Protein Structure and Analysis

Optimization of separation and digestion conditions in immune complexome analysis

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Short title: Optimization of immune complexome analysis

Abbreviations: CICs, circulating immune complexes; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry; PF4, platelet factor 4; RA, rheumatoid arthritis; TSP-1, thrombospondin-1

Abstract

Immune complexome analysis is a method for identifying and profiling of antigens in circulating immune complexes (CICs); it involves separation of immune complexes from serum, direct tryptic digestion of these complexes, and protein analysis via nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). In order to improve this method, we initially investigated the effects of two factors — the gradient elution program and nano-LC column type (C18-packed, C8-packed or packed spray capillary column) — on the numbers of peptides and proteins identified. Longer gradient elution times resulted higher identification capability throughout the range of 25 to 400 min. Moreover, the packed spray capillary column supported identification of more peptides and proteins than did any other column. Additionally, microwave-assisted digestion was compared with conventional digestion, which involved incubation overnight at 37 °C. Microwave-assisted digestion produced more partially digested peptides than did conventional digestion. However, the percentages of miscleaved peptides in all the identified peptides in microwave-assisted digestion of immune complexes (a protein mixture) were lower than those in the physical stimulation-assisted digestion of a model protein. Microwave-assisted digestion is slightly inferior to or as effective as conventional digestion, but drastically reduces the digestion time.

Keywords: gradient elution time; immune complexome analysis; microwave-assisted digestion; nano-liquid chromatography-tandem mass spectrometry; tryptic digestion

Introduction

Immune complexes are products of reactions that involve noncovalent interaction between foreign antigens or autoantigens and antibody molecules. Circulating immune complexes (CICs) are the direct and real-time products of an immune response; therefore, the antigens incorporated into CICs may be useful as biomarkers if CICs in serum samples could be assess efficiently. Based on this concept, we developed a method, designated “immune complexome analysis”, for the identification and profiling of antigens in CICs; specifically, CICs are separated from serum and the constituent proteins, including diagnostic antigens, are subjected to direct tryptic digestion and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) [1]. Initially, we applied this strategy to the study of rheumatoid arthritis (RA), which is a representative autoimmune disease, and analyzed the CICs in established RA patients and controls. We found that CICs containing two antigens — thrombospondin-1 (TSP-1) and platelet factor 4 (PF4) — were present in the serum of 81% and 52%, respectively, of RA patients, but neither antigen was found in any serum sample from a control subject [1]. Then, we evaluated the diagnostic potential of those CICs that included TSP-1 or PF4 for early RA patients. TSP-1 incorporation into CICs was specific (100%) and sensitive (54%) for early RA [2]. Based on this study of RA, we proposed that immune complexome analysis may be widely applicable to the study of the relationships between CICs and certain diseases associated with immune responses.

The potential of immune complexome analysis would be enhanced with improvements in chromatographic performance, which would increase ionization efficiency and consequently lead to higher MS intensity and more MS/MS spectra files; together, these improvements would result in an increase in the number of peptides, proteins [3], and therefore diagnostic antigens identified. The

gradient elution program in LC separation plays an important role in chromatographic performance; and more peptides and proteins are identified in LC-MS/MS analyses with longer gradient elution times than in those with shorter gradient elution times [3, 4]. Moreover, the peptide separation and is significantly different by column type [5].

One important bottleneck in proteomic analyses is the time required for protein digestion. Generally, in-solution digestion of a protein requires incubation periods greater than 12 h; therefore, reducing the digestion time without loss of digestion efficiency is a major challenge in proteomic studies [6-11]. Several methods have been used to reduce digestion time; for example, digestion reactions have been accelerated in microwave ovens [8, 9], with vortex mixing [10] or via ultrasound treatments [11]; each of these methods causes the digestion to be completed in minutes. In our previous studies [1, 2], we used conventional overnight digestion at 37˚C. However, we used magnetic beads covered with immobilized protein G to collect CICs, and these beads are present throughout the tryptic digestion of CICs; moreover, these magnetic beads efficiently absorb microwave irradiation [12]; therefore, we expected that microwave-assisted enzymatic digestion, which would reduce digestion times.

The aim of this study was to optimize the separation and the digestion conditions in immune complexome analysis by assessing the effects of gradient elution program and of nano-LC column types on the number of peptides and proteins identified and evaluating the usefulness of microwave-assisted tryptic digestion.

Material and methods

Reagents and apparatus

Magnetic beads with immobilized protein G was purchased from Millipore (St. Louise, MO, USA). Trypsin was obtained from Promega (Madison,WI, USA). Dithiothretiol, formic acid, water, cytochrome c and phosphate buffered saline (PBS, pH 7.4), for HPLC grade, were obtained from Wako (Osaka, Japan). Acetonitrile for LC-MS grade was obtained from Merck (Darmstadt, Germany). Myoglobin was obtained from Sigma (St. Louise, MO, USA). Iodoacetamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid was purchased from Nacalai Chemicals (Kyoto, Japan). Three types of nano-LC columns, *i.e.* C18-packed (75 μm i.d. x 150 mm, 3 μm particle, 100 Å pore size, Acclaim, PepMap100C18, Dionex, Sunnyvale, CA, USA), C8-packed (75 μm i.d. x 150 mm, 3 μm particle, 100 Å pore size, Acclaim, PepMap100C8, Dionex), and packed spray capillaly columns (C18, 75 μm i.d. x 125 mm, 3 μm particle, 100 Å pore size, Nano HPLC Capillary Column, Nikkyo Technos, Tokyo, Japan) were evaluated. LC-electrospray ionization-tandem MS (LTQ XL, Thermo Fisher Scientific, Weltham, MA, USA) equipped with the custom nano-LC system consisting of a Shimadzu LC pump (Kyoto, Japan) with an LC flow splitter (Dionex) and an HCT PAL autosampler (CTC Analytics, Zwingen, Switzerland). The separation was performed by using the gradient elution with mobile phase A (0.1% formic acid) and B (0.1% formic acid in 90% acetonitrile).

Sample preparation

CICs were purified with magnetic, protein G-coated beads. Each serum mixture (140 μL), which contained a solution (40 μL) of the bead suspension and a solution (100 μL) of serum diluted

10-fold in PBS, was incubated for 30 min with gentle mixing in a 1.5-ml Eppendorf microtube. After the 30-minute incubation period, each tube was placed into a magnetic rack, which caused the beads to adhere to the side of the tube, and the solution was removed with a pipette. The beads with bound CICs were recovered and washed 3 times with 500 μL of PBS in each wash. The beads were resuspended in 100 μL of 10 mM dithiothreitol and incubated at 56 °C for 45 min; 100 μL of 55 mM iodoacetamide was added to each of these mixtures, and each mixture was incubated at room temperature in the dark for 30 min. Subsequently, trypsin in 0.05% acetic acid was added to achieve a final concentration of 0.5 g of trypsin/L, and each mixture was incubated overnight at 37 °C or heated briefly (15, 30, 60, or 120 sec) in a microwave oven (output power 500 W). After completion of the respective tryptic digestion procedure, 100 mM trifluoroacetic acid was added to stop the digestion, and the supernatant, which contained peptide digests that included antigens and antibodies, was recovered. Finally, each peptide mixture was concentrated under reduced pressure to a final volume of approximately 80 μL.

Protein identification by nano-LC-MS/MS

The peptide mixture (1 μl) was subjected to an LC-electrospray ionization-tandem MS, in which the sample was loaded onto a nano-precolumn (300 μm i.d. x 5 mm, L-C-18, Chemicals and Evaluation and Research Institute, Tokyo, Japan) in the injection loop and washed using 0.1% trifluoroacetic acid in 2% acetonitrile. Peptides were then separated by a nano-LC column (*i.e.* C18-packed, C8-packed, silica monolith, or packed spray capillaly column), and ion-sprayed into MS with a spray voltage of 1.5 kV. Separation was performed employing a gradient elution from 5% to 33% mobile phase B over a variable period of 25~400 min ; 33–100% mobile phase B in 5

min; 100% mobile phase B held for 10 min. The mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by progressing from a full scan of the sample to three tandem MS scans of the three most intense precursor masses (as determined by Xcaliber® software [Thermo Fisher Scientific] in real time). The collision energy was normalized to 35%. All the spectra were measured with an overall mass/charge ratio range of 400-1500. The transfer capillary temperature was set at 200 °C. MS/MS data were extracted using Proteome Discoverer (Thermo Fisher Scientific). Spectra were searched against a human or an equine subdatabase from the public non-redundant protein database of International Protein Index version 3.87 presented by The European Bioinformatics Institute (human) or NCBI (equine, 2011.12.20 download) with the following search parameters: mass type, monoisotopic precursor and fragments; enzyme, trypsin (KR); enzyme limits, full enzymatic cleavage allowing up to two missed cleavages; peptide tolerance, 1.2 Da; fragment ion tolerance, 0.8 Da; ion and ion series calculated, B and Y ions; static modification, C (carbamidomethylation); differential modifications, M (oxidation), N and Q (deamidation). Peptide identifications were made according to standard criteria previously described [13]. All peptides must have a ΔCn of at least 0.1 and cross correlation values of at least 1.9 (+1 charge), 2.5 (+2 charge) and 3.5 (+3 charge). Protein assignments were only made if the protein had five or greater MS/MS hits for at least two different peptides passing the above criteria. Last, manual validation of at least one MS/MS spectrum per protein was completed as a final confirmation of peptide and thus protein identification. All the results were obtained by duplicate analyses. All the peptides and proteins found in the first and/or second analysis were counted in the numbers of identified peptides and proteins in the following sections. At the beginning of each day measurement, the performance of nano-LC-MS/MS system was checked by confirming the

sequence coverage (>70%) of fully digested peptides (20 fmol) derived from bovine serum albumin.

Results and discussion

Effects of gradient elution time and column type on the number of peptides and proteins identified

We examined the effects of gradient elution times (between 25 and 400 min) on the number of peptides and proteins identified via LC-MS/MS analyses; we used linear gradients of 5% to 33% of mobile phase B. We also compared the performance of three nano-LC columns (C18-packed, C8-packed or packed spray capillary columns) in these analyses. The gradient elution time had a substantial impact on the number of peptides and proteins identified (Fig. 1a and 1b). As expected, an increase in the gradient time for the range between 25 and 100 min increased LC-MS/MS performance, specifically the number of peptides and proteins identified. However, additional increases in gradient times (for times between 100 and 400 min) only resulted in slight increases in LC-MS/MS performance. Longer gradient times caused the width of each elution peak to broaden, and this broadening might have reduced the number of peaks over the MS intensity threshold required for the switch from MS to MS/MS scans [3, 14]. Fig. 2 shows the total ion chromatograms for three separate analyses of 1-μL samples of tryptic digest; packed spray capillary column and three different gradient elution times (50, 100, or 200 min) were used in these analyses.

Several groups have compared the performance of HPLC columns for peptide separation, and most groups have compared C8-packed columns with C18-packed columns [5]. Because of the long (C-18) alkyl chains, conventional C18 stationary phase columns may reduce the recovery of hydrophobic peptides and therefore affect the MS identification capability. Nice *et al*. reported

that recovery of small peptides was poor on C18 columns and recommended that shorter alkyl chains were used in columns when separating peptides [15]. With a gradient time of 200 min, 59 proteins and 367 peptides were identified on C18-packed column, 58 proteins and 401 peptides were identified on C8-packed column (Fig. 1). The largest numbers of peptides and proteins were identified with the packed spray capillary column (86 proteins and 630 peptides); this column had zero post-column dead volume and, therefore, generated the smallest peak widths and the highest performance [16, 17]. To further compare performance among the columns, each identified protein was categorized according to the number of identified peptides per protein (Fig. 3). Proteins to which 2-4 identified peptides were assigned were among the most frequent class of protein identified with all kind of columns. Of all proteins identified with the packed spray capillary column, 22 proteins were matched with more than 11 peptides; in contrast, only 11 proteins identified with the other types of column were matched with more than 11 peptides. A more accurate identification of a protein is attained when many peptides are assigned to that protein; therefore, the performance of the packed spray capillary column, which consistently resulted in more peptide-protein matches per protein, should be superior to that of any other type of column. The proteins identified by using packed spray capillary with 100 min gradient elution are summarized in Table S1. We concluded that the packed spray capillary column was the best of the columns with respect to both analytical comprehensiveness (number of peptides and proteins) and accuracy of identification (number of peptides assigned to a protein) even though it is shorter (120 mm) than C18 and C8 packed columns (150 mm).

Comparison of microwave-assisted digestion and conventional digestion

Microwave irradiation is an efficient heating source for chemical reactions, and such reactions are often completed in minutes and with high yields [18-20]. Several groups have applied microwave irradiation to protein digestion and, thereby, reduced protein digestion times to a few minutes [8, 21-23]. In addition to microwave-assisted digestion, vortex-assisted and ultrasound-assisted digestions have been used to reduce digestion time [10, 11]. However, the effectiveness of each of these three techniques has been evaluated mainly via digestion of a single model protein or a mixture of a few proteins. The information about the effectiveness of these methods in the context of the digestion of a real, complex biological sample is very limited [24]; therefore, it is unclear that these methods are useful for immune complexome analysis. We used magnetic beads to collect CICs in these immune complexome analyses. The CICs that are collected on the surface of the magnetic beads are then directly digested with trypsin. Therefore, immune complexome analysis may be particularly suited to the use of microwave-assisted digestion because these magnetic beads absorb microwave irradiation efficiently. We evaluated microwave-assisted tryptic digestion of serum-derived CICs by comparing conventional overnight digestion at 37 °C with microwave-assisted digestion. Both digestion procedures are described in the Materials and methods section. The numbers of identified proteins, identified peptides, and miscleaved peptides are listed in Table 1.

Any digestion method that involves physical stimulation, such as microwave irradiation, generally yields miscleaved peptides due to incomplete digestion [25], which leads to wide variation in the peptides present in the digests. For example, if the peptide GTTVIVSSASTKGPSVFPLAPSSK is completely digested via trypsin, only two peptides —

GTTVIVSSASTK and GPSVFPLAPSSK — will be present in the digests; however, in the case of partial or incomplete digestion, three peptides — GTTVIVSSASTKGPSVFPLAPSSK, GTTVIVSSASTK, and GPSVFPLAPSSK — will be present, and these three can each be detected via nano-LC-MS/MS. Therefore, because microwave-assisted digestion results in more incomplete cleavages, more peptide species are generated from each of the protein species in the sample; consequently, the probability of identifying each protein species will also increase. On the other hand, the occurrence of miscleaved peptides naturally decreases their peak intensity in MS chromatogram because they are derived from a certain amount of a protein, which reduces the number of peaks over the MS intensity threshold required for the switch from MS to MS/MS scans. The number of identified peptides and proteins increased when the time of microwave irradiation was increased from 15 to 30 sec, and this number then decreased with irradiation times of 60 or 120 sec (Table 1). The temperature at the surface of magnetic beads is expected to be high because the beads absorb microwave irradiation [12]. Such high temperatures might induce aggregation of peptides and proteins [26].

Notably, the number of successfully digested peptides (*i.e.*, peptides that were not miscleaved) increased with the time increase from 15 to 30 sec, but gradually decreased as microwave irradiation time increased (15 sec, 216; 30 sec, 275; 60 sec, 223; 120 sec, 209). Increasing microwave irradiation time may lead to non-specific digestion of the successfully digested peptides. To confirm this, we spiked two standard proteins (myoglobin, cytochrome c) into the sample prior to digestion procedure and then, checked the peptides derived from the standard proteins after the digestion. When the peptides obtained by the irradiation time of 15 sec or 120 sec were compered,11 peptides were identified in the time of 15 sec, and among them, three

peptides could not be detected in that of 120 sec. It was found out that increasing microwave irradiation time decreased the successfully digested peptides because of further non-specific digestion. For the miscleaved peptides, extending microwave irradiation can also lead to non-specific digestion of these large peptides, while undigested proteins can be digested to form new miscleaved peptides by increasing the microwave irradiation time; slight decrease of the number of miscleaved peptides was observed. To compare the digestion efficiency of a 30-sec microwave-assisted digestion with a conventional digestion, the identified peptides were categorized by the number of amino acids per peptide (Fig. 4). Short peptides with less than five amino acids were not detected because they were too small (less than 500 Da) to be adequately retained on the reversed-phase LC column [27]. In previous studies of model proteins (bovine serum albumin and myoglobin), digestion procedures that included physical stimulation — such as ultrasound, vortex, or pressure — were more likely to produce longer digestion products (peptides) than were conventional digestion procedures [10, 28, 29]. Our findings were unlike those from these previous studies; specifically, the peptides that were 11 to 15 amino acids long were most common in all digests regardless of the digestion method (Fig. 4a). Among the fully digested peptides, the peptides that were 11 to 15 amino acids long were the most common regardless of digestion method (Fig. 4b). Furthermore, when the fully digested peptides and peptides with 1 or 2 missed cleavage sites were compared, the dominant length was shifted from 11-15 amino acids to 16-20 amino acids (Fig. 4c). Additionally, for these miscleaved peptides, much more peptides that comprised more than 21 amino acids were identified from the microwave-assisted digests than from conventional digests (Fig. 4c). The percentages of miscleaved peptides in all the identified peptides obtained by the several irradiation times were in the range of 40.3 - 43.8%; these were

smaller than these obtained by the physical stimulation-assisted digestion of model protein digestion [28, 29]. Taken together, these observations indicate that digestion methods that include physical stimulation might produce fully digested peptides when a protein mixture is digested, but these methods are likely to produce miscleaved peptides when a single protein species is digested. The sequence coverage of a single protein with ultrasound-assisted digestion is much higher than that with conventional digestion [29]; however, an average of the sequence coverages of all the proteins identified in this study did not largely differ between microwave-assisted digestion (26.2%) and conventional digestion (30.1%). Based on these results, the microwave-assisted digestion is slightly inferior to or as effective as conventional digestion in the digestion of protein mixture, but drastically reduces the digestion time.

In the present study, as shown in Table S1, several proteins were identified by using a low mass resolution MS. However, some large peptides with several missed-cleavage sites should not be detected because of low MS performance. When a high mass resolution MS (e.g. Orbitrap) is employed for the analysis, more accurate identification will be obtained and middle-down proteome analysis, where an analysis of the large peptides gives high sequence coverage, will be able to be performed. Since such high quality MS analysis provides better results, immune complexome analysis employing such high spec MS instrument will increase the number of identified proteins and sequence coverage, compared with the present study.

Conclusions

In this study, we systematically investigated the effects of three parameters — gradient elution time, column type, and tryptic digestion procedure — on the numbers of peptides and

proteins identified via immune complexome analysis. As expected, an increase in gradient elution time led to an increase in the number of peptides and proteins that were successfully identified. Among the different types of columns, the packed spray capillary column yielded the highest number of identified peptides and proteins. Of the four digestion times evaluated (15, 30, 60, and 120 sec) for microwave-assisted tryptic digestion, the 30-sec digestion time yielded the highest number of identified peptides. Microwave-assisted digestion method can be an alternative to overnight digestion with reducing digestion time.

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References

[1] Ohyama K, Ueki Y, Kawakami A, Kishikawa N, Tamai M, Kuroda N (2011) Clin Chem 57: 905–909

[2] Ohyama K, Kawakami A, Tamai M, Baba M, Kishikawa N, Kuroda N (2012) Ann Rheum Dis 71:1916-1917

[3] Iwasaki M, Miwa S, Ikegami T, Tomita M, Tanaka N, Ishihama Y (2010) Anal Chem 82:2616–2620

[4] Köcher T, Swart R, Mechtler K (2011) Anal Chem 83:2699-2704

[5] Wilson KJ, Honegger A, Stotzel RP, Hughes GJ (1981) Biochem J 199: 31-41

[6] López-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp II DG, Smith RD (2008) J Proteome Res 7: 3276-3281

[7] Switzar L, Giera M, Niessen WMA (2013) J Proteome Res 12: 1067-1077

[8] Pramanik BN, Mirza UA, Ing YH, Liu Y, Bartner PL, Weber PC, Bose AK (2002) Protein Science 11: 2676-2687

[9] Chen WY, Chen YC (2007) Anal Chem 79: 2394-2401

[10] Yang HJ, Shin S, Kim J, Hong J, Lee S, Kim J (2011) Rapid Commun Mass Spectrom 25: 88-92

[11] López-Ferrer D, Capelo JL, Vázquez J (2005) J Proteome Res 4: 1569-1574

[12] Walkeiwicz JW, Clark AE, Mcgill SL (1988) Miner Metall Proc 124: 247−252

[13] Garcia BA, Smalley DM, Cho H, Shabanowitz J, Ley K, Hunt DF (2005) J Proteome Res 4: 1516-1521

[14] Xu P, Duong DM, Peng J (2009) J Proteome Res 8: 3944-3950

[15] Nice EC, Capp MW, Cooke N, O'Hare MJ (1981) J Chromatogr 218: 569-580

[16] Martin SE, Shabanowitz J, Hunt DF, Marto JA (2000) Anal Chem 72:4266-4274

[17] Gygi SP, Rist B, Griffin TJ, Eng J, Aebersold R (2002) J Proteome Res 1: 47-54.

[18] Lidström P, Tierney J, Wathey B, Westman J (2001) Tetrahedron 57: 9225-9283.

[19] Larhed M, Hallberg A (2001) Drug Discovery Today 6: 406-416.

[20] Elander N, Jones JR, Lu SY, Stone-Elander S (2000) Chem Soc Rev 29: 239-249.

[21] Sun W, Gao SJ, Wang LJ, Chen Y, Wu SZ, Wang XR, Zhang DX, Gao YH (2006) Mol Cell Proteomics 5: 769-775

[22] Juan HF, Chang SC, Huang HC, Chen ST (2005) Proteomics 5: 840-842

[23] Zhong HY, Zhang Y, Wen ZH (2004) Nat Biotechnol 22: 1291-1296

[24] Lin S, Yao G, Qi D, Li Y, Deng C, Yang P, Zhang X (2008) Anal Chem 80: 3655-3665

[25] Reddy PM, Hsu WY, Hu JF, Ho YP (2010) J Am Soc Mass Spectrom 21:421-424

[26] Kosters HA, Wierenga PA, de Vries R, Gruppen H (2011) Biomacromolecules 12: 2159-2170

[27] Wu SL, Kim J, Hancock WS, Karger B (2005) J Proteome Res 4: 1155-1170

[28] Yang H, Hong J, Lee S, Shin S, Kim J, Kim J. (2010) Rapid Commun Mass Spectom 24: 901-8

[29] Shin S, Yang HJ, Kim J., Kim J (2011) Anal Biochem 414: 125-130

Figure captions

Fig. 1 Effects of gradient elution time and column type on the number of (a) peptides and (b) proteins identified. A two-step linear gradient of 5%-33% (mobile phase B) delivered over each of several time periods (25~400 min) was followed by a gradient of 33%-100% B delivered over 5 min and then by 100% B for 10 min. Other conditions are described in the Material and methods section.

Fig. 2 Total ion chromatograms from the immune complexome analysis conducted with the packed spray capillary column and gradient times of (a) 50 min, (b) 100 min, or (c) 200 min.

Fig. 3 Distributions of the numbers of peptides assigned to a protein from different types of nano-LC columns; for each case the gradient time was 100 min. A histogram is provided for comparisons between C18-packed, C8-packed, and packed spray capillary columns. Other conditions are described in the Material and methods section.

Fig. 4 The identified peptides were categorized by the respective number of amino acids; this categorization facilitates the comparison between the digestion efficiency of microwave-assisted digestion (30 sec) and conventional overnight digestion. (a) number of identified peptides; (b) number of successfully digested peptides; (c) number of miscleaved peptides. The LC-MS/MS analysis was conducted with the packed spray capillary column and gradient time of 100 min.

Fig. 1 (a)

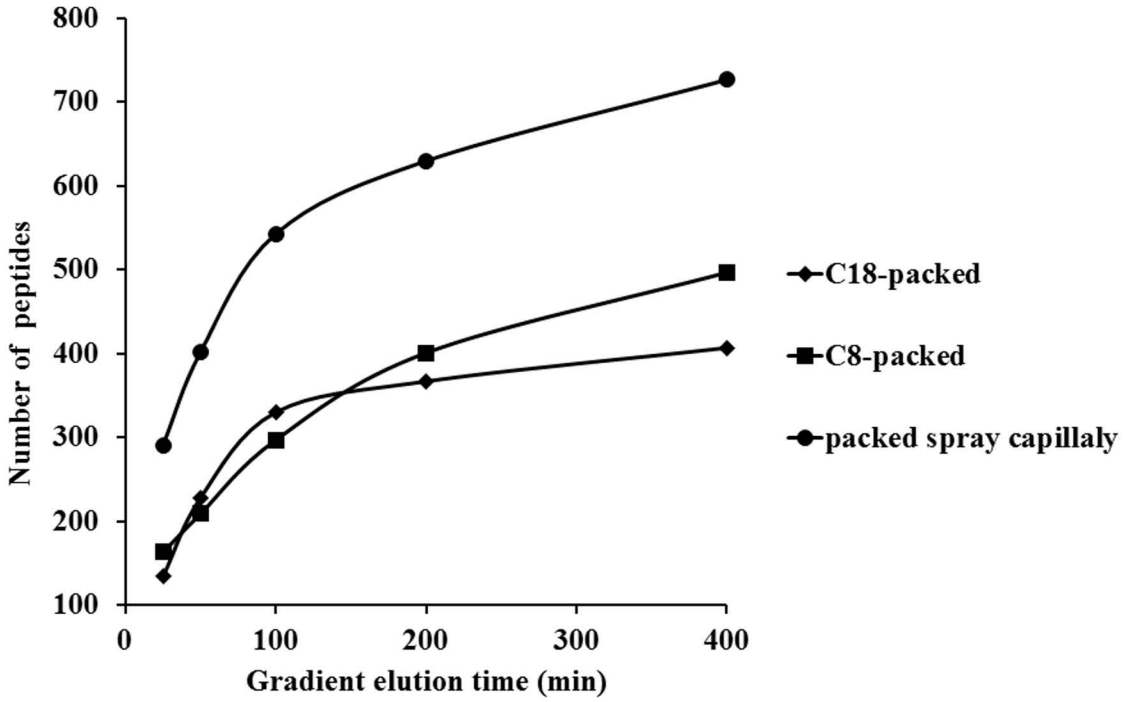

800
700
600
500
400
300
200
100
Number of peptides
0
100
200
300
400
Gradient elution time (min)
C18-packed
C8-packed
packed spray capillaly

Fig. 1 (b)

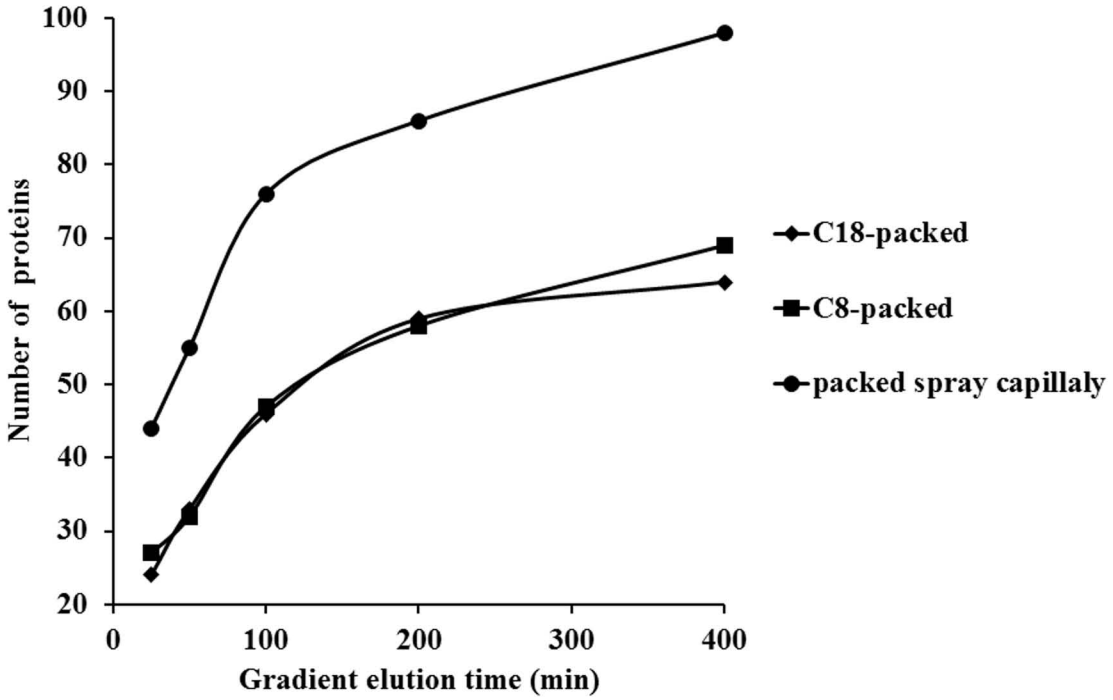

100
90
80
70
60
50
40
30
20
Number of proteins
0
100
200
300
400
Gradient elution time (min)
C18-packed
C8-packed
packed spray capillaly

Fig. 2

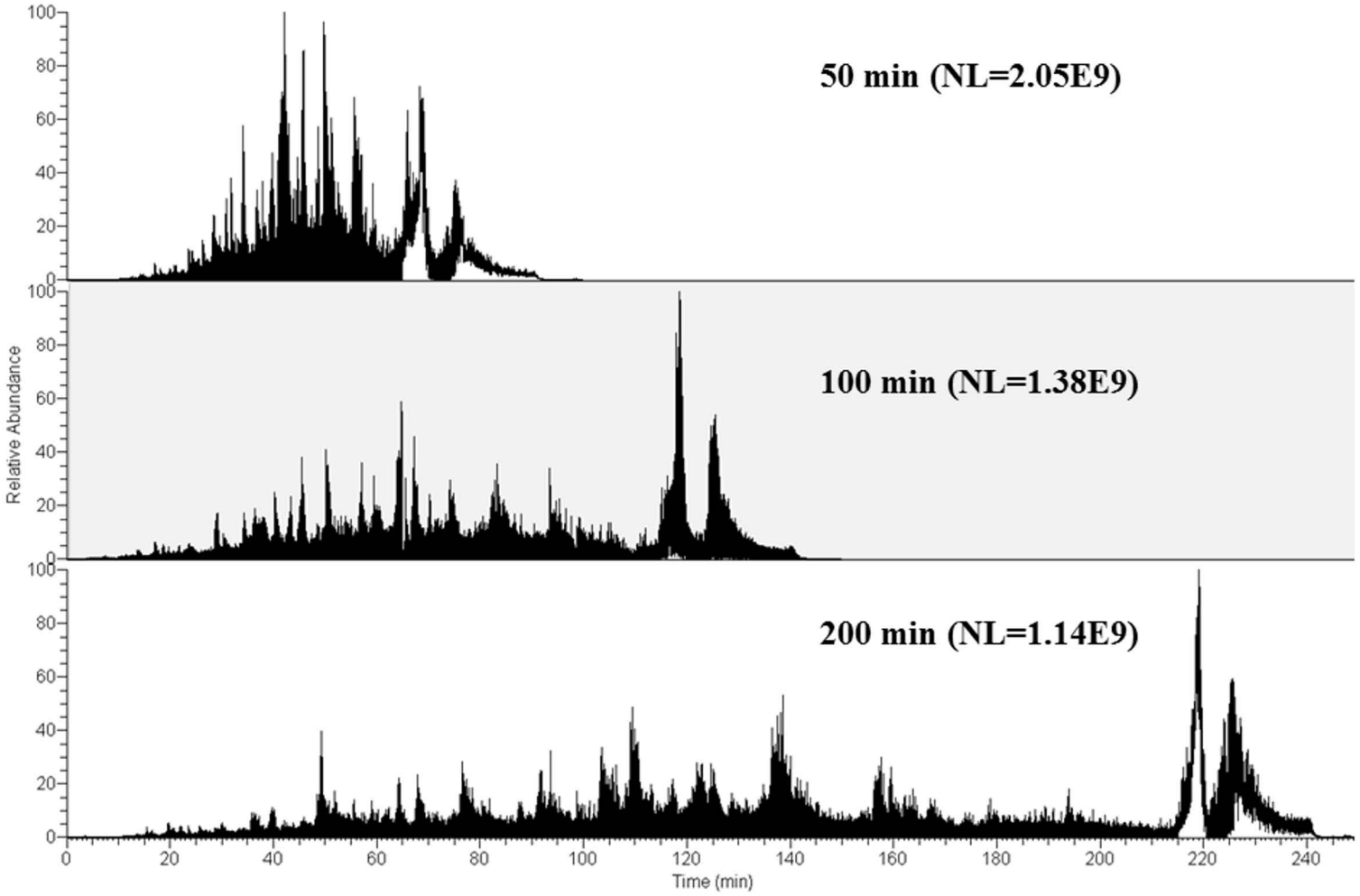
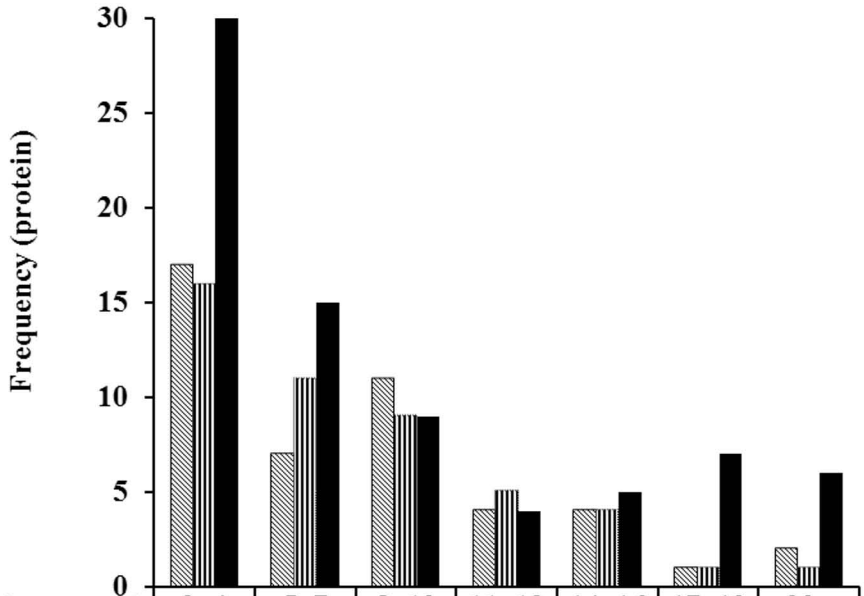
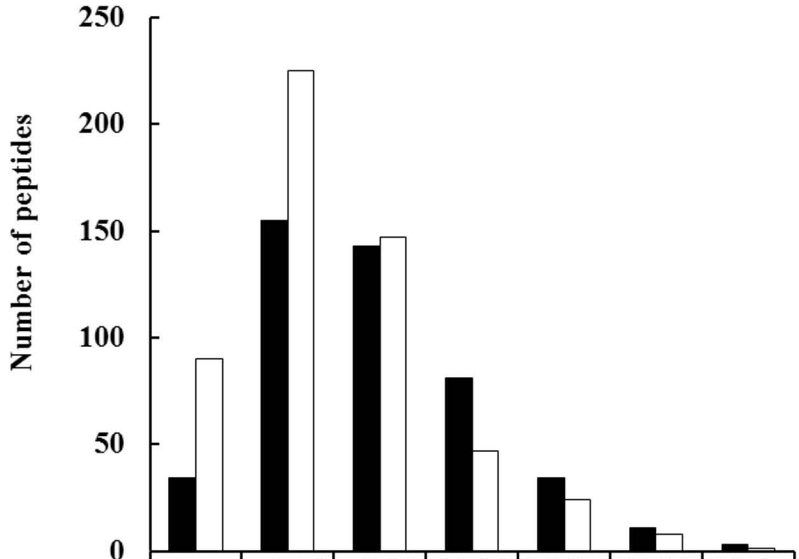

50 min (NL=2.05E9)
100 min (NL=1.38E9)
200 min (NL=1.14E9)
Relative Abundance
Time (min)
0
20
40
60
80
100
120
140
160
180
200
220
240

Fig. 3



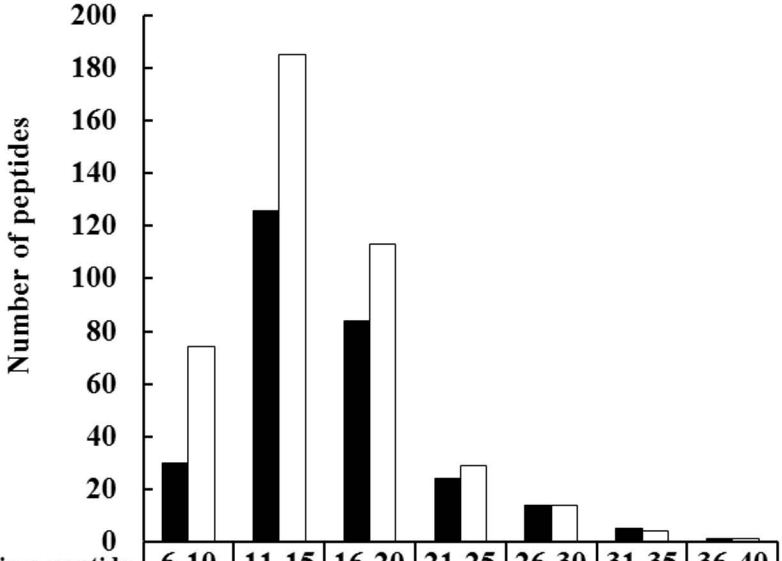
Number of peptides in a protein	2~4	5~7	8~10	11~13	14~16	17~19	20~
C18-packed	17	7	11	4	4	1	2
C8-packed	16	11	9	5	4	1	1
Packed spray capillaly	30	15	9	4	5	7	6

Fig. 4 (a)



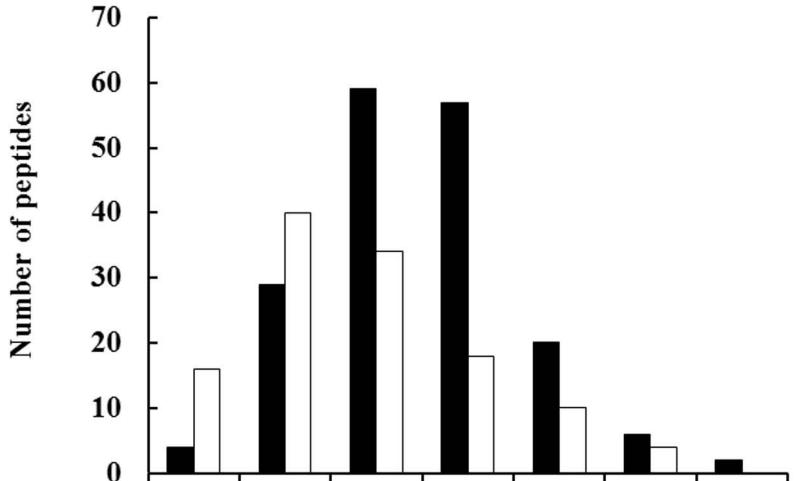
Number of amino acids in a peptide	6-10	11-15	16-20	21-25	26-30	31-35	36-40
■ microwave-assisted (30 sec)	34	155	143	81	34	11	3
□ conventional method (overnight)	90	225	147	47	24	8	1

Fig. 4 (b)



Number of amino acids in a peptide	6-10	11-15	16-20	21-25	26-30	31-35	36-40
■ microwave-assisted (30 sec)	30	126	84	24	14	5	1
□ conventional method (overnight)	74	185	113	29	14	4	1

Fig. 4 (c)



Number of amino acids in a peptide	6-10	11-15	16-20	21-25	26-30	31-35	36-40
■ microwave-assisted (30 sec)	4	29	59	57	20	6	2
□ conventional method (overnight)	16	40	34	18	10	4	0

Table 1 Comparison between microwave-assisted digestion and conventional overnight digestion[a)]

	Overnight digestion	Microwave-assisted digestion			
		15 sec	30 sec	60 sec	120 sec
Number of identified proteins	**76**	**59**	**68**	**57**	**54**
Number of identified peptides	**542**	**373**	**461**	**395**	**372**
Number of successfully digested peptides	**420**	**216**	**284**	**223**	**209**
Number of miscleaved peptides	**122**	**157**	**177**	**172**	**163**
Percentage of miscleaved peptides in identified peptides	**22.5%**	**42.1%**	**38.4%**	**43.5%**	**43.8%**

a) nano-LC-MS/MS analysis was performed by using packed spray capillary column and 100 min gradient (5-33%B).