

Quantitative profiling of plasma peptides in asthmatic mice using liquid chromatography and mass spectrometry

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Asthma is increasing in prevalence worldwide as a result of factors associated with a Western lifestyle. However, simple and reliable diagnostic and prognostic markers are yet to be found. In an attempt to identify protein biomarker profiles among small molecular weight ranges, we employed an approach combining liquid chromatography with mass spectrometry, instead of two-dimensional gel electrophoresis (2-DE), which has previously been used to analyze protein expression patterns. Here we described its application to compare plasma peptides from control and chronic asthma mice. Peptides were quantitatively profiled as a multidimensional peptide mass fingerprint by a combination of reverse-phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. They were identified by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry. In this study, we quantitatively identified the fragment f of complement 3 (C3f), which is important in inflammation. C3f was significantly higher in controls than chronic asthma mice. Our strategy allowed the detection and identification of different plasma peptides between control and chronic asthma mice on a proteomic scale. Therefore, these results suggest that native small peptides detected by non-2-DE techniques may be useful and specific biomarkers of disease.

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1 Introduction

Asthma is a major public health problem worldwide, with morbidity and mortality increasing in the past two decades [1, 2]. Even though antigen-induced IgE production, airway inflammation, and airway hyper-responsiveness [3–5] are well documented in patients with allergic asthma and in animal models [6, 7], simple and reliable diagnostic and prognostic markers are still not developed for the detection of the different types of asthma.

In the levels of mRNA and protein, protein changes do not always parallel corresponding mRNA expression and are frequently altered without similar mRNA changes [8, 9]. In the largest sense, phenotypes are determined by proteins [10]. A protein-based approach to reveal heterogeneous gene expression is advantageous because it identifies biologically relevant phenotypes and may advance the understanding of the pathology of asthma. The latter may also enable the development of new diagnostic and prognostic markers [11].

At present, the standard proteomic tool is 2-DE in combination with MS. However, detection is restricted to *pI* between 4 and 10 and proteins within an *M_r* range of 10–200 kDa [12]. Thus, peptides or small proteins (0.5–10 kDa), such as hormones, chemokines, and growth factors, which are central to biological processes, are not detected by 2-DE. Although Tris-Tricine gels help separate small proteins and peptides [13], this method cannot display

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Abbreviations: C3, complement 3; C3f, fragment f of complement 3; H&E, hematoxylin and eosin; Insulin B, insulin chain B, oxidized; OVA, chicken egg ovalbumin

the whole low molecular weight proteome or peptidome. However, chromatographic separation is a powerful technique for protein purification, and for low molecular weight proteome display. In particular, HPLC is suitable for separating peptides and small proteins with good resolution [14]. A nonporous RP-HPLC technique was employed for the proteomic analysis of premalignant and cancer cell lines [15]. RP-HPLC methods used in the above studies are especially attractive for the fractionation of complex biological samples for MALDI-TOF MS based proteomic studies. Recently, BioVision (Hannover, Germany) established the field of peptidomics, defined as the comprehensive qualitative and quantitative description of peptides in biological samples, and developed a new concept for the analysis of peptides, Differential Peptide Display (DPD) [14]. For example, comparison of peptide patterns between controls and patients with lymphoma revealed obvious differences in peptide content. DPD extends 2-DE: the x-, y-, and z-axes indicate molecular mass, chromatographic fraction, and mass spectrometric intensity, respectively [16, 17]. Escoubas *et al.* generalized the combination of HPLC and MALDI-TOF MS to evaluate quantitative and qualitative variation in tarantula spider venom [18]. Although their HPLC venom profiling appeared to be highly reproducible, MALDI-TOF MS analysis could not be considered quantitative under their experimental conditions and *m/z* values were less accurate.

Accordingly, our main goal is differential comparative analysis of small proteins and identification of specific biomarkers for disease detection by a 'gel-free' format using a non-2-DE method. Here we demonstrate the use of liquid chromatography combined with MALDI-TOF MS as part of a technology for the analysis of relatively differential peptides in control and chronic asthma mice.

Asthma is a chronic inflammatory disease of the airway that arises as a result of inappropriate acquired immunological responses to common environmental antigens [19]. Several biological interactions in the complement pathway that are likely to contribute to the development of asthma involve a potent proinflammatory mediator generated early in inflammatory reactions by proteolytic cleavage of complement 3 (C3) [20]. Elevated levels of complement anaphylatoxin peptides have been observed in the lungs of asthmatic patients [21]. Specifically, using C3-deficient mice as an asthmatic model has forced the importance of complement in asthma [22]. Complement proteins in blood plasma are major proteins in terms of concentration and function, and are very important in innate and adaptive immunity. Among complement proteins, C3 has been deposited covalently as C3b on a membrane. Proteolytic processing of C3 may result in the

generation of fragments including C3a and C3f. To our knowledge, the generation of C3f fragments has not been previously associated with chronic asthma. However, we found that C3f fragments were produced less abundantly in chronic asthma mice than in control mice.

2 Materials and methods

2.1 Animals

Female BALB/c mice (5 weeks old) purchased from Japan SLC (Hamamatsu, Japan) were maintained in the animal facility at the National Genome Research Institute of Korea National Institute of Health. Mice were treated in accordance with standard guidelines for laboratory animal care. The experiments were approved by the Animal Care Committee, Korea National Institute of Health.

2.2 Antigen sensitization and challenge

The numbers of control and chronic asthma mice were prepared 8 and 8, respectively. Mice were sensitized on days 0 and 6 of the experiment by two intraperitoneal injections of 20 µg chicken egg ovalbumin (OVA, grade V, Sigma, St. Louis, MI, USA) emulsified in 1 mg of aluminum hydroxide (Pierce, Rockford, IL, USA) in a total volume of 200 µL. Two weeks after the last sensitization, mice were exposed to 1% w/v OVA aerosolized for 30 min/day, 3 days/week for 6 weeks. OVA challenge was performed by placing mice in a Plexiglas box (29 × 22 × 18 cm) and aerosolizing OVA using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan) driven by compressed air for 30 min. Mice challenged with PBS served as controls. Experimental groups were sacrificed 48 h after the last exposure.

2.3 Mouse plasma

All mice were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneal) and then bled by retro-orbital puncture using heparinized capillary tubes. Blood samples were centrifuged at 16 100 × g for 10 min at 4°C, and then plasma samples were stored at –70°C until subsequent use.

2.4 Measurement of total IgE in plasma

All plasma samples from each experimental group were analyzed for total IgE. Complementary capture and detection antibody pairs for mouse IgE antibodies were purchased from PharMingen (San Diego, CA, USA), and

the IgE ELISA was performed according to the manufacturer's directions. Duplicate plasma samples were diluted 1:100. IgE levels in each sample were measured from optical density readings at 405 nm, and IgE concentrations were calculated from a standard curve generated using recombinant IgE (5–2000 pg/mL).

2.5 Histologic examination

After removal of blood, lungs were slowly perfused with 10 mL PBS *via* the right ventricle, and then were perfused with 4% w/v paraformaldehyde and immersed in fixative solution overnight. Lungs were embedded in paraffin, and 5 μ m sections were stained with hematoxylin and eosin (H&E). The slides were evaluated by microscopy. Inflammation was scored by two independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere [23]. A value of 0 was assigned when no inflammation was detectable, a value of 1 indicated occasional cuffing with inflammatory cells, a value of 2 indicated most bronchi or vessels were surrounded by a thin layer (one to five cells) of inflammatory cells and a value of 3 indicated most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores.

2.6 Liquid chromatography

Plasma samples stored at -70°C were pooled from 4 mice *per* group. The samples were diluted 1:1 with normal saline solution. The diluted samples were filtrated through a 50 kDa molecular mass centrifugal filter devices (Centricon YM-50, Millipore, Billerica, MA, USA). Peptides amounts in the filtrated samples were measured using the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands). The samples were analyzed by the Biocad-Vision™ workstation (Applied Biosystems, Framingham, MS, USA) using a 4.6×250 mm Symmetry300 RP-HPLC column (Waters, Milford, MA, USA). Two mobile phases, A (0.1% v/v TFA) and B (0.1% v/v TFA, 90% v/v ACN), were used for all samples. All mobile phases were filtered (0.22 μ m filter for organic solvent; Millipore). All solvents were HPLC-grade. The filtered samples were injected (90 μ L) with a linear gradient of 0% to 90% ACN (0–100% mobile phase B), a running time of 75 min and 32 fractions were collected at a flow rate of 1 mL/min. Protein absorption was monitored at 254 nm. All separated components were automatically collected in 2 mL micro-centrifuge tubes, and vacuum-evaporated to dryness using a Speed Vacuum Concentrator (Heto-Holten,

Camberley, UK). The dried components were dissolved in 6 μ L of 0.1% v/v aqueous TFA, and stored at 4°C until use. Specific separated components containing the proteins of interest were identified by MALDI-TOF/TOF-MS/MS.

2.7 Mass spectrometry

The dissolved components were resuspended (1:2 v/v) in a mixture of 5 mg CHCA (matrix) and 5 mg L-fucose (co-matrix) in 60% v/v ACN/0.1% v/v TFA with 400 nm bovine insulin (all purchased from Sigma). Samples were deposited in total volume of 1 μ L on the MALDI target, followed by ambient temperature air drying. External calibration was performed with calibration mixture 2 (Sequazyme™ Peptide Mass Standards Kit, Applied Biosystems, Foster City, CA, USA). One hundred laser shots were collected from each location and twenty different locations were collected to generate the MALDI-TOF spectrum. The accelerated ions were analyzed in a MALDI-TOF MS (Voyager-DE STR, Applied Biosystems) in linear mode with delayed extraction. After the Noise Filter/Smooth and Centroid, the mean areas of the mass spectra were obtained using Data Explorer software V4.0 in MALDI-TOF MS.

2.8 Differential peptide display

All data were statistically analyzed, including peak area recognition and visualization by Microsoft Excel (Microsoft Corp.). The x-, y-, and z-axes were represented by the molecular mass, chromatographic fraction and mass spectrometric peak area, respectively. All different mass spectrometric peak areas were obtained to compare differentially expressed patterns between two groups. Bovine insulin (400 nm) was used as an internal standard. The z-axis was determined by comparing the peak area ratio of each peak to insulin. In the chart, every mass peak is depicted as the size of bubble for visual analysis. This comparative analysis was confirmed at least four times. Some peptides that appeared with more than 2-fold expression differences between the experimental groups were analyzed by MALDI-TOF/TOF-MS/MS (4700 Proteomics Analyzer, Applied Biosystems) in reflectron mode with delayed extraction. This TOF/TOF instrument was equipped with an Nd:YAG laser set at a wavelength of 355 nm and a repetition rate of 200 Hz in MS/MS mode. All measurements were made in automatic mode. For protein identification, the mass spectra were saved in Mascot generic file format and submitted to the Mascot database search engine (www.matrixscience.com). The Mascot search parameters were as follows: (1) enzyme; none; (2) species; *mus musculus*; (3) allowed number of missed cleavages; 1; (4) possible post-translational

modifications; *N*-acetyl, oxidation of methionine, phosphorylation of serine, threonine and tyrosine; (5) peptide tolerance; ± 50 ppm; (6) MS/MS tolerance; ± 0.5 Da; and (7) peptide charge; 1+.

2.9 Quantitation

To evaluate quantitatively the data, insulin B (bovine insulin chain B, oxidized) spiked with insulin was dose-dependently measured and intensities compared. Insulin B (1 μ M to 5 μ M) was resuspended (1:2 v/v) in a mixture of 5 mg CHCA and 5 mg L-fucose in 60% v/v ACN/0.1% v/v TFA with 400 nm insulin and deposited in a final volume of 1 μ L on the MALDI target. The amounts of insulin B were determined by comparing the peak area ratios of insulin B to insulin. The intensity of dose-dependent insulin B from 20 spectra was represented by mean $\pm$ SD.

2.10 Statistical analysis

Values are expressed as the mean $\pm$ SEM. Differences between the groups (Control and chronic asthma mice) were determined by Student's *t*-test. A *p* value < 0.05 was considered statistically significant.

3 Results

3.1 Total plasma IgE and histologic examination in control and chronic asthma mice

Levels of total IgE were determined by ELISA 48 h after the last challenge. The levels of total plasma IgE were higher for all chronic asthma mice than all control mice. The concentrations of total IgE in the control and chronic asthma mice were 0.17 ± 0.05 and 3.15 ± 0.42 , respectively (mean $\pm$ SEM, $n = 8$, $p < 0.05$) (data not shown). To evaluate airway inflammation, we stained lung sections with H&E. Peribronchial and perivascular inflammatory

infiltrates were clearly observed in airways of chronic asthma mice relative to control mice (Fig. 1). Inflammation scores were calculated for each experimental group as described in Section 2.5. The score of total lung inflammation was statistically higher in chronic asthma mice than in control mice. The scores in the control and chronic asthma mice were 0.26 ± 0.04 ($n = 4$) and 1.92 ± 0.01 ($n = 4$), respectively, with significantly different inflammation scores ($p < 0.05$).

3.2 Analysis of differential plasma peptides

Plasma from each mouse was pooled into control and chronic asthma group. A cut-off membrane was used to remove very abundant plasma proteins such as albumin, immunoglobulin G, and lipid droplets that could interfere with chromatography (Fig. 2A). The filtered plasma contained low molecular weight proteins or peptides, corresponding to the peptidome [24]. Peptidome was separated into 32 fractions by the C18 RP-HPLC column (Fig. 2B). The retention times of major peptide peaks from repeatedly loaded plasma filtrates were identical when monitored by UV observance. The *m/z* and peak areas of major peptides in each fraction were measured using MALDI-TOF MS (Fig. 2C). The same *m/z* peptide peaks (tolerance; ± 0.5 Dalton difference) within fractions and in nearby fractions were considered as identical peptides. The *m/z* and peak areas of major peptides contained in each fraction were highly reproducible under our conditions (data not shown). All different mass spectrometric signals were statistically analyzed to confirm differentially expressed patterns between two groups. All mass peaks were displayed in virtual bubble form (Fig. 2D). The arrows in Fig. 2D indicate differentially expressed peptides in the 20th fraction. The 2D display data of the virtual bubble were subjected to *de novo* sequencing using MALDI-TOF/TOF-MS/MS (Fig. 2E). The *de novo* sequencing data were analyzed using the Mascot program and corresponding databases. In the 20th

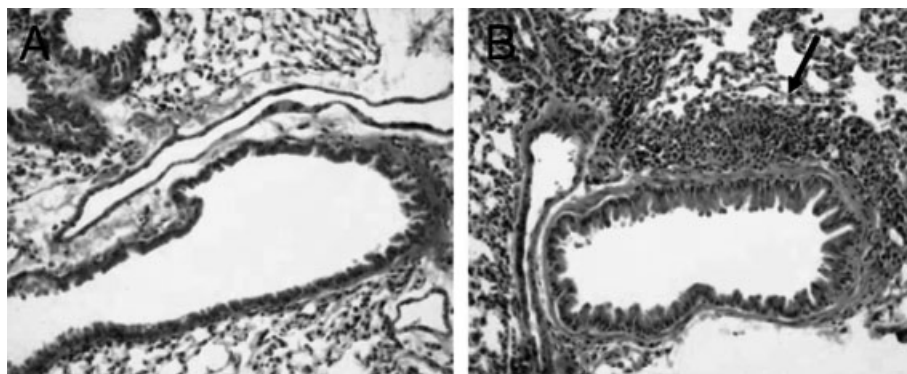


Figure 1. Representative photomicrographs of H&E-stained whole-lung sections from A, control mice and B, chronic asthma mice. Objective lens $\times 200$. Arrow indicates areas of peribronchial inflammation.

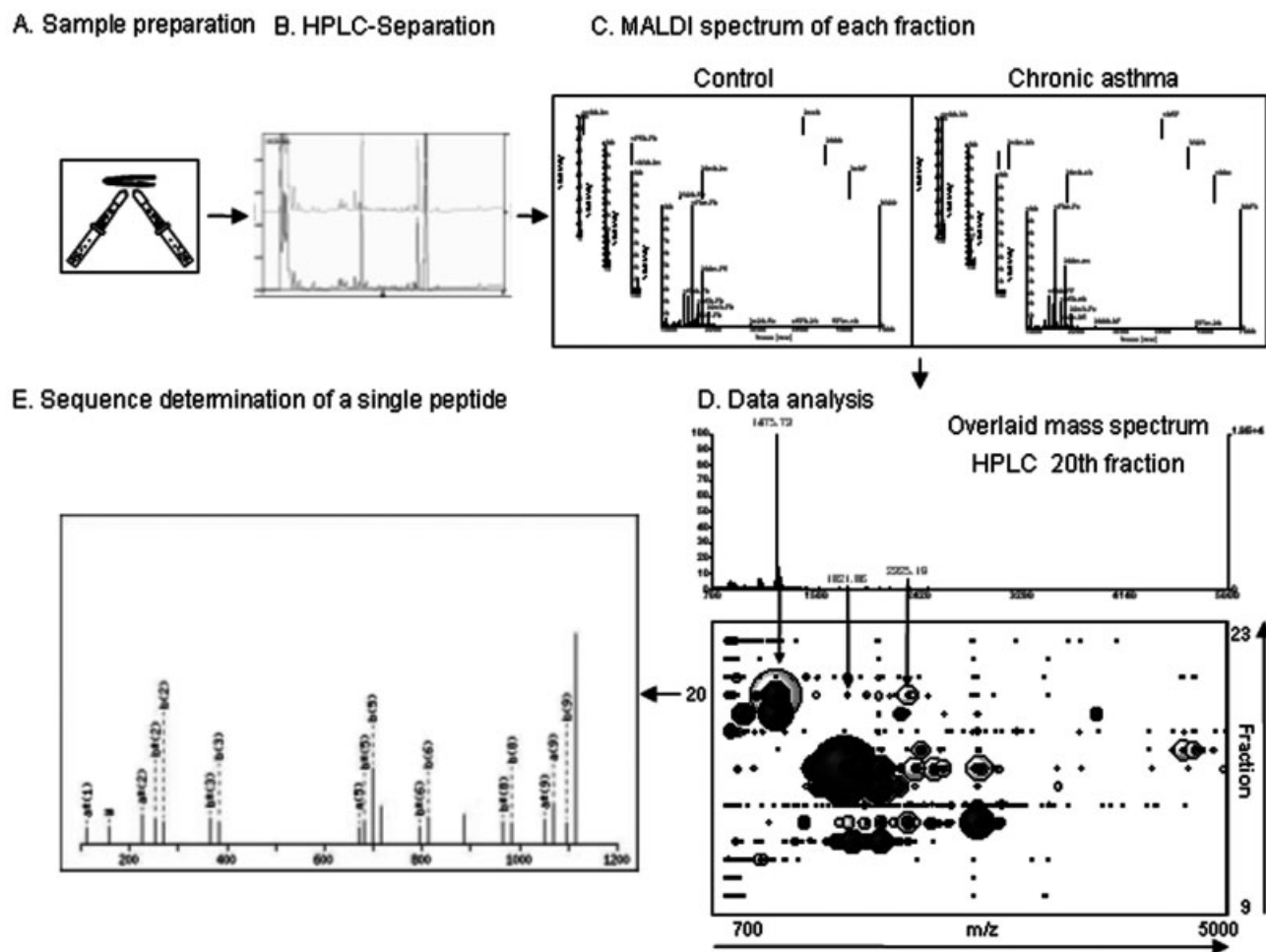


Figure 2. Schematic representation of the differential peptide display process. (A) Peptides from plasma samples of each experimental group were prepared through a 50 kDa cut-off membrane. (B) The peptides were separated by RP-HPLC into 32 fractions. (C) Each chromatographic fraction was analyzed by MALDI-TOF MS to generate data. (D) All 32 mass spectra were displayed in bubble form of the Chart Option in Microsoft Excel. This chart displayed the abundance of differential peptides by each different color. (E) Peptides from differential spots from the two charts were identified by MALDI-TOF/TOF-MS/MS.

fraction, a peptide with an observed molecular mass of 1227.65 Da was identified as the C3f-cleaved peptide (RLLWENGNTLL) and its MALDI-TOF/TOF spectrum is presented in Fig. 2E.

3.3 Quantitative comparison of relative peptide concentrations

To quantitatively evaluate the ionization efficiency of the different peptides, we compared relative concentrations of insulin B to insulin (Fig. 3A). To compensate for varying laser intensities and spot-to-spot differences in sample composition that give rise to fluctuations in analyte ion signals, twenty 100-laser-shot MALDI-TOF spectra were taken from each sample. The spectra acquired at different

positions on a single sample spot showed minimal variation in peak intensity and flight times, and the influence of matrix crystallization appeared negligible. The normalized integral was dose-dependently represented by insulin B (Fig. 3B). These results show that MALDI-TOF spectra are highly reproducible within samples, and the concentration range is scanned with good linearity.

3.4 Comparison and Identification of peptides differentially expressed in control and chronic asthma mice

For comparative analysis, the two groups were overlaid in a single chart. The m/z range was limited to 0.8–7 kDa. The mass chart shows differential peptide expression in

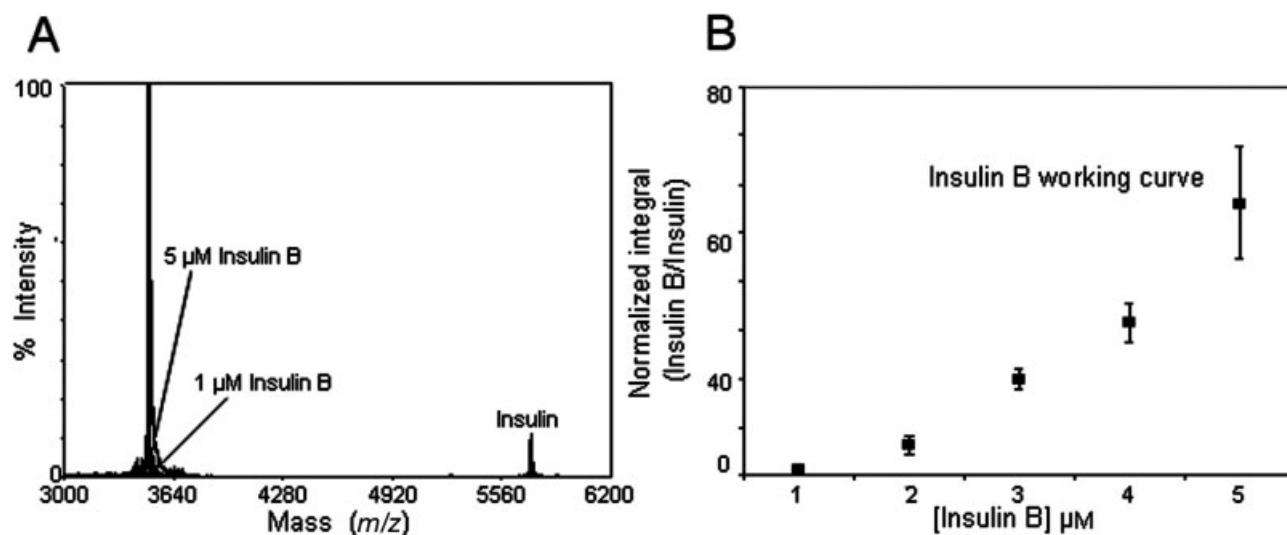


Figure 3. Standard curve for the quantification of insulin B ($M_r = 3496.9$), with insulin ($M_r = 5734.6$) as the internal standard. (A) Representative spectra of data used to generate the working curve. Insulin B concentrations of 1–5 μM were investigated. Insulin (400 nm) was used as an internal standard. (B) Working curve generated from the data represented in (A). The serial range was highly reproducible, with only small variations in five repetitive trials. Error bars reflect the standard deviations of 20 repetitive 100-laser-shot spectra taken from each concentration.

two groups. Areas in the chart correspond to the peptide amounts *per* each peak (Fig. 4). We proceeded to sequence the distinctive peaks in both control and chronic asthma mice using MALDI-TOF/TOF-MS/MS.

The abundant peaks in both groups were common sera peptides, such as fibrinogen and C3. The fragments of fibrinogen observed as common peptides are shown in Table 1. MALDI-TOF/TOF analysis recorded the presence

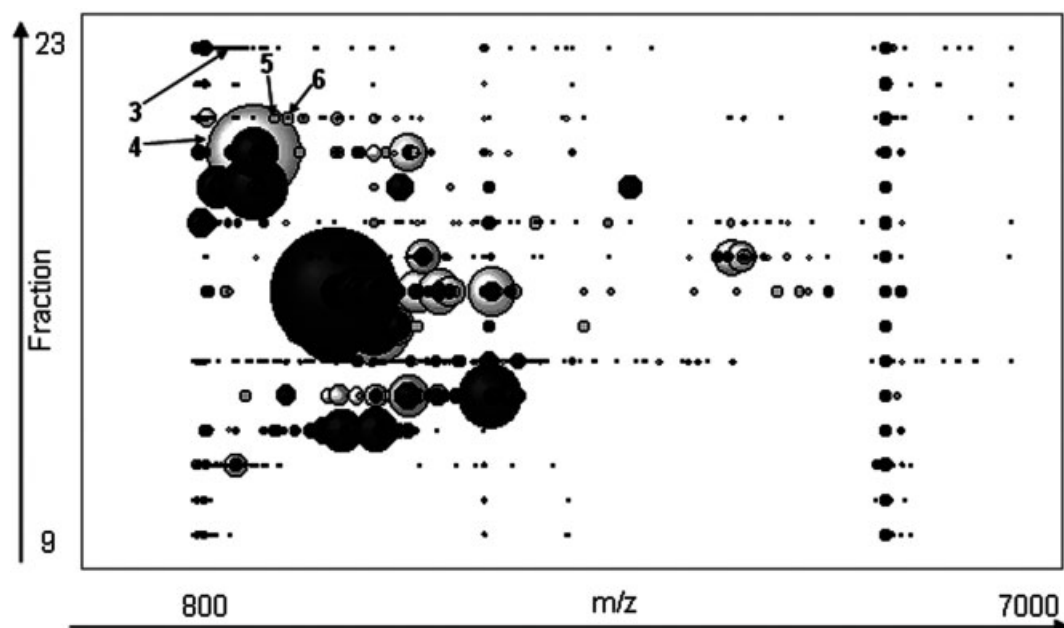


Figure 4. Virtual two-dimensional chart. The differential peptide chart was generated from plasma samples of control and chronic asthma mice. Each horizontal line represents an HPLC fraction and each bubble represents mean area of a peptide, and displayed in different colors (control mice, white; and chronic asthma mice, black). The arrows indicated examples of differentially expressed peptides. The peptide masses are given in Table 2.

Table 1. Fragment series of fibrinogen identified by MALDI-TOF/TOF-MS/MS

No.	Protein name	M_r fragment (observed)	Amino acid sequence
1	Fibrinogen A alpha polypeptide	1479.64	EDKGEFLSEGGGV
2	Fibrinogen A alpha polypeptide	1580.69	TEDKGEFLSEGGGV
3	Fibrinogen A alpha polypeptide	1640.67	TDTEKGEFLSEGGGV
4	Fibrinogen A alpha polypeptide	1695.72	DTEDKGEFLSEGGGV
5	Fibrinogen A alpha polypeptide	1727.89	SEAHREGETRNTKRG
6	Fibrinogen A alpha polypeptide	1758.82	DEAGSEAHREGETRNT
7	Fibrinogen A alpha polypeptide	1796.78	TDTEKGEFLSEGGGV
8	Fibrinogen A alpha polypeptide	1829.85	ADEAGSEAHREGETRNT
9	Fibrinogen A alpha polypeptide	1855.95	AGSEAHREGETRNTKRG
10	Fibrinogen A alpha polypeptide	1960.91	MADEAGSEAHREGETRNT
11	Fibrinogen A alpha polypeptide	1969.96	GDFATRGPGSKAENPTNPGP
12	Fibrinogen A alpha polypeptide	1984.99	EAGSEAHREGETRNTKRG
13	Fibrinogen A alpha polypeptide	2100.04	DEAGSEAHREGETRNTKRG
14	Fibrinogen A alpha polypeptide	2273.01	SQLQEAPPEWKALTEMRQM
15	Fibrinogen A alpha polypeptide	2327.19	DEAGSEAHREGETRNTKRGRA
16	Fibrinogen A alpha polypeptide	2529.29	MADEAGSEAHREGETRNTKRGRA
17	Fibrinogen A alpha polypeptide	2908.58	DEAGSEAHREGETRNTKRGRARAPPT
18	Fibrinogen B beta polypeptide	1820.87	EEPPSLRPAPPPISGGGY
19	Fibrinogen B beta polypeptide	1949.01	KEEPPSLRPAPPPISGGGY
20	Fibrinogen B beta polypeptide	2105.12	RKEEPPSLRPAPPPISGGGY
21	Fibrinogen gamma polypeptide	2433.12	AIQVYYNPDQPPKPGMIDSATQ

of the fibrinogen A alpha peptides and B beta peptides progressively missing amino acids from their *N* termini, and a fibrinogen gamma peptide. The known amino acid sequence of C3f is SSATTFRLWENGNNLL, but the peptide sequences we observed were partially degraded forms of C3f resulting from progressive loss of amino acids from its *N*- or *C*-terminus (Table 2). The C3f fragments were digested progressively from the *N*-terminus by the action of *N*-terminal exopeptidase [25], and *C*-terminal arginines were digested by carboxypeptidase N [26]. The C3f fragments were significantly higher (over 2-fold) in control mice than chronic asthma mice in Fig. 5A. Several differences in the bubble chart (Fig. 5A) were confirmed by referring to the mass spectra (Fig. 5B). The

concentrations of peptides with *m/z* of 1227.65, 1374.69, 1475.73, and 1821.86 Da in the 21st fraction differed significantly and were identified as fragments of C3f with the sequences RLLWENGNNLL, FRLWENGNNLL, TFRLLWENGNNLL, and SSATTFRLWENGNNLL, respectively. The C3f fragment (1071.52 Da) was also abundant in plasma samples from control mice, but the 845.36 and 958.44 Da C3f fragments did not vary significantly under our experimental conditions. We also found other C3 fragments (ILDISMMTG, SEETKQNEAFSLTAKGKG(R)).

4 Discussion

We used HPLC combined with MALDI-TOF MS as an approach to quantitatively analyze differential peptide expression in control and chronic asthma mice. This technique is simple, reproducible, and inexpensive to develop into a working assay. In addition, this procedure is less time-consuming than 2-DE. Modern proteomic methodologies have advanced the large-scale identification of proteins, but are restricted to the analysis of proteins with higher molecular masses (>10 kDa). Identification of low molecular weight peptides and small proteins is necessary, since hormones, cytokines and growth factors play an important role in many biological processes. Peptides or small proteins are generated by activation or

Table 2. *N*-terminal deletion series of C3f identified by MALDI-TOF/TOF-MS/MS

No.	M_r fragment (observed)	Amino acid sequence
1	845.36	WENGNNLL
2	958.44	LWENGNNLL
3	1071.52	LLWNGNNLL
4	1227.65	RLLWENGNNLL
5	1374.69	FRLWENGNNLL
6	1475.73	TFRLLWENGNNLL
7	1821.86	SSATTFRLWENGNNLL

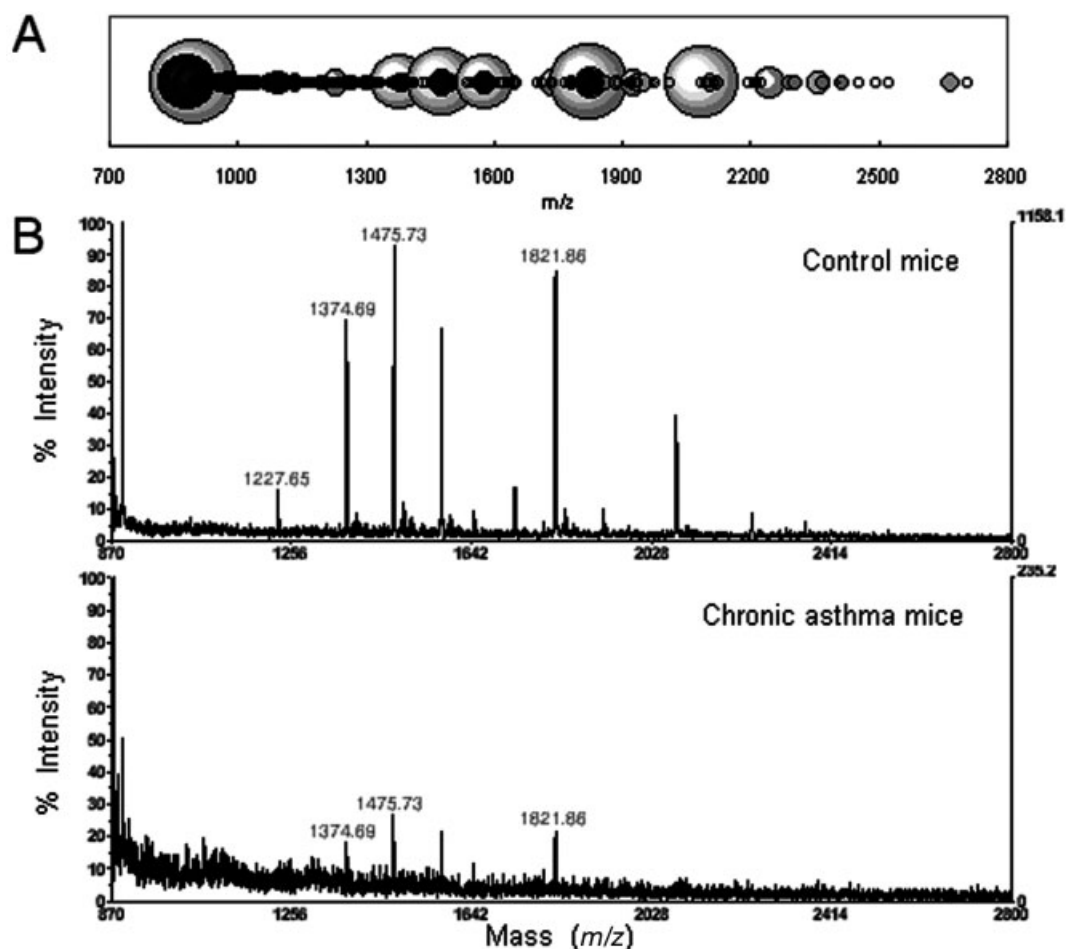


Figure 5. Detection of differentially expressed fragments of C3f. (A) The bubble from overlaid peptides of both control (white) and chronic asthma (black) mice in the 21st fraction. (B) The peaks with m/z of 1227.65, 1374.69, 1475.73, and 1821.86 were abundant in the control mice but were detected only at low levels in chronic asthma mice.

inactivation processes on cell surfaces or secreted proteins. Most rapidly responding physiological processes, including blood clotting and complement activation selected zymogen cascade systems, leave huge amounts of peptides in plasma. In order to comprehensively analyze all peptides or small proteins in a whole organism or a subsystem (peptidome), other technologies than 2-DE are necessary.

Mass spectrometry in combination with LC has already been used in peptidomics research. Heine *et al.* used LC ESI MS for the analysis of peptides in human urine samples [27]. A few peptides like granulin, urodilatin, and angiotensin-I as well as fragments from larger proteins were detected. Another study used online nano LC MALDI-quadrupole-TOF MS to analyze differences in neuropeptide expression between the motor cortex, the striatum, and the thalamus [28]. Here, we combined

HPLC with MALDI-TOF MS in place of 2-DE to quantitatively analyze proteins. A 2D BioVision AG diagram had previously been made by combining HPLC with MALDI-TOF MS for peptides analysis. Data were subjected to statistical analysis and peak recognition, and were visualized with the in-house developed software package Spectromania™. Selected peptides were analyzed by ESI-quadrupole-TOF [10, 14, 17]. However, we generated a simple 2D diagram using Microsoft Excel, and used an internal standard to compensate for varying laser intensities and spot-to-spot differences in sample composition [29]. Furthermore, in contrast to ESI, the MALDI ionization procedure mostly results in singly charged molecules, making the spectra easier to interpret and compare. As a control experiment, we compared relative concentrations of insulin B to insulin (Fig. 3). Previously, Stoeckli *et al.* compared ionization efficiencies of amyloid β -(1–40) and amyloid β -(1–42) to insulin in a concentration range from

50 fmol/ μ L to 1 pmol/ μ L [30]. Thus, direct comparison of signal intensities enables determination of relative peptide concentrations from sample to sample. Further, when internal standards are used and the peak area instead of peak height is selected, quantification errors occur in the range of 2–3% [31].

Using this quantitative method we tested plasma between control and chronic asthma mice. We found that C3f fragments were produced less abundantly in chronic asthma mice than in control mice. Complement may impact key features of asthma. Recently a lot of evidence has demonstrated that complement is contributed to the pathogenesis of asthma. Many features of asthma exacerbation such as smooth muscle contraction, mucus hypersecretion, and recruitment of inflammatory cells are consistent with actions of C3a [32, 33]. C3 is cleaved into two fragments, C3a and C3b. Regulation of the cleavage of C3 is critical. Serine esterase factor I cleaves C3 into an inactive product, iC3b, releasing a small peptide, C3f [34]. A key to the regulation of complement activation is competition between factor B and factor H for binding to C3b on membranes [35]. When C3b attached to the non-host cell surface, including the pathogen or xenotransplanted cell surface, C3b was not degraded by factor I. C3 is cleaved and bound to the membrane in an amplification step. This process requires another complement protein, factor B. But when the cell attached to C3b is a host cell, C3b is degraded into iC3b by factor I with factor H [36]. Therefore, factor B may be bound predominantly to C3b rather than factor H for lasting inflammatory responses, as in the case of chronic asthma, and then the C3b could participate longer in the formation of a C3 convertase enzyme. The C3f portion of C3b and the alpha fibrinogen peptide A were much more predominant in sera of myocardial infarction patients compared to control patients [37]. However, we observed that the C3f peptide is generated and degraded continuously in normal plasma. In particular, the levels of C3f fragments were increased in control mice compared with asthmatic mice. To our best knowledge, this is the first report to suggest that C3 is continuously activated, degraded into C3f, digested further and removed from the blood. C3f production almost completely disappears in asthmatic mice. The mechanism by which the continuous degradation is terminated is unknown. This question should be addressed in the near future. Many peptides, fibrinogens and other complements, were detected but were not significantly different between both groups. Although there were several different peptides, most peptides of over 3 kDa were not identified in this study, because of detection limitations of the MALDI-TOF/TOF-MS/MS and differences in peptide ionization. Therefore, a more developed technical approach is required to identify these peptides.

5 Concluding remarks

In summary, this result showed that measuring small proteins in plasma can be identified with the advanced technique of MALDI-TOF MS. Furthermore, we suggest that C3f fragments might be diagnostic markers for asthma, and that the analysis of specific C3f fragment changes might provide new insights into the mechanisms of this disease. Further more experiments are needed to prove that C3f fragments might be biomarkers for asthma. This peptidomic method may assist in the development of new biomarkers for various diseases like cancer, and should be applicable in many fields.

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6 References

- [1] Li, X. M., Huang, C. K., Zhang, T. F., Teper, A. A. *et al.*, *J. Allergy Clin. Immunol.* 2000, 106, 660–668.
- [2] Beasley, R., Crane, J., Lai, C. K., Pearce, N., *J. Allergy Clin. Immunol.* 2000, 105, 466–472.
- [3] Pauwels, R., *Clin. Exp. Allergy* 1989, 19, 395–398.
- [4] Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., Vignola, A. M., *Am. J. Respir. Crit. Care Med.* 2000, 161, 1720–1745.
- [5] Gleich, G. J., *J. Allergy Clin. Immunol.* 2000, 105, 651–663.
- [6] Renz, H., Bradley, K., Saloga, J., Loader, J. *et al.*, *J. Exp. Med.* 1993, 177, 1175–1180.
- [7] Wills-Karp, M., *Annu. Rev. Immunol.* 1999, 17, 255–281.
- [8] Russell, A., Thompson, M. A., Hendley, J., Trute, L. *et al.*, *Oncogene* 1999, 18, 1983–1991.
- [9] Ozturk, M., Bolkent, S., Yilmazer, S., Kaner, G., Unal, H., *Anal. Cell. Pathol.* 1998, 16, 201–209.
- [10] Klose, J., *Electrophoresis* 1999, 20, 643–652.
- [11] Tammen, H., Kreipe, H., Hess, R., Kellmann, M. *et al.*, *Breast Cancer Res. Treat.* 2003, 79, 83–93.
- [12] Clynen, E., De Loof, A., Schoofs, L., *Gen. Comp. Endocrinol.* 2003, 132, 1–9.
- [13] Le, Q. T., Katunuma, N., *Anal. Biochem.* 2004, 324, 237–240.
- [14] Schulz-Knappe, P., Zucht, H. D., Heine, G., Jurgens, M. *et al.*, *Comb. Chem. High Throughput Screen.* 2001, 4, 207–217.
- [15] Chong, B. E., Hamler, R. L., Lubman, D. M., Ethier, S. P. *et al.*, *Anal. Chem.* 2001, 73, 1219–1227.
- [16] Tammen, H., Hess, R., Uckert, S., Becker, A. J. *et al.*, *Urology* 2002, 59, 784–789.
- [17] Heine, G., Zucht, H. D., Schuhmann, M. U., Burger, K. *et al.*, *J. Chromatogr. B* 2002, 782, 353–361.
- [18] Escoubas, P., Corzo, G., Whiteley, B. J., Celerier, M. L., Nakajima, T., *Rapid Commun. Mass Spectrom.* 2002, 16, 403–413.

- [19] Krug, N., Tschernig, T., Erpenbeck, V. J., Hohlfeld, J. M., Kohl, J., *Am. J. Respir. Crit. Care Med.* 2001, **164**, 1841–1843.
- [20] Nakano, Y., Morita, S., Kawamoto, A., Suda, T. *et al.*, *J. Allergy Clin. Immunol.* 2003, **112**, 525–530.
- [21] Drouin, S. M., Corry, D. B., Kildsgaard, J., Wetsel, R. A., *J. Immunol.* 2001, **167**, 4141–4145.
- [22] Humbles, A. A., Lu, B., Nilsson, C. A., Lilly, C., Israel, E. *et al.*, *Nature* 2000, **406**, 998–1001.
- [23] Kwak, Y. G., Song, C. H., Yi, H. K., Hwang, P. H. *et al.*, *J. Clin. Invest.* 2003, **111**, 1083–1092.
- [24] Pieper, R., Gatlin, C. L., Makusky, A. J., Russo, P. S. *et al.*, *Proteomics* 2003, **3**, 1345–1364.
- [25] Sanderink, G. J., Artur, Y., Siest, G., *J. Clin. Chem. Clin. Biochem.* 1988, **26**, 795–807.
- [26] Matthews, K. W., Mueller-Ortiz, S. L., Wetsel, R. A., *Mol. Immunol.* 2004, **40**, 785–793.
- [27] Heine, G., Raida, M., Forssmann, W. G., *J. Chromatogr. A* 1997, **776**, 117–124.
- [28] Skold, K., Svensson, M., Kaplan, A., Bjorkestén, L. *et al.*, *Proteomics* 2002, **2**, 447–454.
- [29] Nelson, R. W., McLean, M. A., Hutchens, T. W., *Anal. Chem.* 1994, **66**, 1408–1415.
- [30] Stoeckli, M., Staab, D., Staufenbiel, M., Wiederhold, K. H., Signor, L., *Anal. Biochem.* 2002, **311**, 33–39.
- [31] Wilkinson, W. R., Gusev, A. I., Proctor, A., Houalla, M., Hercules, D. M., *Fresenius' J. Anal. Chem.* 1997, **357**, 241–248.
- [32] Van de Graaf, E. A., Jansen, H. M., Bakker, M. M., Alberts, C. *et al.*, *J. Immunol. Methods* 1992, **147**, 241–250.
- [33] Drouin, S. M., Corry, D. B., Hollman, T. J., Kildsgaard, J., Wetsel, R. A., *J. Immunol.* 2002, **169**, 5926–5933.
- [34] Marshall, J., Kupchak, P., Zhu, W., Yantha, J. *et al.*, *J. Proteome Res.* 2003, **2**, 361–372.
- [35] Pangburn, M. K., *Immunopharmacology* 2000, **49**, 149–157.
- [36] Walport, M. J., *N. Engl. J. Med.* 2001, **344**, 1140–1144.
- [37] Klein, J., Hořejší, V., *Immunology*, Blackwell Science Ltd., New York 1997, pp. 348–392.