. CTSL readily digested the recombinant CgA, and the extent of digestion was time dependent.

(A)

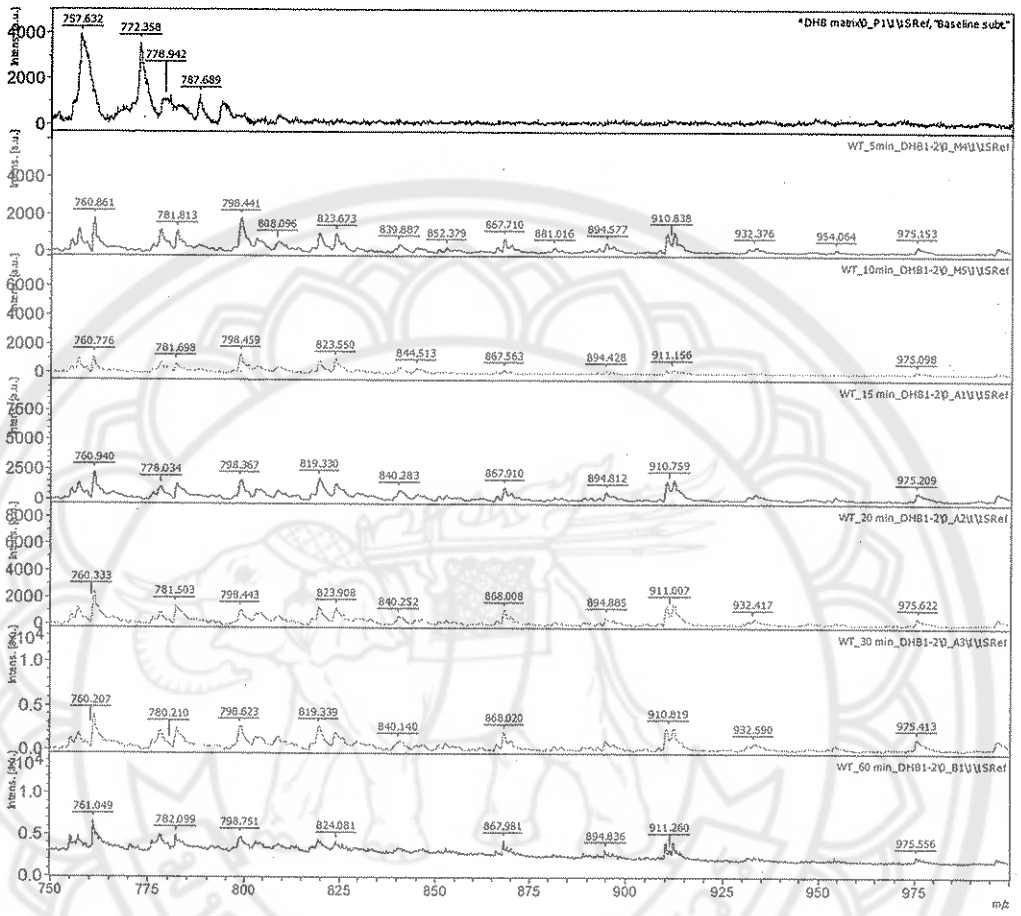


Figure 35 Digestion of wild-type CgA by CTSL. Wild-type of CgA (10 µg/reaction) was digested with 1 ng of CTSL for 5, 10, 15, 20, 30 and 60 min and CTSL digests were subjected to MALDI-TOF analysis.

(A) Mass between 800-1000 m/z. (B) Mass between 800-2000 m/z.

(B)

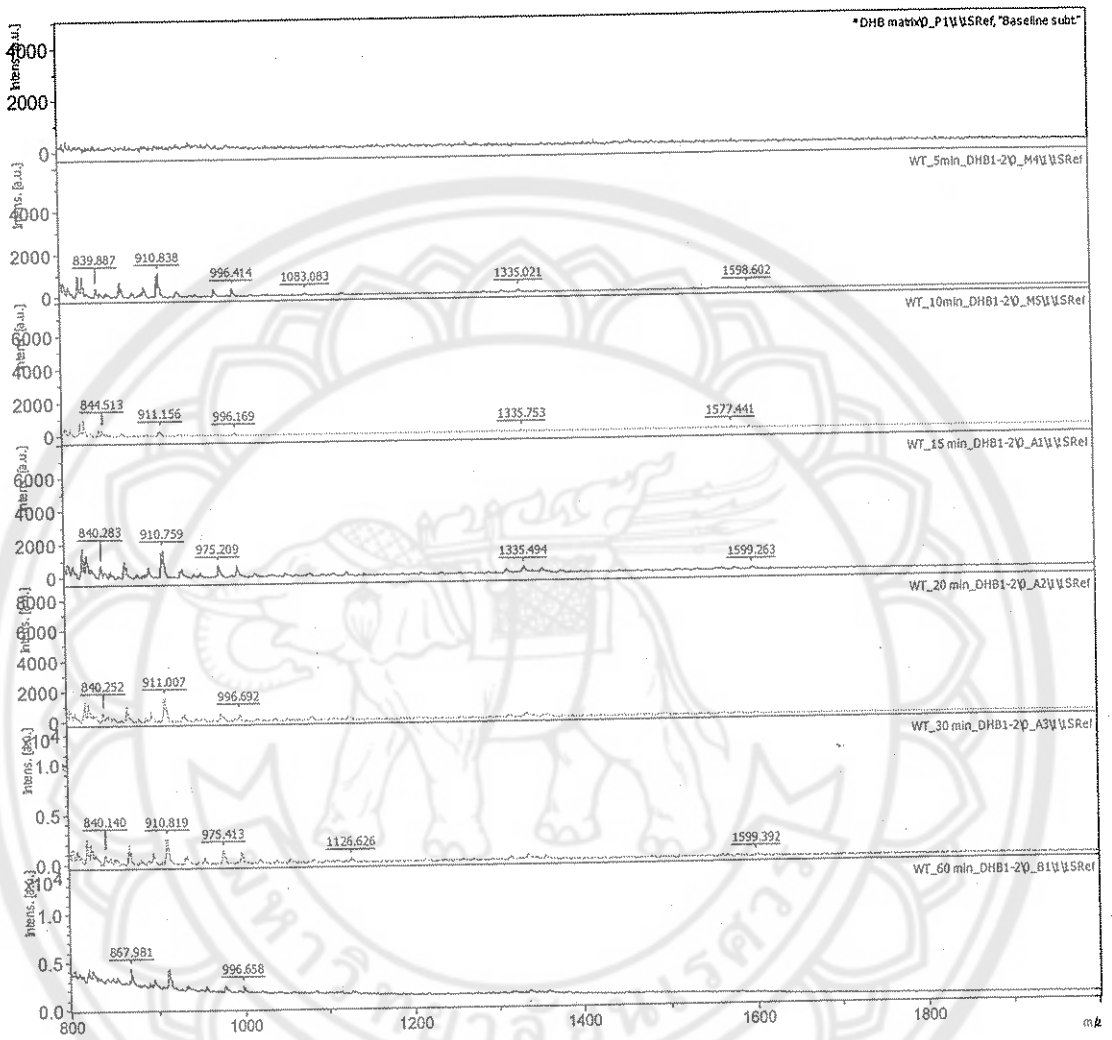


Figure 35 (Cont.)

(A)

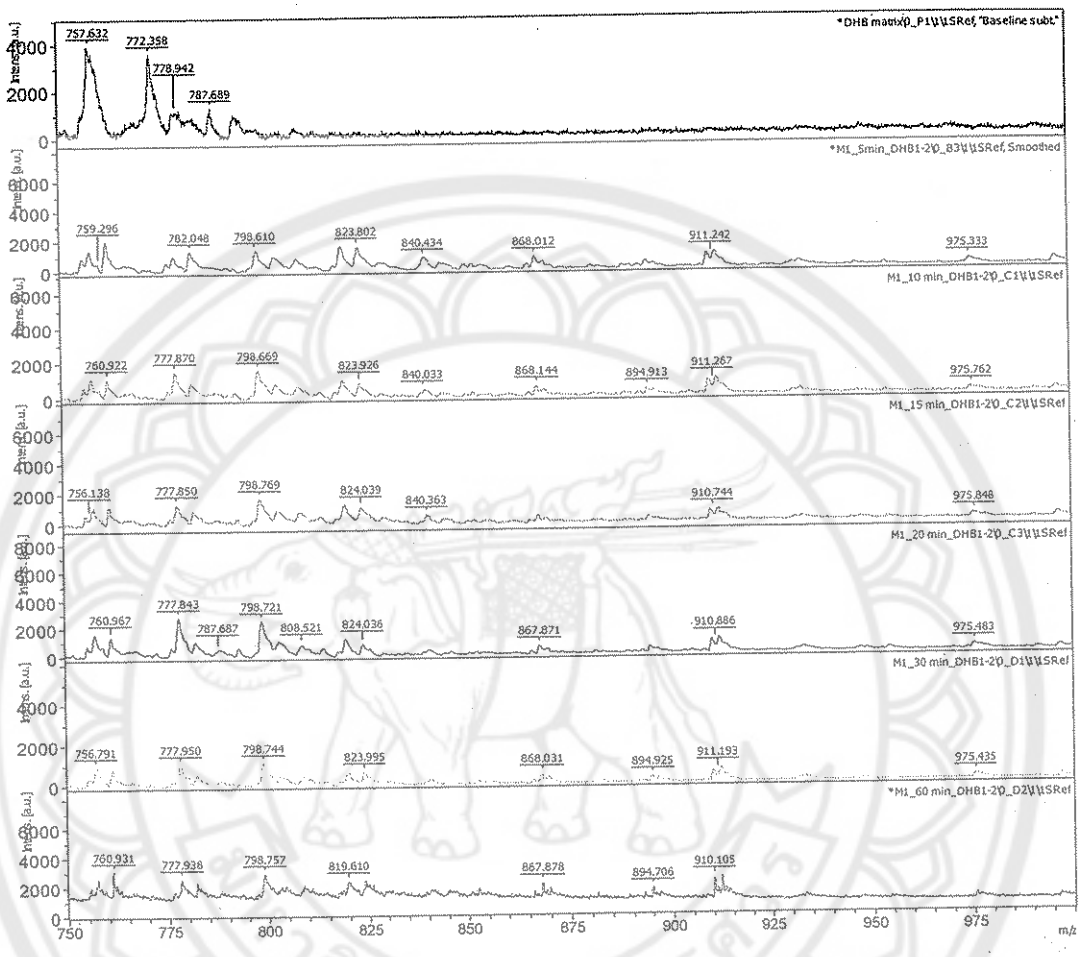


Figure 36 Digestion of mutant form of CgA by CTSL. The mutant at the position $Arg_{253}Trp$ of CgA (10 µg/reaction) was digested with 1 ng of CTSL for 5, 10, 15, 20, 30 and 60 min and CTSL digests were subjected to MALDI-TOF analysis.

(A) Mass between 800-1000 m/z. (B) Mass between 800-2000 m/z.

(B)

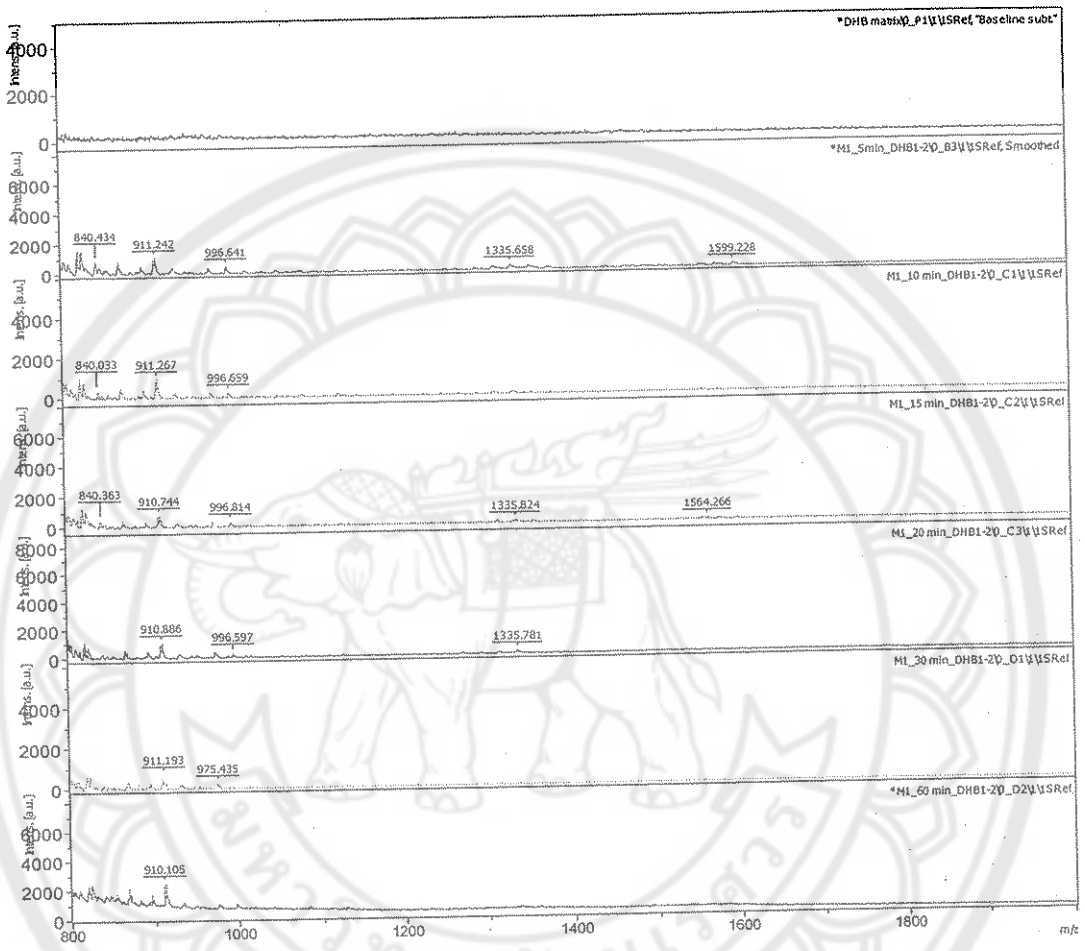


Figure 36 (Cont.)

(A)

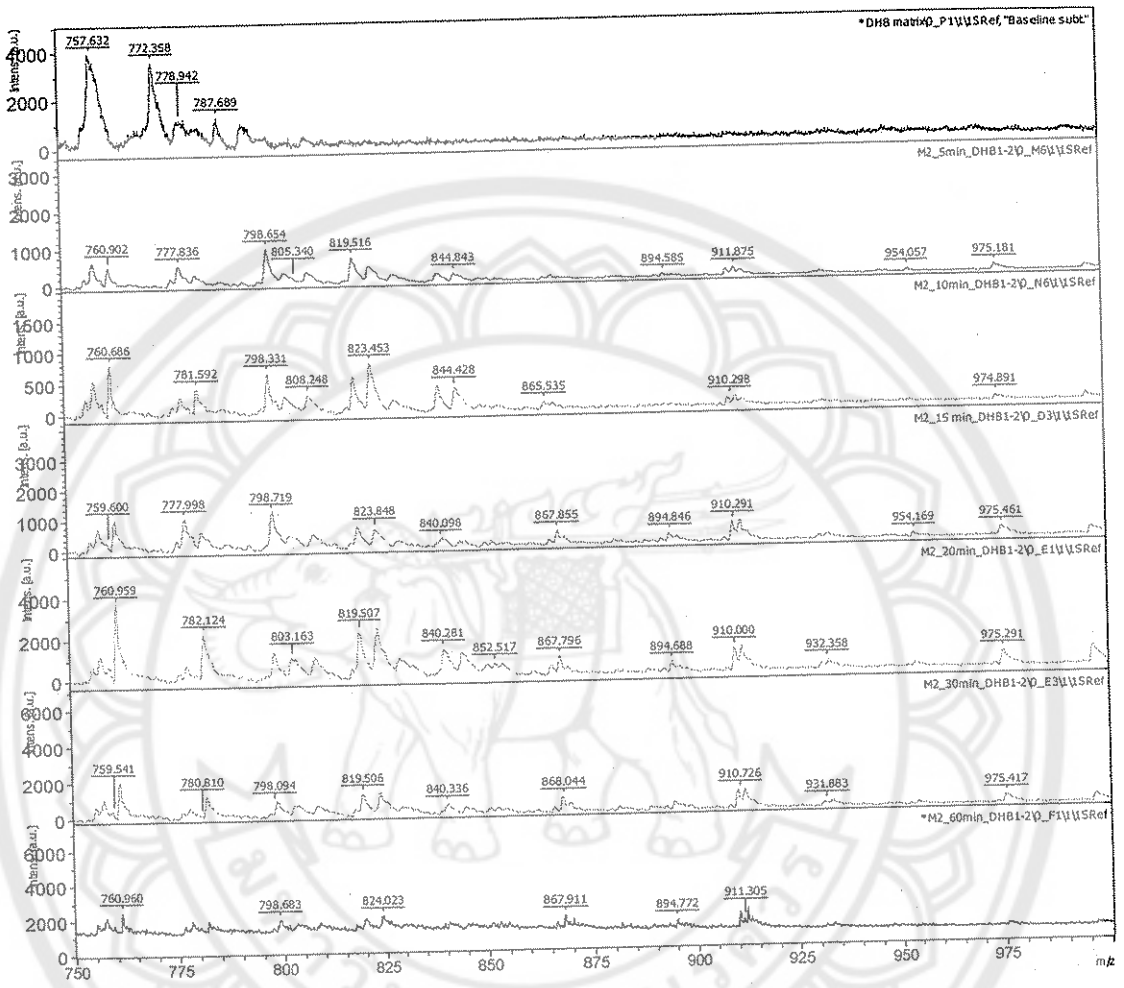


Figure 37 Digestion of mutant form of CgA by CTSL. The mutation at position Arg_{374}Gln of CgA (10 µg/reaction) was digested with 1 ng of CTSL for 5, 10, 15, 20, 30 and 60 min and CTSL digests were subjected to MALDI-TOF analysis.

(A) Mass between 800-1000 m/z. (B) Mass between 800-2000 m/z.

(B)

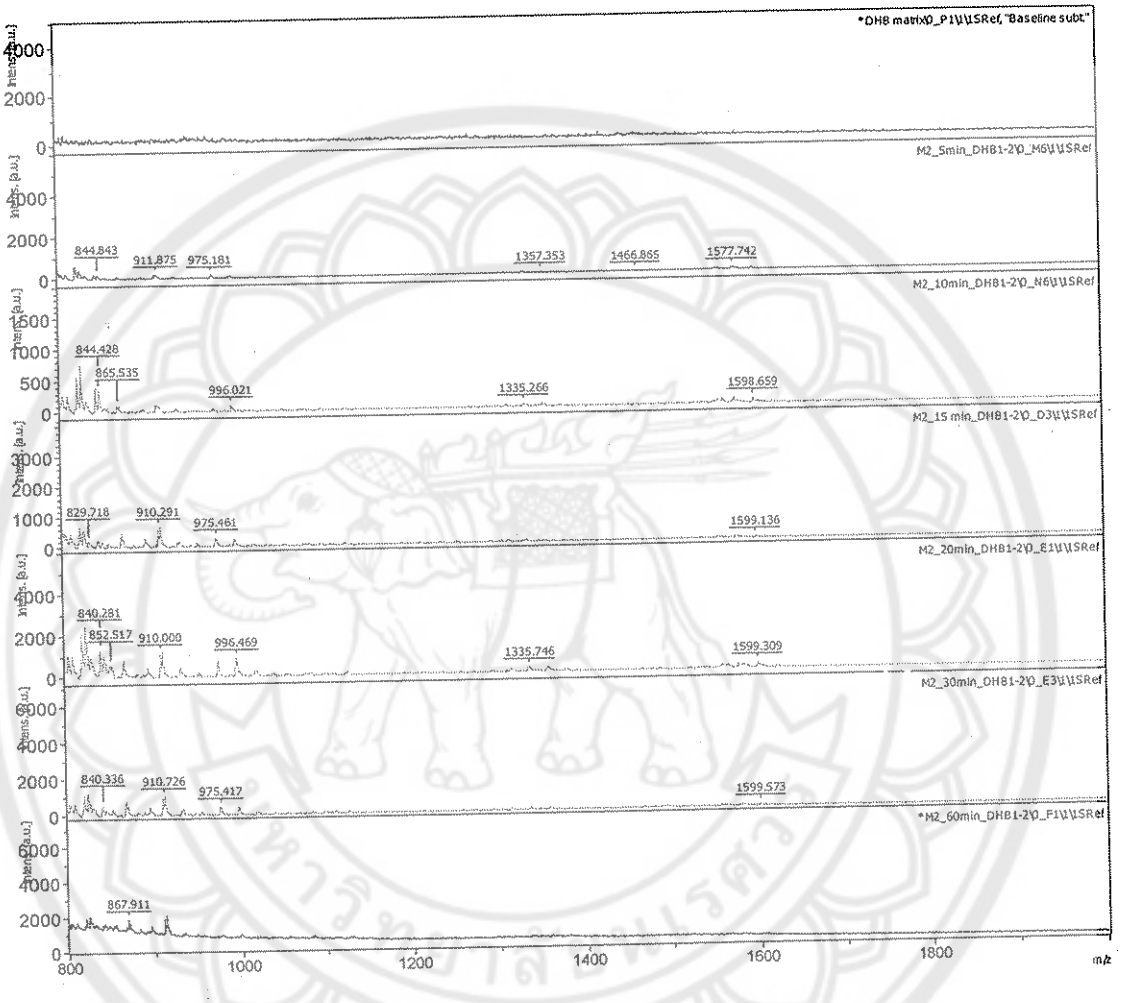


Figure 37 (Cont.)

(A)

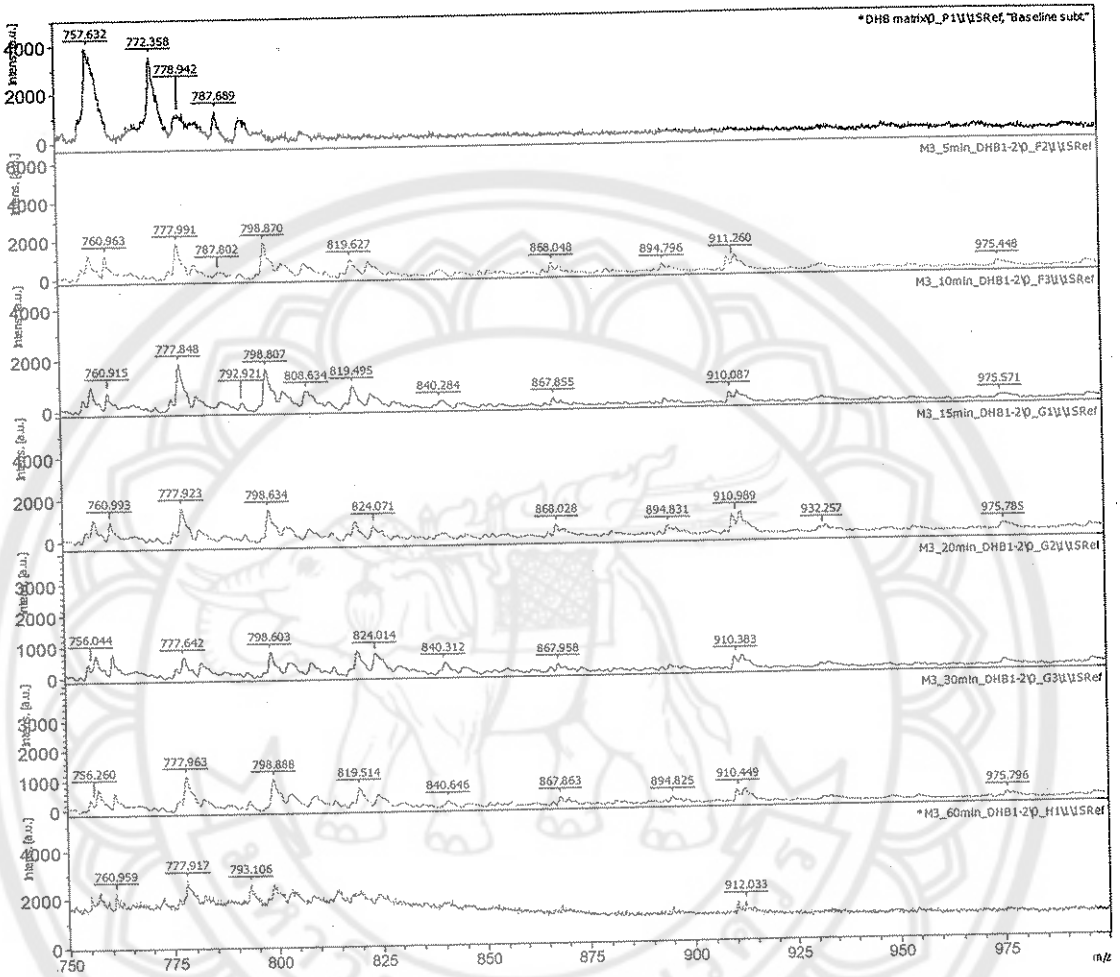


Figure 38 Digestion of mutant form of CgA by CTSL. The mutation at position Arg_{381}Trp of CgA (10 µg/reaction) was digested with 1 ng of CTSL for 5, 10, 15, 20, 30 and 60 min and CTSL digests were subjected to MALDI-TOF analysis.

(A) Mass between 800-1000 m/z. (B) Mass between 800-2000 m/z.

(B)

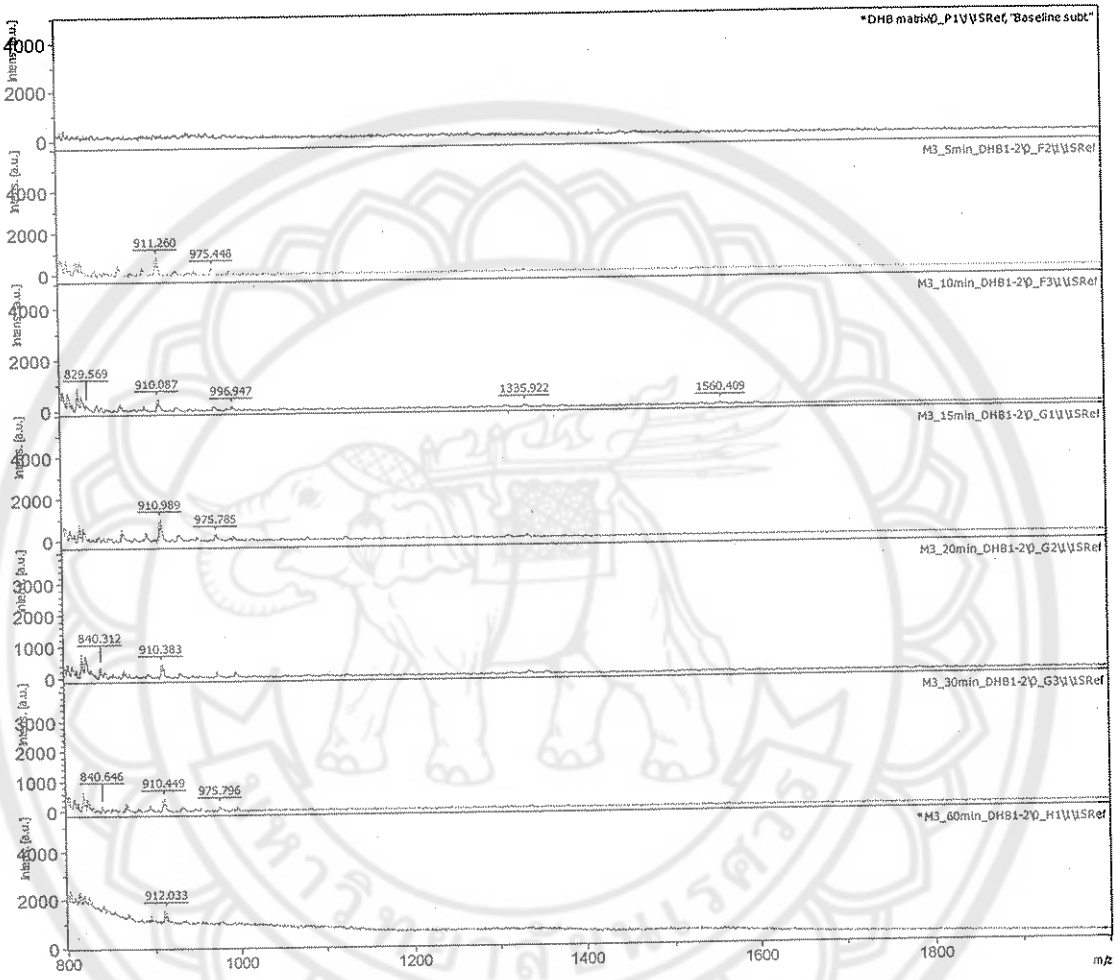


Figure 38 (Cont.)

(A)

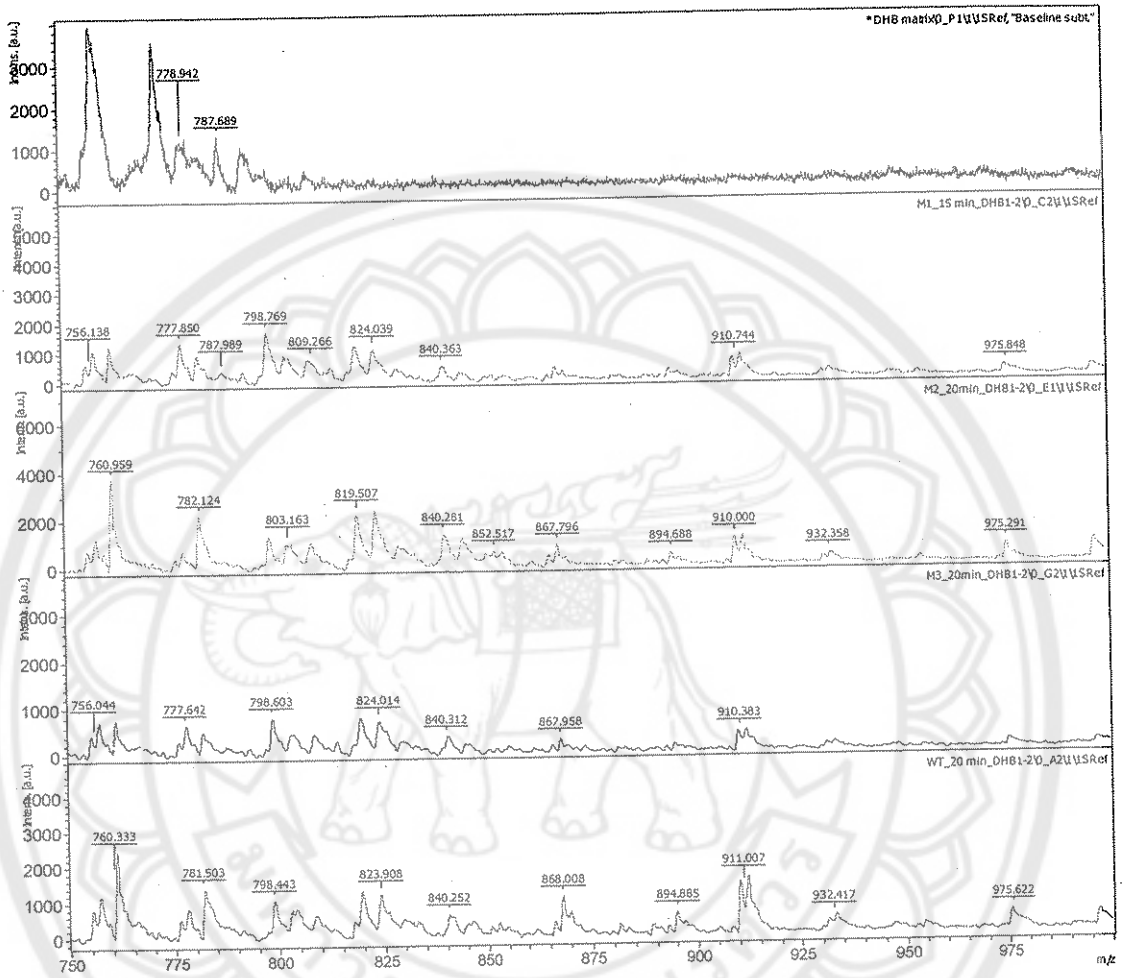


Figure 39 Comparison of recombinant human chromogranin A both wild-type and mutant forms after digested with CTSL for 20 min at 30°C and CTSL digests were subjected to MALDI-TOF analysis.

(A) Mass between 800-1000 m/z. (B) Mass between 800-2000 m/z.

(B)

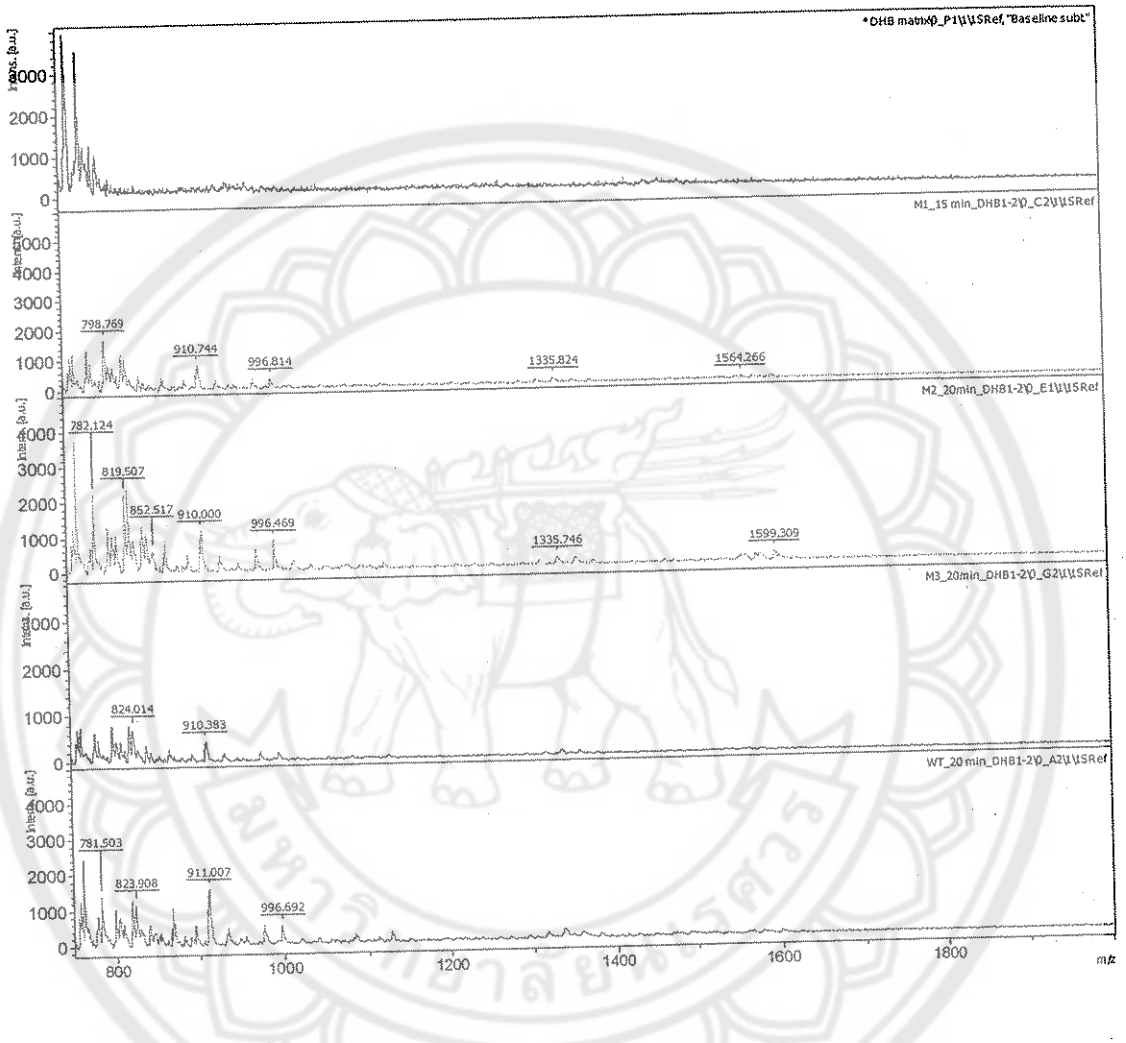


Figure 39 (Cont.)

Time-dependent, specific cleavage of recombinant chromogranin A in *vitro*

Purified recombinant human chromogranin A and its variants were digested with CTSL for various periods of time (5, 10, 15, 20, 30 and 60 min) at 30 ^{0}C and the reaction products were analyzed by MALDI-TOF. The fragments from wild-type and variants generated in the full-length are listed in the table 12.

Digestion of CgA containing naturally occurring variants by CTSL

Analysis of the mass spectrometric data revealed generation of the short fragments with m/z 840, 845, 868, 881, 894, 911, 932, 975, 996, 1083, 1126, 1335, 1577 and 1598 from the wild-type CgA. Digestion of Arg_{253}Trp, Arg_{374}Gln and Arg_{381}Trp generate the similar mass with m/z 840, 845 and 975 that compare with the wild-type.

Potential fragments generating from the full-length of human chromogranin A as identified by protein prospector program were listed in the table below.

Table 12 Digestion of the full-length of human chromogranin A as identified by protein prospector program

MS-Digest Search Results		Residues CgA
m/z (mi)	m/z (av)	
840.4475	840.9659	360-366
845.4377	845.9418	375-381
846.468	846.9645	334-339
850.4305	850.9524	396-402
880.5112	881.0314	367-374
891.3803	891.8747	403-410
896.4697	896.9872	72-78
935.4316	935.9688	117-124
949.4374	950.0015	318-324
975.4629	976.0341	102-110
1001.5388	1002.1304	375-382
1070.4861	1071.0979	277-285
1143.5793	1144.2801	198-208
1185.6474	1186.3586	60-70
1205.5281	1206.2153	304-313
1347.5659	1348.3303	340 -350
1426.7536	1427.607	382-394
1762.8316	1763.9759	286-300
2074.9676	2076.1973	176-195
2186.0823	2187.3798	410-428
2226.0521	2227.3607	254-276
2476.1474	2477.6166	79-100
3008.4777	3010.5396	17-43
3319.4469	3321.3831	145-175
3980.7567	3983.1348	210-245

Table 13 Digestion of the wild-type of human chromogranin A compare with the data as identified by protein prospector program

MS-Digest Search Results(m/z)	Wild-type	Residues CGA
840.9659	840.252	360-366
845.9418	845.379	375-381
846.9645	868.008	334-339
850.9524		396-402
881.0314	881.016	367-374
891.8747		403-410
896.9872	894.885	72-78
935.9688	911.007	117-124
950.0015	932.417	318-324
976.0341	975.622	102-110
1002.1304	996.692	375-382
1071.0979	1083.083	277-285
1071.0979	1083.083	277-285
1144.2801	1126.222	198-208
1186.3586	1335.021	60-70
1206.2153	1577.326	304-313
1348.3303	1598.602	340 -350
1427.607		382-394
1763.9759		286-300
2076.1973		176-195
2187.3798		410-428
2227.3607		254-276
2477.6166		79-100
3010.5396		17-43
3321.3831		145-175

Table 14 Digestion of the $Arg_{253}Trp$ of human chromogranin A compare with the data as identified by protein prospector program

MS-Digest Search Results (m/z)	$Arg_{253}Trp$	Residues CGA
840.9659	840.363	360-366
845.9418	845	375-381
846.9645	868	334-339
850.9524	894	396-402
881.0314	910.744	367-374
891.8747		403-410
896.9872		72-78
935.9688		117-124
950.0015		318-324
976.0341	975.848	102-110
1002.1304	996.814	375-382
1071.0979	1335.824	277-285
1144.2801	1564.266	198-208
1186.3586	1598.927	60-70
1206.2153		304-313
1348.3303		340 -350
1427.607		382-394
1763.9759		286-300
2076.1973		176-195
2187.3798		410-428
2227.3607		254-276
2477.6166		79-100
3010.5396		17-43
3321.3831		145-175

Table 15 **Digestion of the $Arg_{374}Gln$ of human chromogranin A compare with the data as identified by protein prospector program**

MS-Digest Search Results (m/z)	$Arg_{374}Gln$	Residues CGA
840.9659	840.281	360-366
845.9418	845.058	375-381
846.9645	867.796	334-339
850.9524	894.688	396-402
881.0314	910	367-374
891.8747	912.212	403-410
896.9872	932.358	72-78
935.9688		117-124
950.0015		318-324
976.0341	975.291	102-110
1002.1304	996.469	375-382
1071.0979	1018.404	277-285
1144.2801	1335.746	198-208
1186.3586	1356.789	60-70
1206.2153	1542.718	304-313
1348.3303	1560.696	340 -350
1427.607	1577.315	382-394
1763.9759	1582.354	286-300
2076.1973	1586.313	176-195
2187.3798	1599.309	410-428
2227.3607		254-276
2477.6166		79-100
3010.5396		17-43
3321.3831		145-175

Table 16 **Digestion of the $Arg_{381}Trp$ of human chromogranin A compare with the data as identified by protein prospector program**

MS-Digest Search Results m/z (av)	$Arg_{381}Trp$	Residues CGA
840.9659	840.312	360-366
845.9418	845	375-381
846.9645	867.958	334-339
850.9524	894.825	396-402
881.0314	910.383	367-374
891.8747		403-410
896.9872		72-78
935.9688		117-124
950.0015		318-324
976.0341	975.918	102-110
1002.1304	996.789	375-382
1071.0979		277-285
1144.2801		198-208
1186.3586		60-70
1206.2153		304-313
1348.3303		340 -350
1427.607		382-394
1763.9759		286-300
2076.1973		176-195
2187.3798		410-428
2227.3607		254-276
2477.6166		79-100
3010.5396		17-43
3321.3831		145-175

Table 17 Comparable the digestion products between wild-type and mutant forms of human chromogranin A

Wild-type	$Arg_{253}Trp$	$Arg_{374}Gln$	$Arg_{381}Trp$	Residues CGA
840.252	840.363	840.281	840.312	360-366
845.379	845	845.058	845	375-381
868.008	868.144	867.796	867.958	367-374
881.016	-	-	-	
894.885	894.827	894.688	894.825	
911.007	910.744	910.000	910.383	
932.417	932.529	932.358	-	
975.622	975.848	975.291	975.918	102-110
996.692	996.814	996.469	996.789	
1083.083		1018.404		
1126.222				
1335.021	1335.824	1335.746	1335.992	
		1356.789		
		1542.718		
	1564.266	1560.696	-	
1577.326	-	1577.315	-	360-373
		1582.354		
		1586.313		
1598.602	1598.927	1599.309		