CHAPTER 4

RESULTS AND DISCUSSION

The proteins in *Bungarus candidus* venom were analyzed using all methods as described in the previous chapter. The results will be shown and discussed in each part of this chapter, respectively.

4.1 Characterization proteins by gel electrophoresis for crude *Bungarus candidus* venom

Crude Malayan krait venom was separated by 2-D gel electrophoresis and the result was shown in Figure 4.1

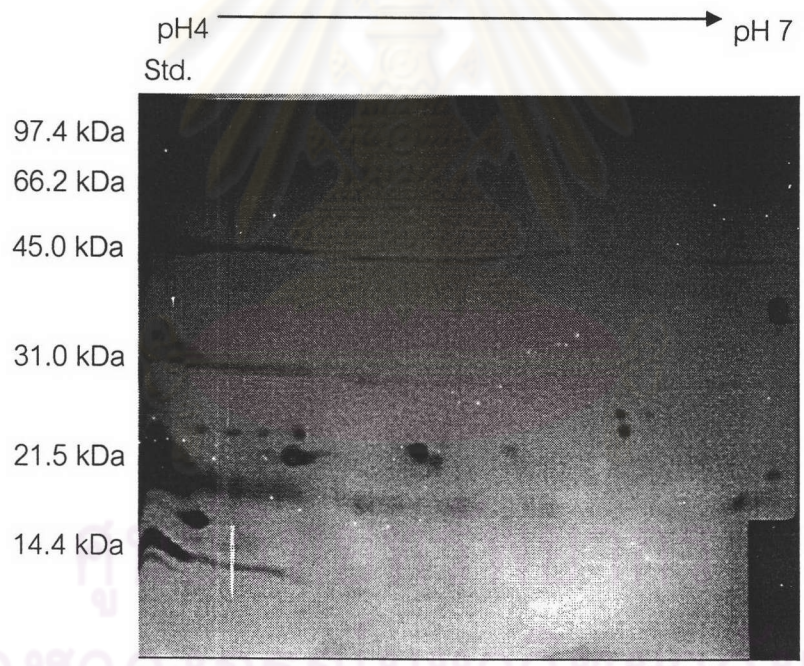

Figure 4.1 2-D SDS-PAGE gel stained by silver.

Each spot in gel represents individual protein which was separated by *pI* in the first dimension and followed by molecular weight separation in the second dimension. (The results were summarized in Table 4.1)

Table 4.1 Molecular weight range of protein spots in silver-stained gel

Molecular weight range (kDa)	Number of spot proteins
45.0-31.0	2
31.0-21.5	14
21.5-14.4	1

There are 17 spots of proteins in silver stained gel. However, silver staining which has glutardialdehyde in staining procedure is affected to the in-gel digestion protocol because glutardialdehyde[27] is caused by proteins cross linking in gel. Another staining technique is coomassie blue G staining. The gel is shown in Figure 4.2

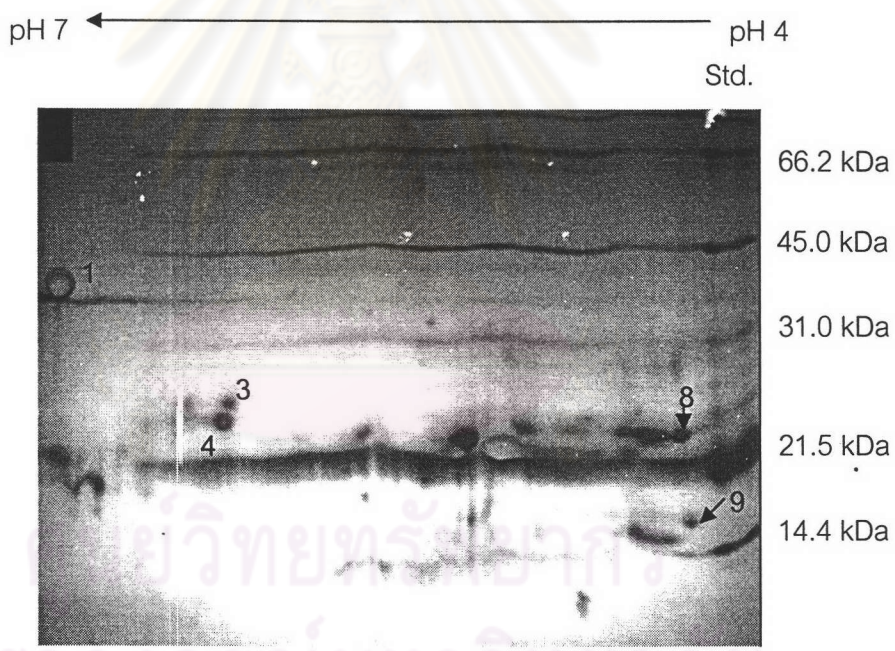

Figure 4.2 2-D gel image of pH 4-7 was stained by coomassie brilliant blue G

The gel was also stained by coomassie brilliant blue G staining technique and shows 36 spots of proteins (Figure 4.2). The abundance of proteins were summarized in Table 4.2.

Table 4.2 Molecular weight range of spot proteins in coomassie blue G -stained gel

Molecular weight range (kDa)	Number of spot proteins
66.2-45.0	4
45.0-31.0	17
31.0-21.5	11
21.5-14.4	2

After proteins were separated by SDS-PAGE and visualized with coomassie staining. The spots were excised and subjected to digestion with trypsin as described in Section 3.4.5. The molecular weight of each tryptic fragment was analyzed by MALDI-TOF MS. However, there are only 5 spots of proteins that obtained mass spectra from analyzed tryptic fragments. The mass spectra are shown in Figure 1-5A in Appendices and the result are shown in Table 4.3

Table 4.3 Mass per charge of in-gel tryptic peptide of each spot (spot numbers were shown in Figure 4.2)

Spot No.	m/z
1	2963.715, 2554.090, 2291.689, 2275.763, 2236.561, 1108.930, 1060.664, 941.204
3	2291.521, 2236.675, 2150.124, 1047.164
4	2291.508, 2275.559, 1168.016, 1060.609, 1022.631
8	2773.507, 2514.979, 2275.345, 2164.935, 1791.919, 1632.447, 1266.906
9	2434.804, 2275.756, 2251.323, 1617.533, 1448.125, 1417.037, 1401.110, 1374.828, 1362.075, 1358.960, 1356.197

And then, the mass spectra were used for performing of protein peptide mass mapping *via* PepIdent (http://au.expasy.org/tools/peptident.html). However, the

searched results were not reasonable, they might be new proteins or the data was not enough for identification of protein. Therefore, the sequencing techniques were required for further proteins identification.

4.2 Characterization proteins by gel electrophoresis, ion exchange chromatography, mass spectrometry and sequencing technique for *Bungarus candidus* venom fraction

Protein mixtures of *Bungarus candidus* fractions No. 2, 6, 7 and 8 (IEX No. 2, 6, 7and 8) were collected from ion exchange chromatography as described in Section 3.1. The ion exchange chromatogram is shown in Figure 4.3 and the concentration of proteins of each fraction is shown in Table 4.4

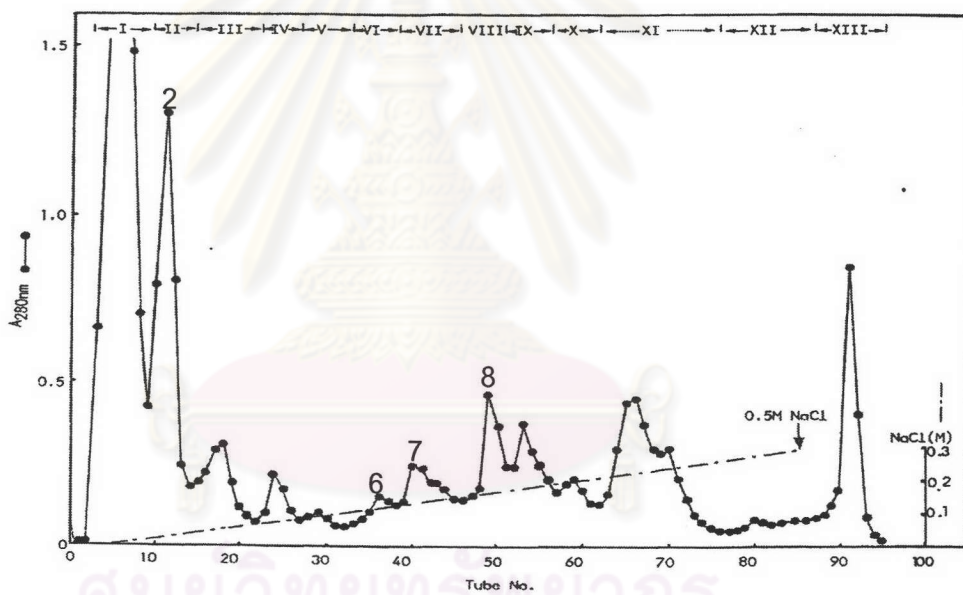

Figure 4.3 Ion exchange chromatogram of *Bungarus candidus* venom.

Table 4.4 The concentration of proteins of each fraction

IEX No.	Concentration (mg/mL)
2	0.4251
6	0.0408
7	0.1067
8	0.1698

4.2.1. Molecular weight of proteins in each fraction by MALDI-TOF MS

The mass spectra of each fraction are shown in Figure 6-9A in Appendices and the results are summarized in Table 4.5

Table 4.5 Mass per charge of each ion exchange fractions 2, 6, 7, 8

IEX No.	m/z
2	9061.21, 8729.17, 7331.15, 4535.81, 4369.77, 3670.03
6	7454.00, 3731.79
7	9072.06, 8807.66, 7477.00, 4540.77, 4410.51, 3741.07
8	6671.32, 3343.54

From Table 4.5, there are 3 proteins in *Bungarus candidus* venom IEX No. 2. The m/z of 4535.81, 4369.77 and 3670.03 is doublet charged species of proteins which yield the molecular weight of 9060.21, 8728.17 and 7330.15, respectively. From of IEX fraction No. 7, there are 3 proteins. The m/z of 4540.77, 4410.51 and 3741.07 is doublet charged species of proteins which yield the molecular weight of 9071.06, 8806.66 and 7476.00, respectively. From IEX fraction No. 6, the m/z of 7454.00 is singlet charge species and 3731.79 is the doublet charged species of the protein so molecular weight is 7453. From the IEX results of fractions 6 and 8, there is only one protein which is easier for further analysis.

4.2.2 1-D gel electrophoresis of fractions 6 and 8

SDS-PAGE of fractions 6 and 8 is shown in Figure 4.4

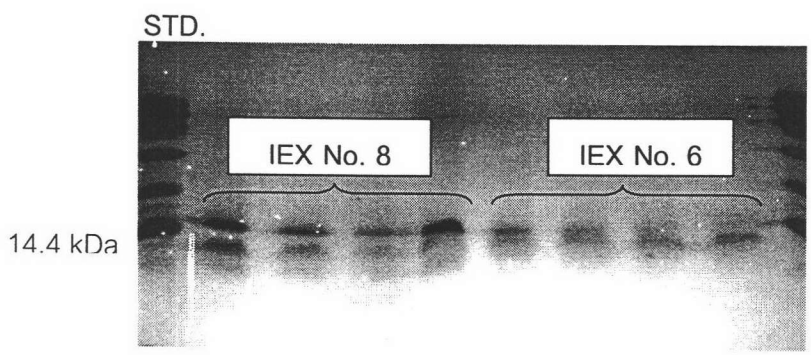

Figure 4.4 One-dimensional coomassie-stained gel of IEX No. 6 and 8.

After that, the bands were excised and subjected to trypsin digestion. The molecular weight of each tryptic peptides were analyzed by MALDI-TOF MS. The mass spectra of tryptic peptides are shown in Figure 10-11A as listed in Table 4.6

Table 4.6 Mass per charge of in-gel tryptic fragment of each band

Band of IEX No.	m/z
6	1916.94, 1813.47, 1776.16, 1709.27, 1674.73, 1495.21, 1477.34, 1436.15, 1322.23, 1120.54, 1022.13
8	1812.95, 1798.79, 1516.85, 1443.21, 1415.12, 1298.30, 1282.39, 1061.43

And then, the molecular weights of in-gel tryptic fragment of IEX fractions No. 6 and 8 were used for protein peptide mass mapping *via* the program MASCOT (http:// www.matrixscience.com). However, the searched results were not reasonable. It might be a new protein or the data was not enough to identify each protein. Sometimes more than one candidate for identity of the protein can be found, whereupon additional information obtained by sequencing techniques is generally used to constrain the search.

4.2.3 N-terminal sequencing by Edman degradation

IEXs No. 6 and 8 were desalted as described in section 3.4.5. The mass spectra of IEXs No. 6 and 8 were shown in Figure 12-13A. The protein which is in IEXs No. 6 and 8 are proteins A and B, respectively. The results of N-terminal sequencing by Edman degradation of protein A and B are shown below.

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Protein A: KTKI-      PEKD-      QKV

Protein B: NLINF      MEMIR      YT
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Results show that, N-terminal sequencing of proteins A and B has 11 residues and 12 residues, respectively.

From database searching, the sequencing data were in protein sequence database *via* the program Blast (http:// www.ncbi.nlm.nih.gov/BLAST/ Blast.cgi). Search parameters were set for vertebrata organism and swiss-prot database. The N-terminal sequencing of protein A matched with weak toxin 1, 2 and 3 (*Bungarus candidus*). The results are shown below.

Weak toxin 1, 2, 3 : ^{28}TCLICPEKDCQKV^{35}

Protein A : ^{1}KTK**I-PEKD-QKV**^{13}

The bold letters are shown similar sequence between protein A and weak toxin 1, 2, 3. The percentage of similarity is 61.54%.

The N-terminal sequencing of protein B matched with phospholipase A2 beta-bungarotoxin A3 chain precursor (*Bungarus multicintus*) and beta-neurotoxin (*Bungarus candidus*) by searched in protein sequence database *via* the program Blast. The percentage of similarity is 100.00%. The N-terminal amino acid sequences are shown below.

Phospholipase A2 : NLINF^{5} MEMIR^{10} YT

Beta-neurotoxin : NLINF^{5} MEMIR^{10} YT

Protein B : **NLINF**^{5} **MEMIR**^{10} **YT**

However, the results of searched database were not reasonable because molecular weight of protein B is 7453, but molecular weight of phospholipase A2 beta-bungarotoxin A3 chain precursor and beta-neurotoxin were 16217 and 10966, respectively. Therefore, protein B was not phospholipase A2 or beta-neurotoxin.

4.2.4 Peptide mass mapped and partial sequencing by ESI-Q-TOF MS of *Bungarus candidus* venom fraction 6

B. candidus venom fraction No. 6 was desalted as described in section 3.4.5. The mass spectrum of protein A was shown in Figure 14A. After digestion, the molecular weight of tryptic fragments were analyzed by MALDI-TOF MS as shown in Figure 4.5 and ESI-Q MS as shown in Figure 12A-13A.

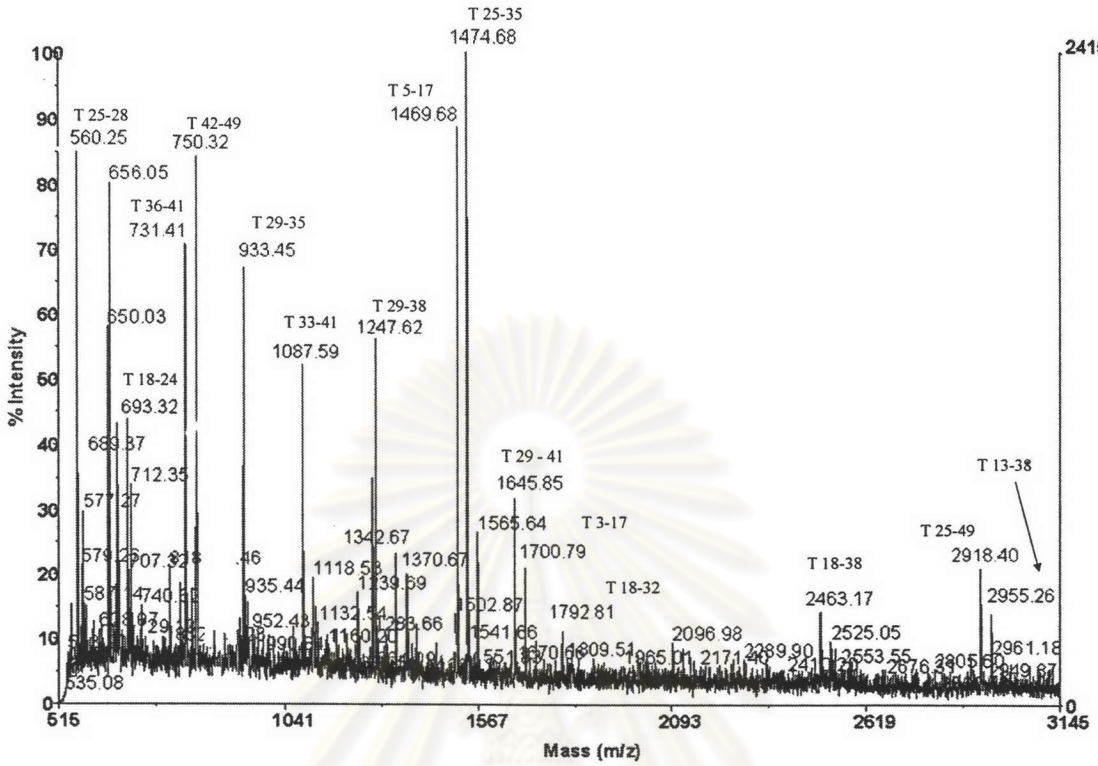

Figure 4.5 MALDI-TOF mass spectrum of tryptic digests of *B. candidus* venom fraction 6.

The molecular weight of each peptide was analyzed by MALDI-TOF. The m/z of a singlet charge of each peptide was used for database searching *via* PepIdent (http://au.expasy.org/tools/peptident.html). Searching parameters were set at 100 ppm of mass tolerance, 2 missed cleavage, iodoacetamide modified cystein and vertebrata specie. The search result of identified protein is candoxin, and each peak of spectrum was the m/z of peptide fragments of candoxin. For example, the m/z of 1474.68 was a singlet charge that assigned to peptide fragment which has twenty fifth to thirty fifth amino acid residues. Similar to peptide fragment which has fifth to seventeenth amino acid residues that are assigned to the singlet charged m/z of 1469.68. From the results, there are 15 peaks of mass spectrum that are assigned to peptide fragments of candoxin which molecular weight was 7344, but molecular weight of protein A was 7453. Therefore, protein A was not candoxin.

Furthermore, the peptides were sequenced by MS/MS with ESI-Q-TOF and the results are shown in Figures 4.5, 4.6 and 4.7

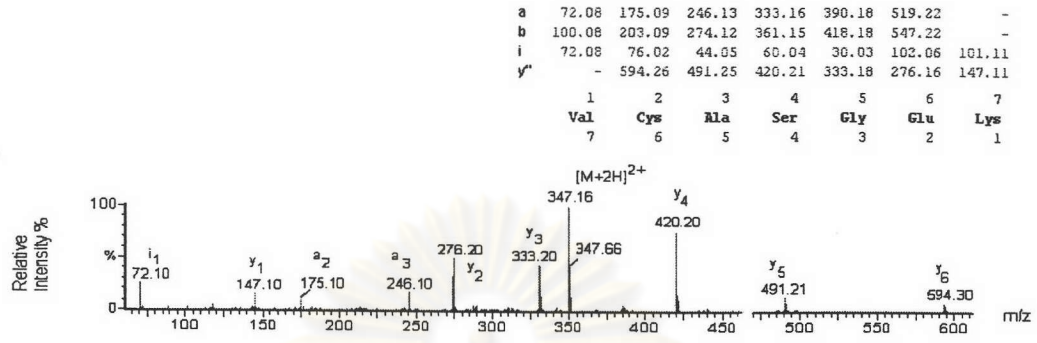

Figure 4.6 ESI-Q-TOF mass spectrum of partial amino acid sequence of precursor ion of m/z of 347.16.

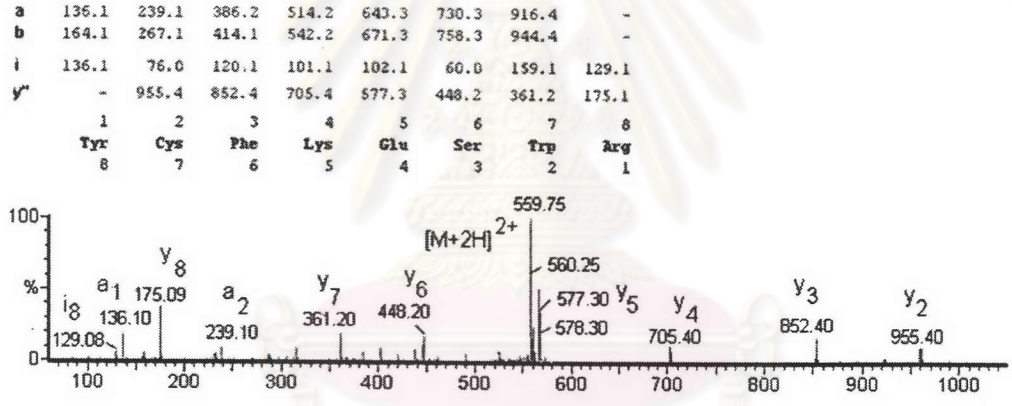

Figure 4.7 ESI-Q-TOF mass spectrum of partial amino acid sequence of precursor ion of m/z of 559.75.

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a	102.06	173.09	329.19	386.22	487.26	-
b	130.05	201.09	357.19	414.21	515.26	-
i	102.06	44.05	129.11	30.03	74.06	129.11
y''	-	560.33	489.29	333.19	276.17	175.12
	1	2	3	4	5	6
	Glu	Ala	Arg	Gly	Thr	Arg
	6	5	4	3	2	1

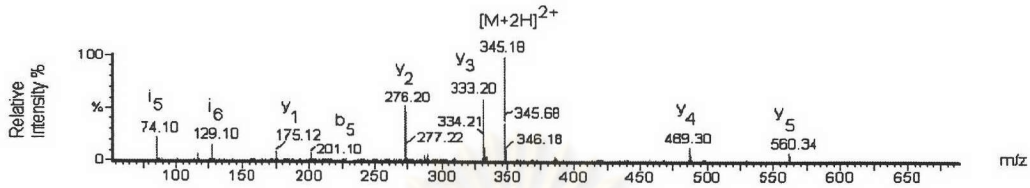

Figure 4.8 ESI-Q-TOF mass spectrum of partial amino acid sequence of precursor ion of m/z of 345.18.

a	76.0	179.0	280.1	381.1	496.2	611.2	714.2	-
b	104.0	207.0	308.1	409.1	524.1	639.2	742.2	-
y''	-	771.2	668.2	567.2	466.1	351.1	236.1	133.1
i	76.0	76.0	74.1	74.1	88.0	88.0	76.0	87.1
	1	2	3	4	5	6	7	8
	Cys	Cys	Thr	Thr	Asp	Asp	Cys	Asn
	8	7	6	5	4	3	2	1

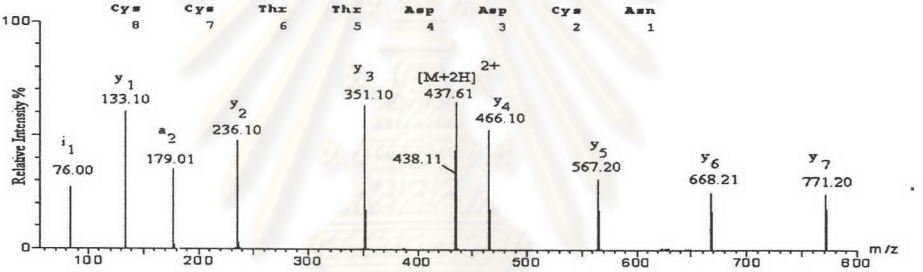

Figure 4.9 ESI-Q-TOF mass spectrum of partial amino acid sequence of precursor ion of m/z of 437.61.

The sequence obtained from each tryptic peptide was used for protein identification *via* Blast (http:// www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi). Search parameters were set to two missed trypsin cleavages, vertebrata organism and nr database. From the result of database searching, partial amino acid sequence of protein A was nearly coincided with partial amino acid sequence of candoxin. A comparison of amino acid sequence between candoxin and protein A is shown below.

Candoxin:	MKCKI[5]	CNFDT[10]	CRAGE[15]	LKVCA[20]	SGEKY[25]	CFKES[30]
Protein A:	KTKI-	PEK**D**-	QKV--	--**VCA**	**SGEKY**	**CFKES**
Candoxin:	WREAR[35]	GTRIE[40]	RGCAA[45]	TCPKG[50]	SVYGL[55]	YVLCC[60]
Protein A:	**WREAR**	**GTR**--	-----	-----	-----	---**CC**

Candoxin: TTDDC[65] N

Protein A: **TTDDC N**

The bold letters are shown similar sequence between protein A and candoxin. The amino acid sequence of first peptide fragment, VCASGEK, was seemed to be the eighteenth to twenty fourth amino acids residues of candoxin. Next, the second peptide, YCFKESWR, as shown in Figure 4.6 was seemed to be the twenty fifth to thirty second amino acid residues. In addition, the third amino acid sequence, EARGTR, as shown in Figure 4.7 was seemed to be the thirty third to thirty eighth amino acid residues. The fourth amino acid sequence, CCTTDDCN, as shown in Figure 4.9 was seemed to be the fifty ninth to sixty sixth amino acid residues. The percentage of identity was 45.5%. However, the result of N-terminal sequencing was not matched with candoxin. Therefore, further analysis of this protein is required to perform a fully sequence.

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