CHAPTER 3

RESULTS AND DISCUSSION

3.1 SDS-Polyacrylamide Gel Electrophoresis of Snake Venom

The SDS-PAGE electrophoresis of *T. macrops* venom was preformed in 12%*T*, 3%*C* discontinuous SDS gel (Figure 3.1). The gel was stained with Coomassie Brilliant Blue. The molecular weights of protein from *T. macrops* venom were estimated. The *T. macrops* venom shows widely molecular mass range from 14 to 66 kDa. Venom from *T. macrops* has two major proteins with approximate molecular weight of 14.4 and 45 kDa.

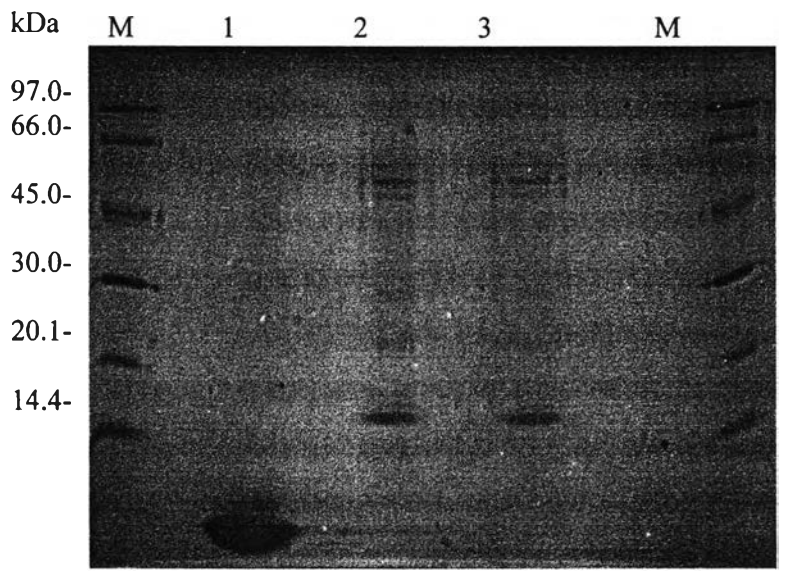

Figure 3.1 SDS-PAGE *T. macrops* Venom. Lane M: The Low Molecular Weight Standard containing phophorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin; lane 1: Insulin (5.7 kDa): lane 2, 3: T. macrops venom 5 μg.

3.2 Two-Dimensional Electrophoresis of Snake Venom

The 2-D gel images of *T. macrops* venom with the different IPG pH and staining methods are demonstrated in Figure 3.2. The 2-D gel images are very similar with many spotting in molecular mass ranges between 14 kDa and 66 kDa. Most of protein spots are grouped in the left region, which is the acidic (low pI) region. These can be presumed that most of proteins from *T. macrops* venom are acidic proteins. Results showed that there were 45 protein spots in Silver-stained 12.5%*T*, pH 4-7 gel (Figure 3.2 A), 37 protein spots in Silver-stained 12.5%*T*, pH 3-10 gel (Figure 3.2 B), and 32 protein spots in Coomassie-stained 12.5%*T*, pH 3-10 gel (Figure 3.2 C). The narrow pI range (pH 4-7) gel image reveals the proteins much clearer than a wide pI range (pH 3-10) gel. Also, the resolution of IEF using narrow pI range (pH 4-7) IPG strip is increasing. There are more protein spots in Silver-stained 12.5%*T*, pH 3-10 gel than Coomassie-stained gel according to the high sensitivity of Silver staining method. The 2-D gel images are presented in Figure 3.2 A-C.

The protein spots of interest (No. 1-7 in Figure 3.2 C) were manually excised and then in-gel digestion with trypsin was performed. The tryptic fragments from each spot were used for protein identification via a peptide mass mapping technique (Section 1.6.2 (2)). The silver stained spots showed no signals in MALDI mass spectrometry. It might be because of the low protein amount in the silver stained spot, thus the detection limits of silver staining can be better than the detection limits of mass spectrometry. Also, the analysis of silver staining gel can be accomplished only with the high-sensitivity mass spectrometry systems, such as nano or micro electrospray mass spectrometry.[18] Therefore, Coomassie stained spots (Figure 3.3 C) were used for the peptide mass mapping technique. MALDI/Tof mass spectra of tryptic fragments are shown in Figure 3.3 A-G. The significant peaks in mass spectra were shown in Table 3.1.

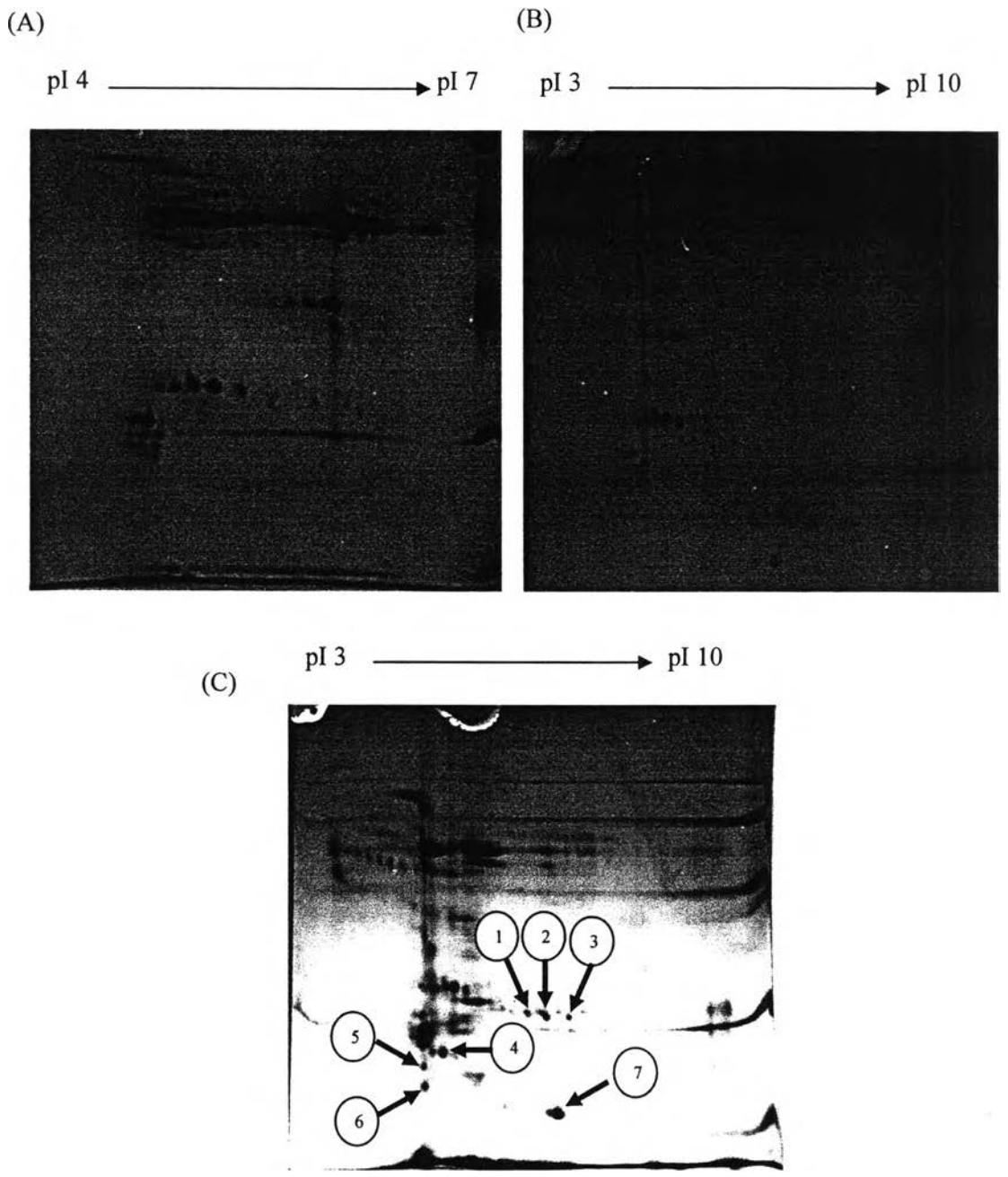

Figure 3.2 2-D Gel Images of *T. macrops* Venom: (A) Silver-stained gel 12.5%*T*, 2.6%*C*, pH4-7; (B) Silver-stained gel 12.5%*T*, 2.6%*C*, pH3-10; (C) Coomassie Stained gel 12.5%*T*, 2.6%*C*, pH3-10. The numbers denote gel spots that were digested.

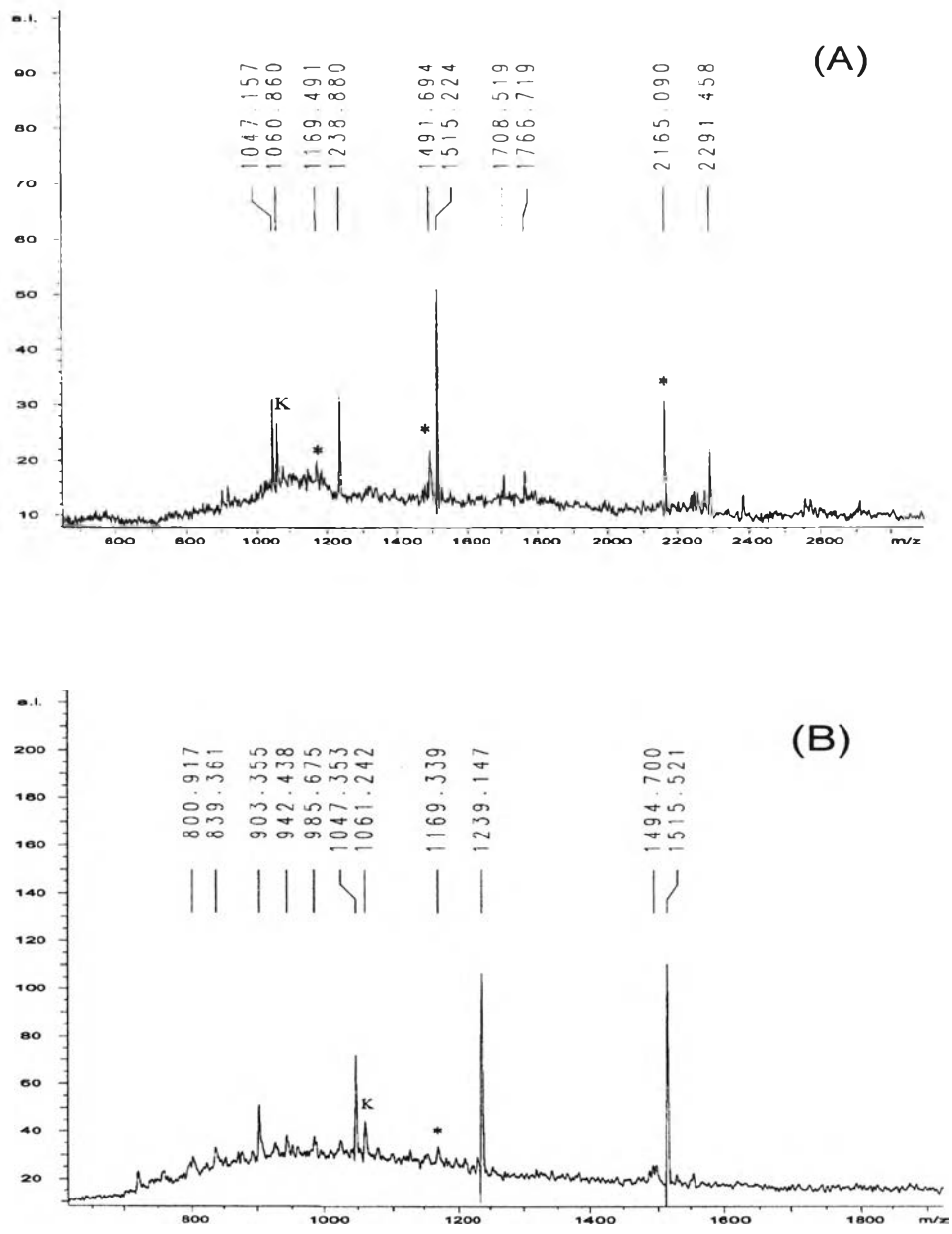

Figure 3.3 Tryptic Fragments Mass Spectra from 7 Protein Spots: (A) Spot No. 1; (B) Spot No. 2; (C) Spot No. 3; (D) Spot No. 4; (E) Spot No. 5; (F) Spot No. 6. Peaks marked with K latter and asterisks are keratin contamination and tryptic autolysis products.

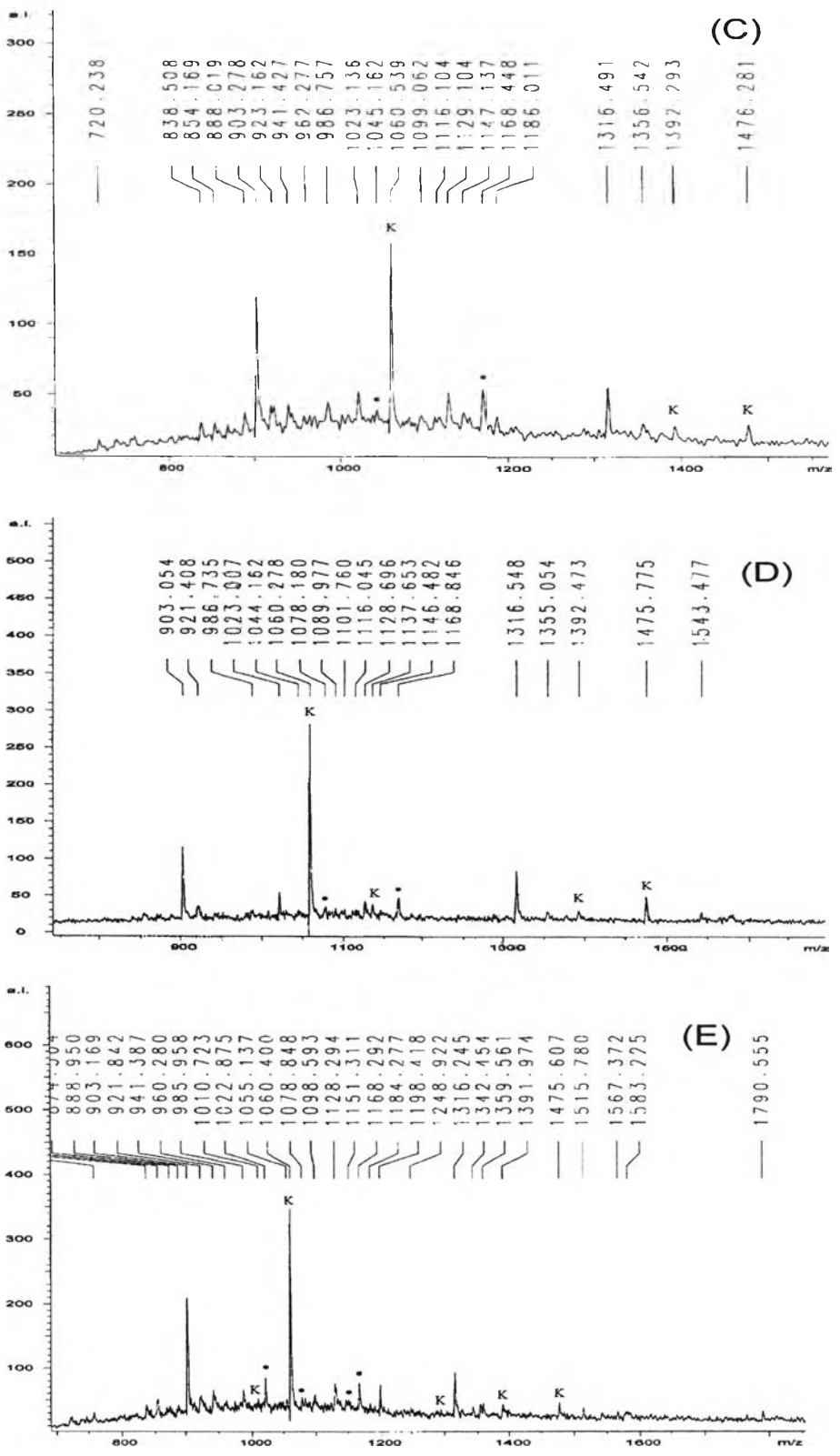

Figure 3.3 Tryptic Fragments Mass Spectra from 7 Protein Spots: (A) Spot No. 1; (B) Spot No. 2; (C) Spot No. 3; (D) Spot No. 4; (E) Spot No. 5; (F) Spot No. 6. Peaks marked with K latter and asterisks are keratin contamination and tryptic autolysis products. (continued)

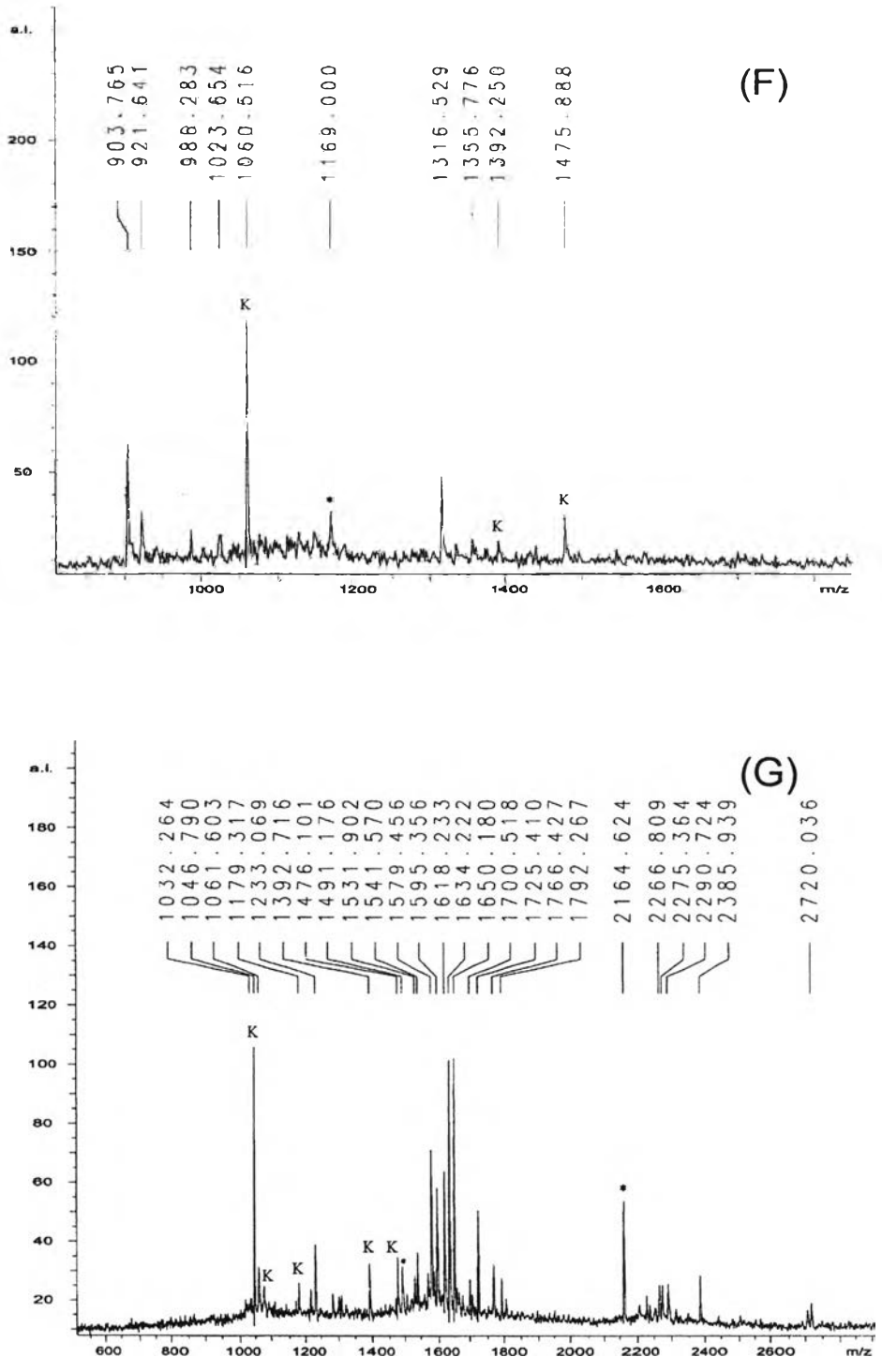

Figure 3.3 Tryptic Fragments Mass Spectra from 7 Protein Spots: (A) Spot No. 1; (B) Spot No. 2; (C) Spot No. 3; (D) Spot No. 4; (E) Spot No. 5; (F) Spot No. 6. Peaks marked with K latter and asterisks are keratin contamination and tryptic autolysis products. (continued)

Table 3.1 Measured Tryptic Fragments Masses from Figure 3.3 A-G.

Spot No. (Figure 3.2 (C))	Observed Mass* (*m/z*)
1	1047.16, 1238.88, 1515.22, 1708.52, 1766.72, 2291.46
2	800.917, 839.36, 903.36, 942.44, 985.68, 1047.35, 1239.15, 1494.70, 1515.52
3	702.24, 838.51, 854.17, 888.019, 903.28, 923.16, 941.43, 962.28, 968.76,1023.14, 1099.062, 1116.10, 1129.10, 1147.14, 1186.01, 1316.49, 1356.54
4	903.05, 921.41, 986.74, 1023.01, 1044.16, 1089.98, 1101.76, 1116.05, 1128.70, 1137.65, 1316.55, 1355.05, 1543.48
5	888.95, 903.17, 921.84, 941.39, 960.28, 985.96, 1022.88, 1098.59, 1128.29, 1198.42, 1248.92, 1316.25, 1342.45, 1359.56, 1515.78, 1567.372, 1585.22, 1790.56
6	903.77, 921.64, 986.28, 1023.65, 1316.53, 1355.776
7	1046.79, 1179.32, 1233.07, 1531.90, 1541.57, 1579.46, 1595.36, 1618.23, 1634.22, 1650.18, 1700.52, 1725.41, 1766.43, 1792.27, 2266.81, 2275.36, 2290.72, 2385.94, 2720.04

* Observed masses omit masses of keratin contamination and trypsin autolysis products.

The MALDI/Tof results in Table 3.1 were performed the database searching via the MASCOT program (www.matrixscience.com). The search parameters included 100 ppm mass tolerance, 2 missed cleavages, modifications, trypsin enzyme, and both of NCBI and SWISS-PROT databases were selected. The search results showed no reasonable matching protein. It might be due to no information of these proteins in database, since there is no record of any protein from *T. macrops* venom in protein database.

3.3 Gel Filtration Chromatography of Snake Venom

T. macrops venom (5.5 mg) was dissolved in 0.7 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. 0.5 ml of supernatant was applied to column (1.6 x 52.5 cm.) of Sephacryl S-100HR equilibrated with the buffer. The column was eluted with the same buffer. There are twelve peaks of protein (I-XII) which eluted from the column (Figure 3.4). All fractions were collected for further measurement of enzymatic activity and determination of molecular masses of proteins in each fraction by MALDI/Tof MS.

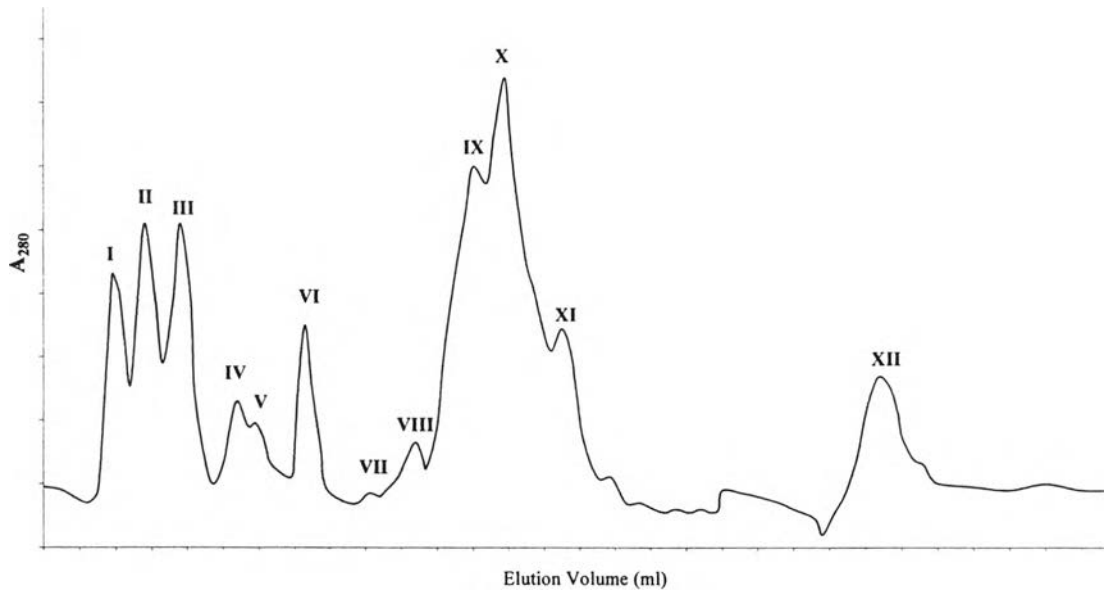

Figure 3.4 Gel Filtration Chromatogram of *T. macrops* Venom on Sephacryl S-100HR Column.

3.4 Proteolytic and Phospholipase A Activities

The twelve Gel Filtration (GF) fractions, which eluted in the top part of all twelve peaks, were used for measurements of proteolytic and phospholipase A activities. The calculated protein concentrations of the twelve GF fractions from fractions I to XII are shown in Table 3.2. The protein concentration of each fraction was measured with the absorbance of 280nm. Table 3.2 summarizes the proteolytic and phospholipase A activity of the twelve GF fractions.

Table 3.2 Summary of Fractionation of *T. macrops* Venom.

Fraction	Protein (mg/ml)	Proteolytic activity*	Phospholipase A activity*
I	0.280	-	-
II	0.737	+	-
III	0.712	+	-
IV	0.041	-	-
V	0.023	-	-
VI	0.034	-	+
VII	0.020	-	-
VII	0.025	-	-
IX	0.056	-	-
X	0.176	-	-
XI	0.064	-	-
XII	0.071	-	-

* (-) no activity, (+) shows that fraction possess activity.

The proteolytic activity was found in the GF fraction peaks II and III with A_{280} 0.0445 and 0.0368, respectively. The specific activity of the GF fraction peaks II and III are calculated to 0.18 and 0.15 unit/ml. These protein fractions with proteolytic activity were the second and third GF peaks, indicating that they are high molecular weight proteins. The hemolytic clear zone on agarose plate indicates phospholipase A activity. Phospholipase A activity was found only in fraction VI which has clear zone of 6 mm.

Due to the results of enzymatic activity measurement, the proteolytic activity was found only in GF fraction peaks II and III. The GF fraction peak VI contained Phospholipase A activity. Therefore, these three fractions (I, II, and VI) were interesting to perform protein identification. The crude *T. macrops* venom and all GF fractions were determinated. The MALDI spectrum of crude *T. macrops* venom has broad peaks with a singly charged ion at *m/z* of 13325.20 and a doubly charged ion at *m/z* of 6670.33 (Figure 3.5). There were three spectra of fractions VI, IX, and X

protein obtained from MALDI/Tof MS of twelve GF fractions. The spectra are shown in Figures 3.6 to 3.8. The singly charged ion at *m/z* of 13732.02 and doubly charged ion at *m/z* of 6873.66 were observed from the fractions VI protein. The *m/z* of singly and doubly charged ion of the fractions IX and X protein spectra are 13255.68, 6633.24, and 13341.09, 6668.08, respectively. Consequently, the molecular weights of the fractions VI, IX, and X protein are 13731.02, 13254.68, and 13340.09 Da.

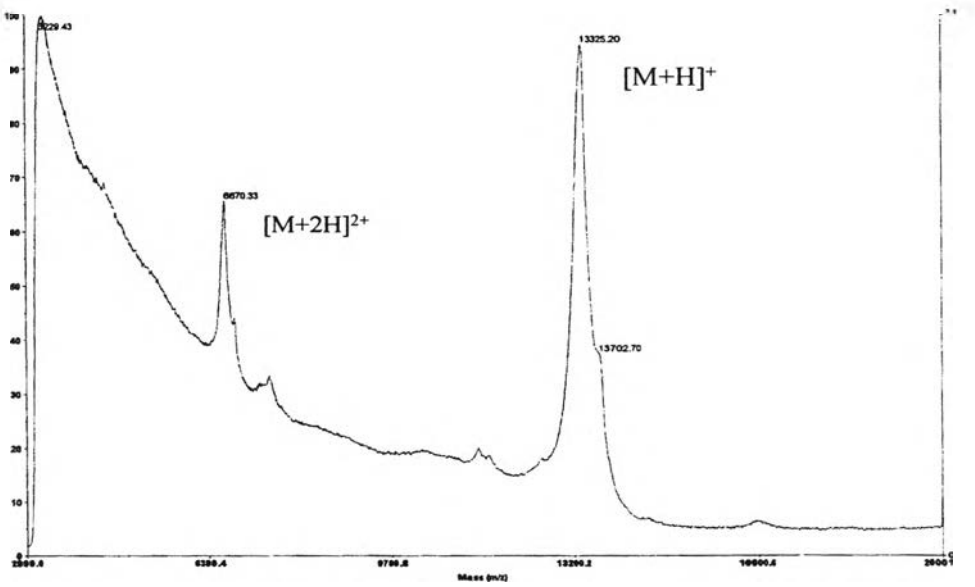

Figure 3.5 MALDI/Tof Mass Spectrum of Crude *T. macrops* Venom.

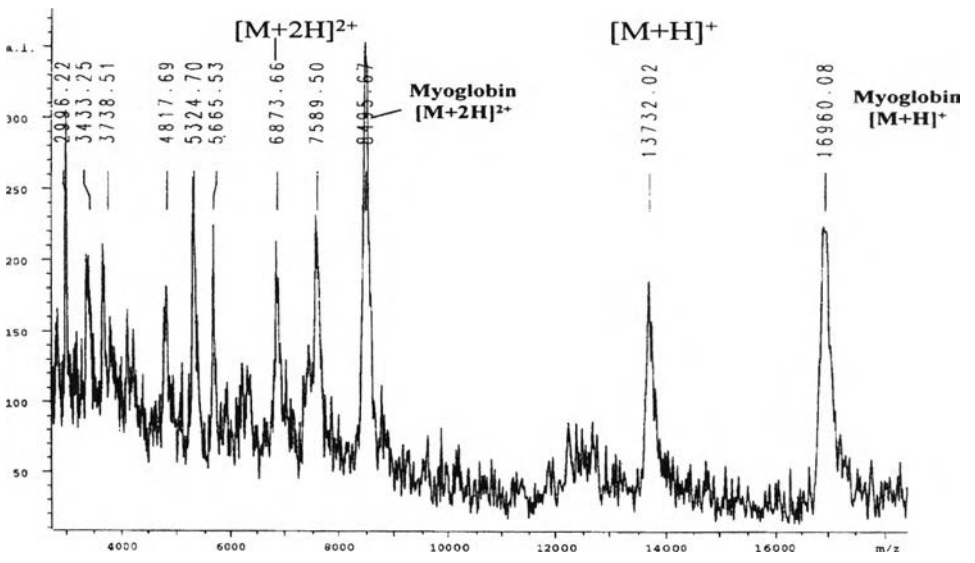

Figure 3.6 MALDI/Tof Mass Spectrum of Fractions VI Protein from Gel Filtration. The internal calibration was performed using myoglobin (16951 Da).

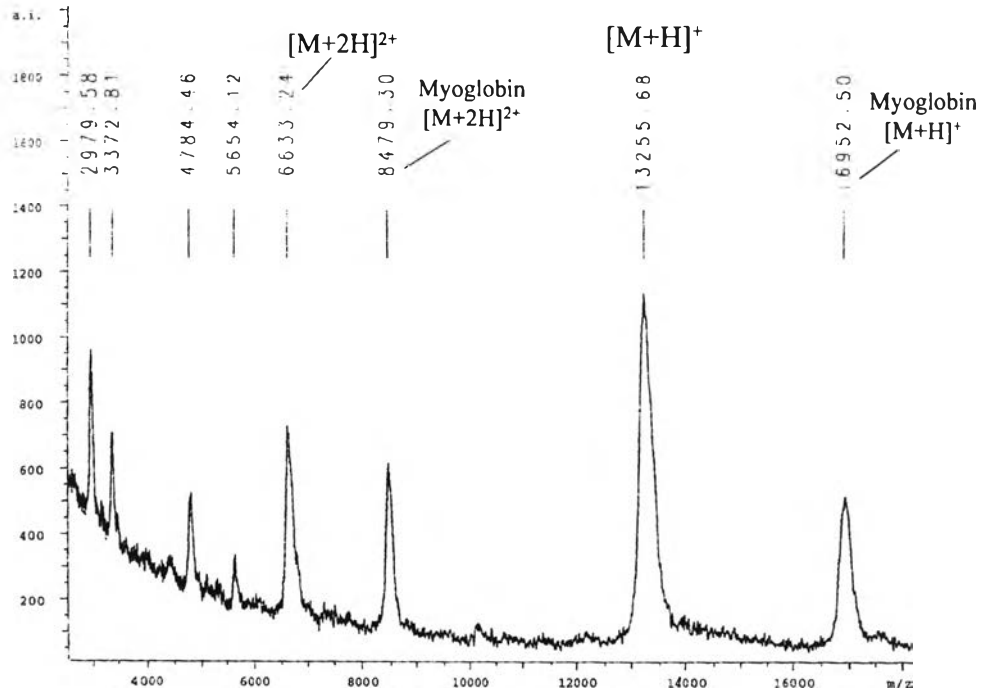

Figure 3.7 MALDI/Tof Mass Spectrum of Fractions IX Protein from Gel Filtration

The internal calibration was performed using myoglobin (16951 Da).

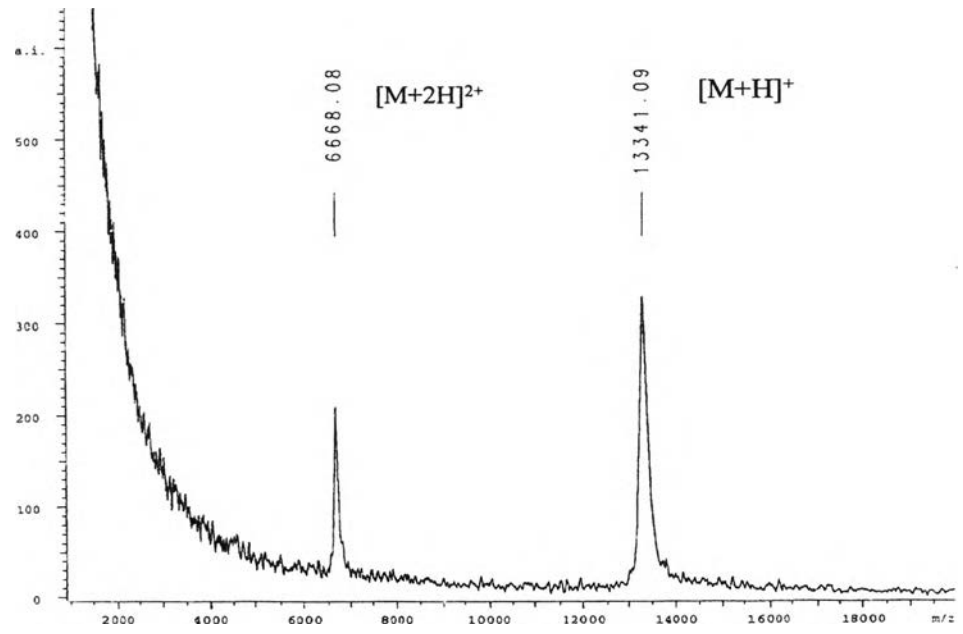

Figure 3.8 MALDI/Tof Mass Spectrum of Fractions X Protein from Gel Filtration.

3.5 N-terminal Sequence Determination

The GF fractionated proteins were analyzed using Edman degradation. The optimal amount of loading on the Edman sequencer is typically 200 picomole of proteins. Due to the limitation of concentration of GF fractionated protein, only N-terminal sequence of GF fraction X protein was acquired. The N-terminal sequence of fraction X protein (13341.09 Da, $[M+H]^+$) is HVLQLGLYIL. This N-terminal sequence was searched using the BLAST program (www.ncbi.nlm.nih.gov/blast/). The result of matched proteins was unreasonable match that no according to molecular mass and source of the protein due to the same reason of peptide mass mapping as described before (Section 3.2).

3.6 Tandem MS

The protein of GF fraction VI was digested with trypsin. The sequences of tryptic fragments were analyzed using ESI-Q-Tof. The conventional mass spectrum is shown in Figure 3.9. The four product ion spectra were obtained from precursor ions at *m/z* of 659.3, 844.3, 984.0, and 1224.0, respectively. The product ion mass spectra results are shown in Figures 3.10 to 3.13. The interpretations of the sequence are generally performed.

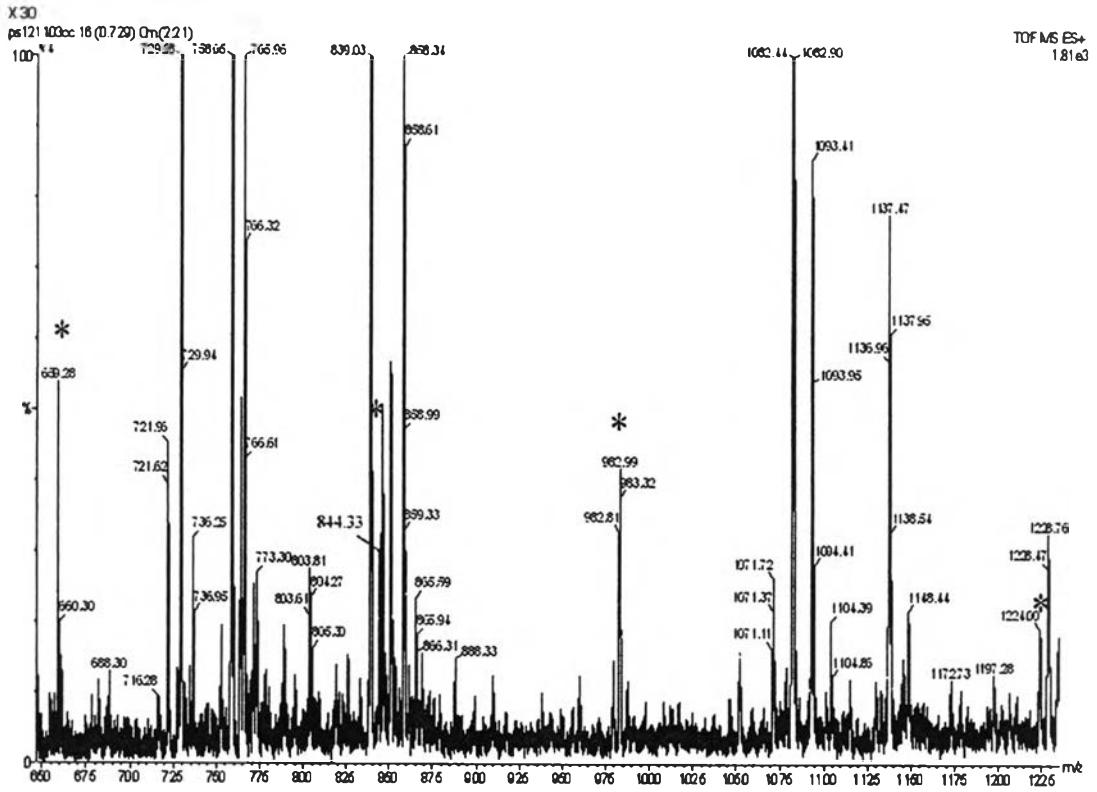

Figure 3.9 The conventional mass spectrum obtains from ESI-Q/Tof.

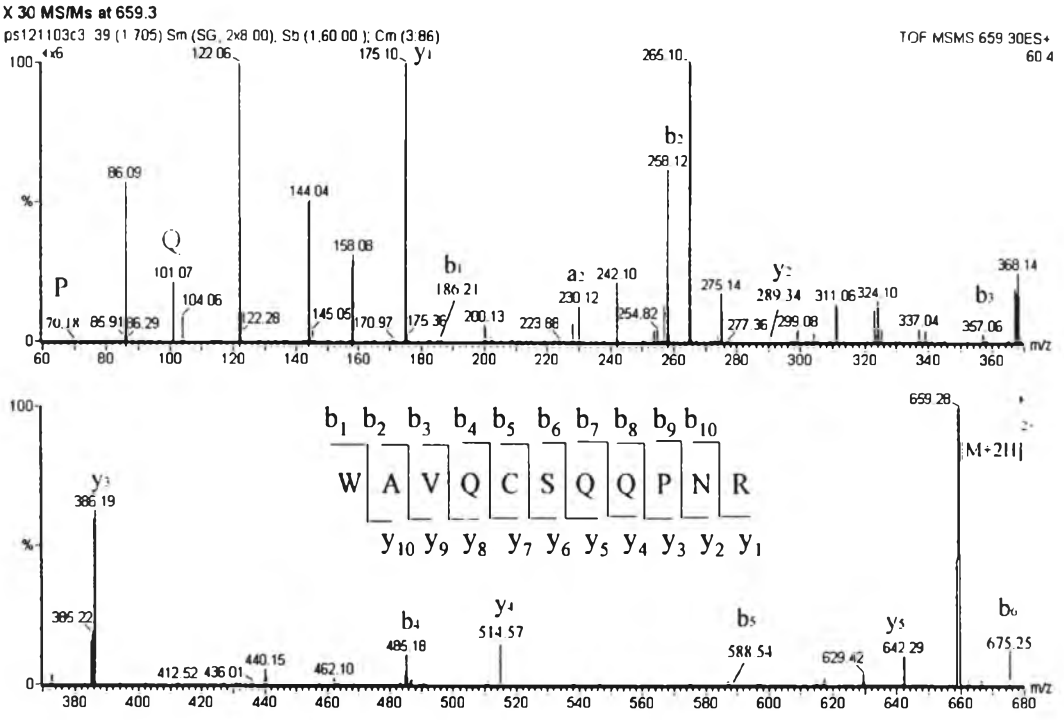

Figure 3.10 The product ion mass spectrum of precursor ion at m/z of 659.3 obtains from ESI-Q/Tof.

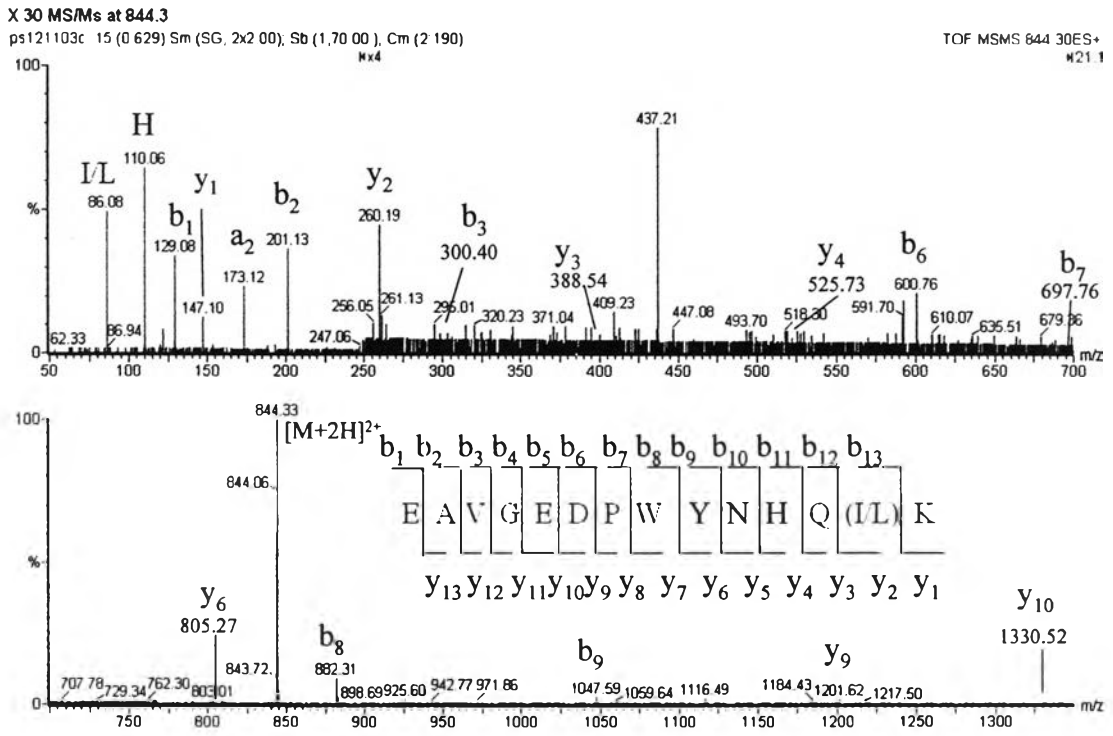

Figure 3.11 The product ion mass spectrum of precursor ion at m/z of 844.3 obtains from ESI-Q/Tof.

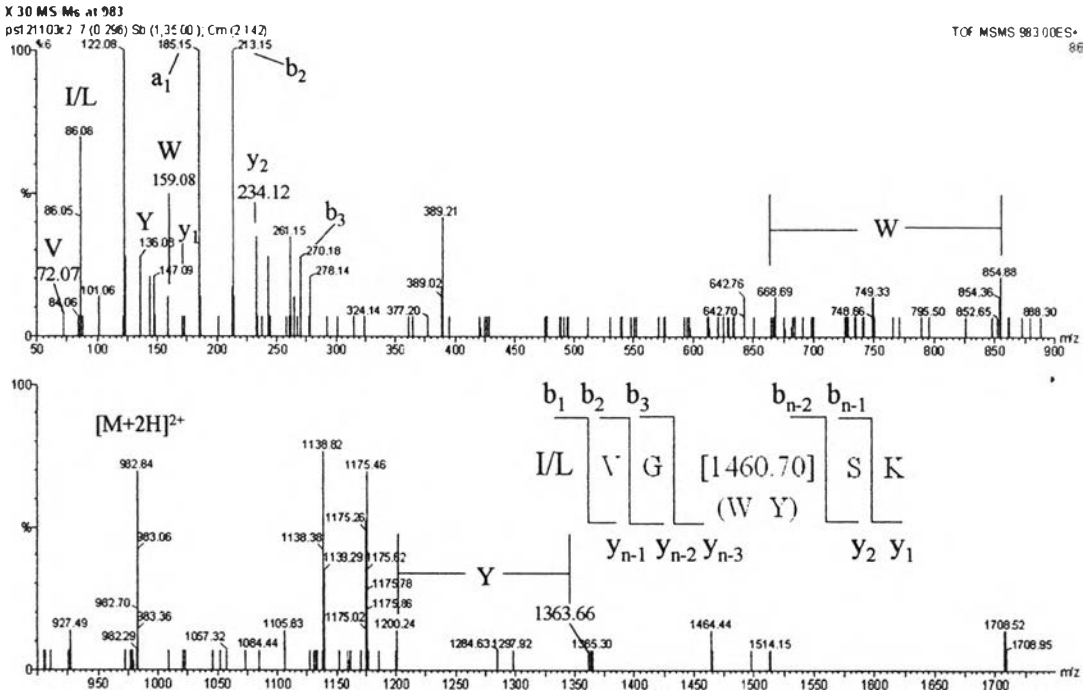

Figure 3.12 The product ion mass spectrum of precursor ion at m/*z* of 984.0 obtains from ESI-Q/Tof.

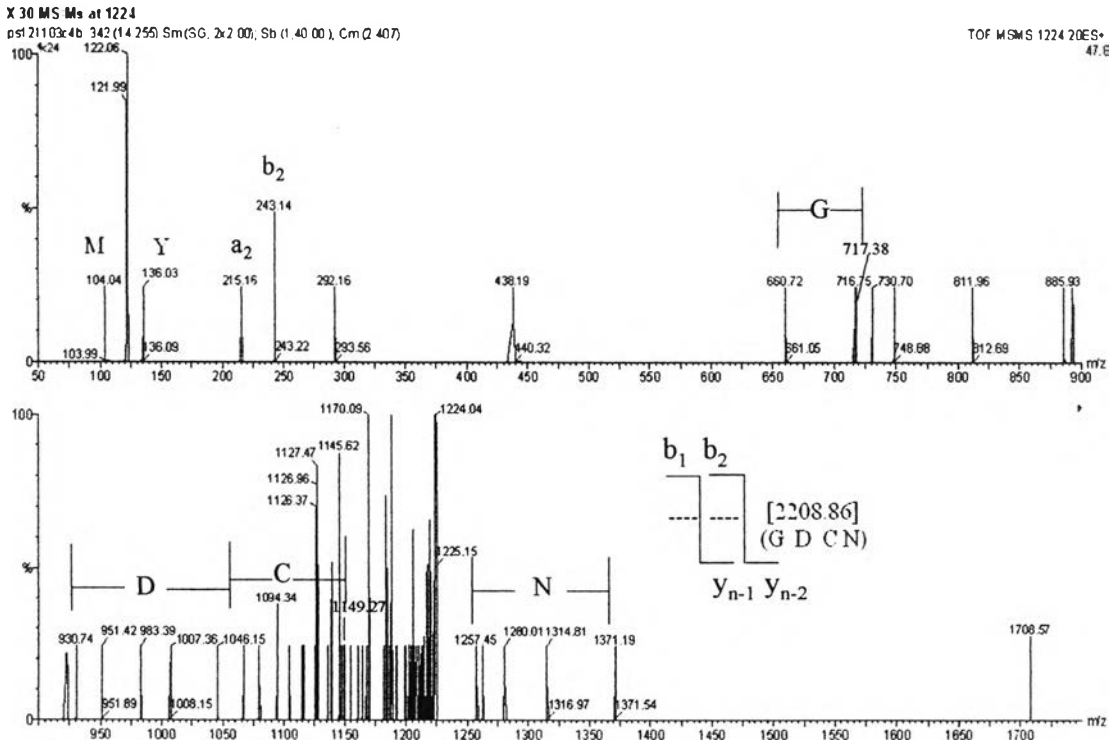

Figure 3.13 The product ion mass spectrum of precursor ion at m/*z* of 1224.0 obtains from ESI-Q/Tof.

The product ion spectrum in the Figure 3.10 was obtained by fragmentation of a doubly charged ion with an *m/z* of 659.3. The measured molecular weight of peptide is 1317.6 Da, $[M+H]^+$. The proposed amino acid sequence is WAVQCSQQPNR. The calculated protonated mass for this peptide is 1316.51 Da which is a good match for the measured mass of 1317.6 Da. From the product ion spectrum shown in the Figure 3.11, the precursor ion is a doubly charged ion with an *m/z* of 844.3. The protonated molecular weight measured in this spectrum is 1687.6 Da, $[M+H]^+$. The proposed amino acid sequence is EAVGEDPWYNHQ(I/L)K. The calculated protonated mass for this peptide is 1686.05 Da which is a good match for the measured mass of 1687.6 Da. The product ion spectra shown in the Figures 3.12 and 3.13 cannot be fully sequencing due to a lack of a complete set of b- and y-ions. Furthermore, the abundance of these product ions is low, which leads to poor product ion spectra. Precursor ion at *m/z* of 984.0 is a doubly charged ion. The molecular mass for the protonated tryptic fragment ($[M+H]^+$) is 1965.0 Da. The sequence that could be deduced from this spectrum is I/L V G [1460.70] SK (Figure 3.12). The value in brackets [1460.70] denotes the remaining mass difference between the assigned ions. The product ion spectrum shown in Figure 3.13 was obtained from a precursor ion at *m/z* of 1224.0 which is a doubly charged ion. The molecular mass for the protonated tryptic fragment ($[M+H]^+$) is 2447.0 Da. This product ion spectrum cannot be interpreted due to the same reason as previously mentioned.