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Research paper

Selective, sensitive and comprehensive detection of immune complex antigens by immune complexome analysis with papain-digestion and elution

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ABSTRACT

Comprehensive identification and profiling of antigens in immune complexes (ICs) in biological fluids, such as serum and cerebrospinal fluid, is useful for developing early diagnostic markers and specific treatments for many diseases. We have developed a method, designated "immune complexome analysis", to comprehensively identify the antigens in ICs. In this method, we first purify ICs from biological fluid by using Protein G- or Protein A-coated beads, then these ICs are subjected to tryptic digestion on the beads and subsequent analysis using nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). We previously used this method to find specific antigens in circulating ICs (CIC-antigens) in serum for autoimmune diseases, infectious disease and cancers. However, this method detects not only CIC-antigens but also antibodies and proteins bound non-specifically to the beads, which restricts the detection of minor peptides released by the digestion of CIC-antigens whose amounts are generally much less than antibodies and the proteins. To selectively detect CIC-antigens with enhanced sensitivity, in this study we compared three methods (Method A, direct tryptic digestion on the beads; Method B, low-pH elution and tryptic digestion; Method C, papain-digestion, elution, and tryptic digestion) and examined which method selectively elutes CIC-antigens from CICs bound to the beads and selectively detects CIC-antigens using nano-LC-MS/MS. We also compared three types of CIC-capturing beads (Protein G-coated magnetic beads, Protein A-coated magnetic beads and Proceptor™-sepharose beads) to examine if parallel use of these beads aids the comprehensive detection of CIC-antigens in immune complexome analysis. Comparison showed that Method C provided the most selective and sensitive detection of CIC-antigens, without interference by antibodies and proteins non-specifically bound to the beads. In addition, using three types of beads allowed the examination of a wide range of CIC-antigens in immune complexome analysis. Therefore, combining Method C with three types of beads should allow the selective and sensitive identification of IC-antigens present in biological fluids from patients with a variety of diseases. The identification of IC-antigens may lead to the development of diagnostic methods and protocols for specific treatments for these diseases.

1. Introduction

Immune complexes (ICs) are formed upon noncovalent interaction between foreign antigens or autoantigens and antibody proteins (Nezlin, 2000). Enhanced formation and defective clearance of ICs occurs in autoimmune diseases, cancers, and infections, which triggers such diseases (Chauhan, 2017). Therefore, comprehensive identification and profiling of antigens present in ICs is useful to find targets for developing early diagnostic markers and specific treatments for these diseases. To validate this concept, we have developed a method, designated "immune complexome analysis", to catalogue antigens in ICs. In this approach, ICs are isolated from biological fluids, such as serum and cerebrospinal fluid, by using Protein G- or Protein A-coated beads that bind the fragment of crystallization (Fc) domain of antibodies, and the ICs are then subjected to tryptic digestion (in which the ICs are directly digested on the beads without eluting them) and analyzed using nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) (Ohyama et al., 2011). We have successfully used this

Abbreviations: CIC, circulating immune complex; IC, Immune complex; Ig, immunoglobulin; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry

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method to identify specific antigens in circulating ICs (CIC-antigens) in serum or cerebrospinal fluid for autoimmune diseases (Ohyama et al., 2011, 2012, 2015a, 2015b; Aibara et al., 2018a), infectious disease (Ohyama et al., 2016), cancers (Ohyama et al., 2017) and liver transplant recipient (Aibara et al., 2018b).

However, our own work and work from other laboratories has demonstrated remarkable interference from abundant proteins and antibodies in identifying antigens from CICs (Trinkle-Mulcahy et al., 2008). In our method, not only CICs but also large amounts of antibodies and proteins that bind non-specifically to beads are detected. Large amounts of peptides from these dominant proteins make the detection of minor peptides from CIC-antigens difficult, resulting in a low number of identified CIC-antigens and incorrect profiling of CIC-antigens by immune complexome analysis. To enhance the selectivity and sensitive detection of CIC-antigens by immune complexome analysis, CICs should be selectively eluted from the beads and then digested prior to MS/MS analysis.

We examined two methods for the selective elution of antigens from CICs on the beads: low-pH elution and papain-digestion elution. Low-pH elution, in which solvent polarity as well as electrostatic and hydrophobic interactions between antigens and antibodies are reduced by using a low pH eluent, is the most frequently used method to separate antigens and antibodies (Yarmush et al., 1992; Li et al., 2007; Gustaw et al., 2008; Kavita et al., 2017). On the other hand, papain selectively cleaves immunoglobulin (Ig) at the heavy chain hinge region into three fragments: one Fc and two identical Fab fragments (Bennett et al., 1997; Moorhouse et al., 1997; Adamczyk et al., 2000). Therefore, we envisioned that papain would cleave antibodies in CICs at the hinge region, followed by selective dissociation of the antigens from CICs without eluting the proteins bound non-specifically to the beads. In this study, we thus compared three methods: Method A, direct tryptic digestion on the beads; Method B, low-pH elution and tryptic digestion; Method C, papain-digestion, elution, and tryptic digestion (Fig. 1). The methods were applied to the analysis of a serum sample as well as an *in vitro* formed immune complex (complex of myoglobin and anti-myoglobin) to examine which method selectively elutes antigens from CICs bound to the beads and allows the sensitive detection of antigens using nano-LC-MS/MS.

We also compared three types of beads (Protein G-coated magnetic beads, Protein A-coated beads, and Proceptor™-sepharose beads) used for CIC isolation. We investigated the profiles of CIC-antigens using each bead type and assessed whether parallel use of these beads aids the comprehensive detection of CIC-antigens in immune complexome analysis.

2. Materials and methods

2.1. Materials and chemicals

Magnetic beads with immobilized Protein G or Protein A were purchased from Millipore (Billerica, WI, USA). Proceptor™-sepharose beads were purchased from ProGen Biologics (Wildwood, MO, USA). Equine myoglobin was from Sigma (St. Louis, MO, USA) and goat anti-myoglobin antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Human pooled serum was from Funakoshi (Tokyo, Japan). Dithiothreitol, formic acid, ultrapure water, acetonitrile, acetic acid, and phosphate-buffered saline (PBS, 9.0 mmol/L Na_2HPO_4, 2.9 mmol/L NaH_2PO_4, 137 mmol/L NaCl, pH 7.4) (all high-performance liquid chromatography (HPLC) grade), ethylenediaminetetraacetic acid (EDTA) and glycine were obtained from Wako (Osaka, Japan). Iodoacetamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ammonium hydrogen carbonate, trifluoroacetic acid (TFA), tris (hydroxymethyl)aminomethane, hydrochloric acid and L-cysteine were obtained from Nacalai Tesque (Kyoto, Japan). Papain was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Trypsin was obtained from Promega (Madison, WI, USA).

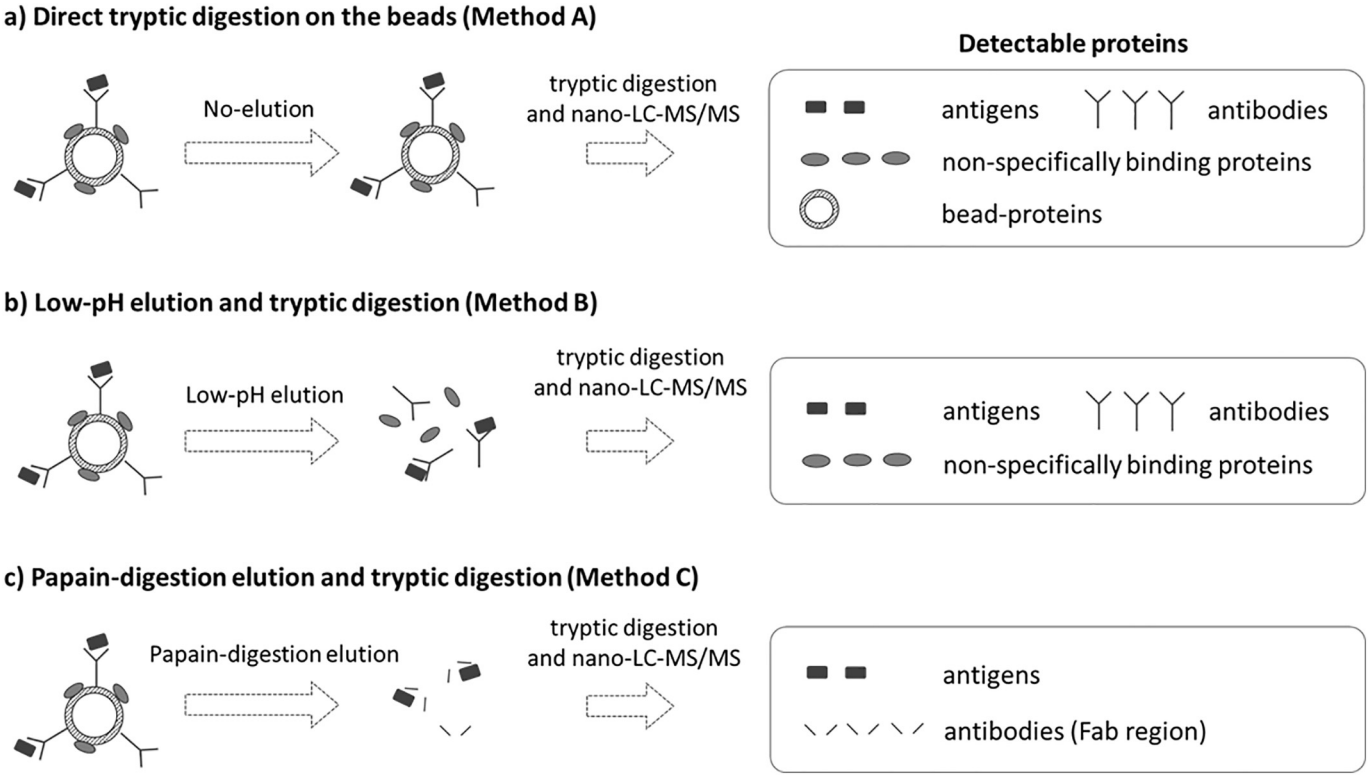

Fig. 1. Proteins likely detected by the three protocols. a) Direct tryptic digestion on the beads, b) Low-pH elution and tryptic digestion, c) Papain-digestion, elution, and tryptic digestion.

2.2. Pretreatment of in vitro formed IC and pooled serum in immune complexome analysis

2.2.1. Preparation of in vitro IC formed from myoglobin and anti-myoglobin

Myoglobin-IC, which is the *in vitro* IC formed from myoglobin and anti-myoglobin, was prepared by combining myoglobin (2.5 pmol/μL, 10 μL) with anti-myoglobin (2.5 pmol/μL, 10 μL) at room temperature for 3 h. Here, we separately used myoglobin, anti-myoglobin and myoglobin-IC to examine the selectivity of the three methods for detecting CICs on Protein G-coated magnetic beads. In this experiment, pretreatment and nano-LC-MS/MS analysis of these models were carried out in the same manner as for pooled human serum, described below.

2.2.2. Separation of CICs from pooled serum

CICs were purified by magnetic beads immobilized with Protein G- or Protein A-coated magnetic beads or by Proceptor™-sepharose beads. Each bead type (40 μL) was incubated with 10 μL of human pooled serum diluted with 90 μL PBS for 30 min with gentle mixing, then the liquid was removed with a pipette. (For Protein G- or Protein A-coated magnetic beads, we used magnetic stand and allowed the beads to migrate toward the magnet, and then removed the supernatant with a pipette. For Proceptor™-sepharose beads, we centrifuged the beads for 1 min and then removed the supernatant with a pipette.) The beads were washed three times with 500 μL PBS. Further processing for tryptic digestion was according to one of the following three procedures (Section 2.2.3.).

2.2.3. Further processing for tryptic digestion

2.2.3.1. Direct tryptic digestion of CICs on the beads (Method A, Fig. 1a). The washed beads were re-suspended in 100 μL of 10 mM dithiothreitol and incubated at 56 °C for 45 min for reduction. Then, 100 μL of 55 mM iodoacetamide was added to the mixtures and incubated at room temperature in the dark for 30 min. Ammonium hydrogen carbonate (100 μL of 50 mM) and 100 μL of ultrapure water were added, followed by trypsin in 0.05% acetic acid to achieve a final concentration of 0.5 g of trypsin/L, then the mixture was incubated overnight at 37 °C. We then added 12 μL of 100 mL/L of TFA and mixed the beads for 2 min to stop the digestion. Next, supernatant containing the peptide digest was transferred to another tube with a pipette, leaving the beads in the first tube. This supernatant (about 400 μL) was vacuum-reduced to approximately 80 μL and stored at 4 °C for subsequent analysis by nano-LC-MS/MS.

2.2.3.2. Low-pH elution of CIC-antigens and in-solution tryptic digestion (Method B, Fig. 1b). We treated the washed beads with 50 μL of 0.05 M glycine/HCl pH 2.5 and mixed them at room temperature for 5 min. The supernatant was transferred to another tube and then we added 50 μL of 0.05 M Tris/HCl pH 9.0 to neutralize the sample. Next, 100 μL of 10 mM dithiothreitol was added and the sample was incubated at 56 °C for 45 min, followed by digestion as described in Section 2.2.3.1.

2.2.3.3. Papain-digestion of CIC-antigens and in-solution tryptic digestion (Method C, Fig. 1c). Washed beads were suspended in 50 μL of papain solution (0.04 M EDTA, 0.04 M L-cysteine) and incubated at 37 °C for 30 min. The supernatant was transferred to another tube, then 50 μL of 0.06 M iodoacetamide dissolved in PBS was added to stop papain digestion. Next, we added 100 μL of 10 mM dithiothreitol and further incubated the sample at 56 °C for 45 min. Subsequent digestion was conducted as described in Section 2.2.3.1.

2.3. Protein identification by nano-LC-MS/MS

The peptide mixture (1 μL) was injected into the injection loop of nano-precolumn and washed using 0.1% TFA in 2% acetonitorile (Baba et al., 2013). An LC–electrospray ionization (ESI)–MS/MS instrument (LTQ XL, Thermo Fisher Scientific, Waltham, MA, USA) was 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) was used for anlaysis. Peptides were separated on a nano-LC column (C18, 75 μm i.d. × 125 mm, 3 μM particle, 100 Å pore size, Nikkyo Technos, Tokyo, Japan) and ion-sprayed into MS with a spray voltage of 1.5 kV. The separation was performed by using the mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid in 90% acetonitrile), employing a gradient elution from 5% to 33% mobile phase B in 100 min, and 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 progressing from a full scan of the sample to three MS/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 of 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 1.3.1.339 (Thermo Fisher Scientific). Spectra were searched against sub-databases from the public nonredundant protein database of UniProt Knowledgebase (human, 2015.01.29 download), National Center for Biotechnology Information (equine, 2011.12.20 download; goat, 2016.5.21 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); and differential modifications, M (oxidation), N, and Q (deamidation). The filter criteria (single, double, and triple charge peptides with a correlation factor [XCorr] and protein probability [P]) were adjusted maintaining the empirically determined protein false discovery rate at 5%. All the proteins were identified by more than one of their unique peptides. In this study, proteins other than antibodies were considered as CIC-antigens. Protein G bead-derived proteins that were detected when treating only Protein G beads using each method were excluded from the detected proteins in the analysis. We performed pretreatment of three pool serum samples and then, we analyzed each of the three pretreated samples in triplicate. Also, we defined the proteins that were detected in one and more than one out of three biological replicates. At the beginning of each day measurement, the performance of the nano-LC–MS/MS system was checked by confirming the sequence coverage (> 70%) of fully digested peptides (20 fmol) derived from bovine serum albumin.

3. Results

3.1. Selectivity and sensitivity of the three methods for the detection of CIC-antigens by immune complexome analysis

3.1.1. Analysis of myoglobin, anti-myoglobin or in vitro formed myoglobin-IC

Myoglobin, anti-myoglobin antibody or myoglobin-IC was respectively mixed with Protein G-coated magnetic beads. We compared the number of peptides from myoglobin and anti-myoglobin identified by each of the three methods (Method A, B and C) using nano-LC-MS/MS (Fig. 2). When we mixed only myoglobin with Protein G-coated magnetic beads and then the beads were subjected to Method A, Method B or Method C, we detected 3–4 peptides, 0–3 peptides or no peptide, respectively (Fig. 2a). When only anti-myoglobin was mixed with Protein G-coated magnetic beads and the beads were subjected to Method A, Method B or Method C, several peptides derived from anti-myoglobin were detected using all three methods (Fig. 2b). When myoglobin-IC was mixed with Protein G-coated magnetic beads, a higher number of peptides from both myoglobin and anti-myoglobin was detected using Method A and Method B compared to Method C (Fig. 2c). Similar results were observed when we used albumin and

		Method A	Method B	Method C
(a) Antigen + Protein G-coated beads	antigen	+++	+	-
	antibody	-	-	-
(b) Antibody + Protein G-coated beads	antigen	-	-	-
	antibody	+++	+++	++
(c) Model IC + Protein G-coated beads	antigen	+++	+++	++
	antibody	+++	+++	++

Fig. 2. Presence or absence of myoglobin (antigen), anti-myoglobin (antibody), and myoglobin-IC by each method. + + +: detected with 3 or more peptides, + +: detected with 2–3 peptides, +: detected with 1–2 peptide, −: not detected (all data are shown as mean values).

alcohol dehydrogenase, which are larger molecules than myoglobin (data not shown).

3.1.2. Analysis of ICs from the serum sample

We examined the differences in CIC-antigens in the human serum sample using Protein G-coated magnetic beads with the three methods. We performed pretreatment of three pool serum samples for each of the three methods and analyzed all the samples in triplicate. There was a statistically significant difference (Tukey-Kramer multiple comparisons test) in numbers of identified antigens (average ± standard error) between Method A (58 ± 4), Method B (42 ± 4), Method C (28 ± 2); Method A > Method B ($P < .01$), Method B > Method C ($P < .01$), Method A > Method C ($P < .001$). We compared the antigens that were detected in one and more than one out of three biological replicates between the three methods, and examined their overlap (Fig. 3). The number of CIC-antigens in human serum detected using each method was as follows: Method A, 207; Method B, 124; and Method C, 53 (the circle of each method in Fig. 3). The number of selectively identified antigens using each method (the proteins that were identified exclusively by a certain method) was as follows: Methods A, 148; Method B, 67; Methods C, 24. Also, the name of the identified antigens is listed in Supplemental data (Table S1).

The nano-LC-MS/MS chromatograms obtained using the three methods were clearly different (Fig. 4). The peaks observed using Method A at around 48 min and 72 min in the chromatogram were not visible using Method B and Method C: these two peaks were observed when Protein G-coated beads were treated using Method A. On the other hand, markedly fewer peaks were obtained using Method C compared to the other methods.

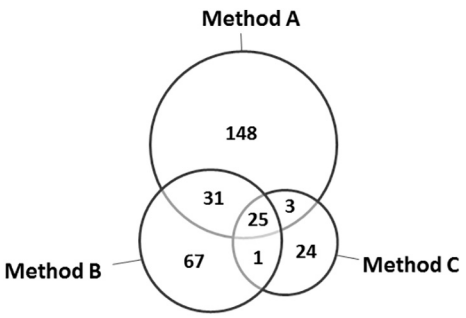

Fig. 3. Comparison of the number of human proteins identified by each method using Protein G-coated magnetic beads.

3.2. Comprehensive analysis for the detection of CIC-antigens using three types of beads

Using Method C, we examined the difference in the number of proteins identified as a CIC-antigen in the analysis of the serum sample when using Protein G- or Protein A-coated magnetic beads or Proceptor™-sepharose beads (Fig. 5). The number of antigens identified when using each bead type was as follows: Protein G-coated magnetic beads, 55; Protein A-coated magnetic beads, 53; Proceptor™-sepharose beads, 16 (the circle of each method in Fig. 5). The number of antigens selectively identified by each bead type (the proteins that were identified exclusively by a certain bead) was as follows: Protein G-coated magnetic beads, 27; Protein A-coated magnetic beads, 28; Proceptor™-sepharose beads, 5. Also, the name of the identified antigens is listed in Supplemental data (Table S2).

4. Discussion

Detecting specific antigens in ICs in samples from patients with a variety of diseases is an important step for developing biomarkers for these diseases. Such efforts could also provide information regarding the pathways that contribute to disease pathology. A major obstacle to these efforts are abundant serum proteins. In this report, we compared three different methods (Method A, Method B and Method C, as seen in Fig. 1) in an attempt to improve both selectivity and sensitivity in the detection of CIC-antigens from serum. We also examined which of three bead types (Protein G-coated magnetic beads, Protein A-coated beads and Proceptor™-sepharose beads) allow CIC-antigens to be detected more comprehensively.

Myoglobin was found to non-specifically bind to Protein G-coated magnetic beads because myoglobin was detected using Method A and Method B when myoglobin alone was mixed with the beads (Fig. 2a). This suggests that Method A and Method B detect proteins that bind

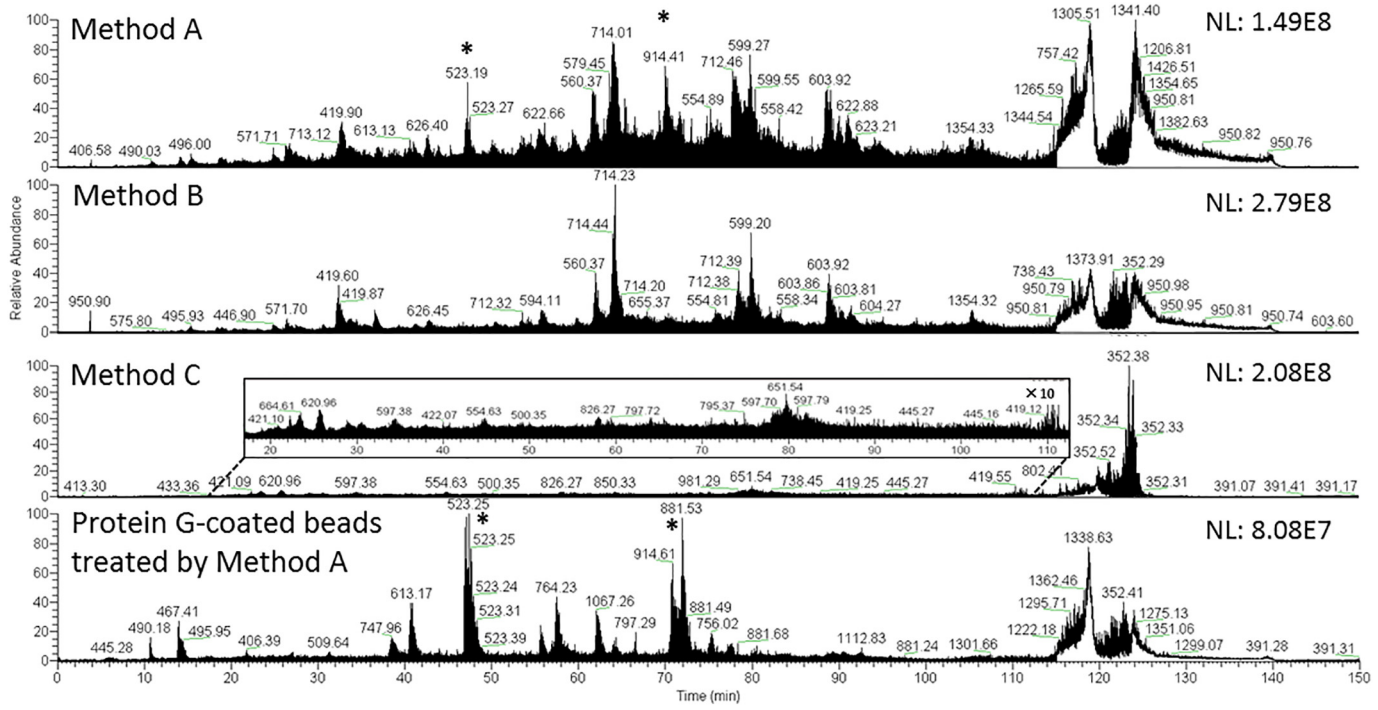

Fig. 4. Total ion chromatograms of pooled serum pretreated using Method A, Method B, Method C, and protein G-coated magnetic beads treated using Method A. The peaks marked by asterisk are derived from bead-proteins.

non-specifically to the beads. On the other hand, myoglobin was not detected by Method C and may have been retained on the beads after papain-digestion and elution. Therefore, we propose that using Method C we can identify CIC-antigens with higher selectivity compared to the other two methods. As the concentration of free antibody is much higher than that of CICs, free antibodies interfere with the detection of CICs in immune complexome analysis. Therefore, excluding antibody peptides from the peptide digests used for nano-LC-MS/MS analysis should improve the sensitivity of immune complexome analysis. Although several peptides derived from the antibody (anti-myoglobin) were detected by all three methods (Fig. 2b), fewer peptides derived from the antibody were detected when using Method C compared to the other methods. The peptides detected when using Method C were limited to the Fab region of the antibody (data not shown), which means that papain digestion in Method C cleaves antibodies at the hinge region, as expected. The application of Method C can therefore decrease the number of peptides derived from antibodies, allowing identification of CIC-antigens with higher sensitivity than the other protocols. When *in vitro* formed myoglobin-IC was mixed with Protein G-coated magnetic beads, the number of peptides from both myoglobin and anti-myoglobin was higher when using Method A and Method B than when using Method C (Fig. 2c). Considering the results obtained when myoglobin (Fig. 2a) or anti-myoglobin (Fig. 2b) was individually mixed with Protein G-coated beads, many non-specifically bound proteins and antibodies were detected when using Method A and Method B and therefore use of these two methods will likely decrease the number of CIC-antigens detected in immune complexome analysis. From these results, we concluded that Method C provided more selective and sensitive detection of CIC-antigens because it can selectively elute antigens from CICs, drastically reducing the number of peptides generated from antibodies and from proteins bound non-specifically to the beads. It would be better to confirm that our method detects the antigens whose corresponding antibodies have been reported to be specifically detected in patients with a certain autoimmune disease. However, it has not been sure that such antibodies really form IC *in vivo* in the disease. Therefore, an artificial IC, like myoglobin-IC used here, should be a target to confirm the method specificity.

The number of CIC-antigens in human serum detected using Method C was statistically smaller than the other two methods (Fig. 3). Considering the results obtained when using *in vitro* formed myoglobin-IC, the CIC-antigens detected when using Method A or Method B should involve a large number of proteins bound non-specifically to the beads, while the number of CIC-antigens detected when using Method C is unaffected by proteins bound non-specifically to the beads. We also

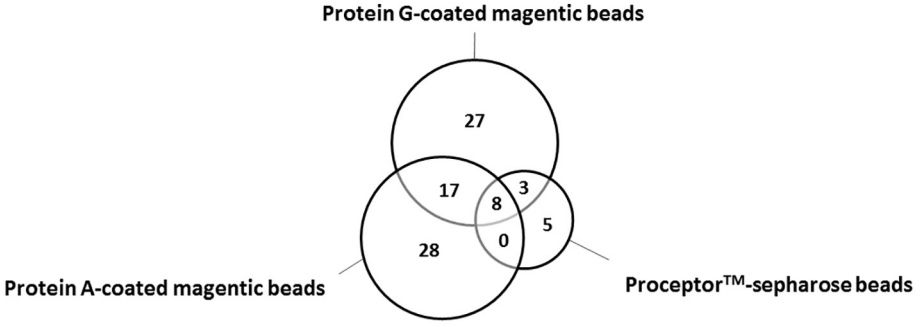

Fig. 5. Comparison of the number of human proteins identified using each of three types of beads with papain-digestion, elution, and tryptic digestion (Method C).

validated the selective elution of IC-antigens from beads by Method C in using serum spiked with myoglobin or myoglobin-IC. The pool serum spiked with myoglobin (2.5 pmol/μL, 10 μL) was used as a negative control, and we confirmed that the added myoglobin was not detected by Method C. Furthermore, we used the pool serum spiked with myoglobin-IC (2.5 pmol/μL, 10 μL) as a positive control, and we confirmed that 1 peptide of myoglobin from this spiked IC was detected by Method C. These results support that Method C selectively elutes and detects IC-antigens.

Comparing the chromatograms obtained by nano-LC-MS/MS analysis using each method, the many peaks observed using Method A (Fig. 4a) disappeared in the chromatograms obtained using Method B (Fig. 4b) and Method C (Fig. 4c). These peaks were observed only in the chromatogram obtained when Protein G-coated beads treated by Method A (Fig. 4d), indicating that these peaks originated from Protein G. On the other hand, markedly fewer peaks were obtained when using Method C compared to the other methods, suggesting that Method C allows the highly sensitive detection of CIC-antigens without interference by non-specifically bound proteins and antibodies and by peptides originating from Protein G.

We found that Method C can detect CIC-antigens more selectively and sensitively than the other methods. Immune complexome analysis of serum samples from patients with several diseases requires comprehensive analysis of CIC-antigens from the serum. In this context, we thus evaluated three types of beads (Protein G-coated magnetic beads, Protein A-magnetic beads and Proceptor™-sepharose beads) to examine if their parallel use is complementary and improves the analysis of CIC-antigens (Fig. 5). Each bead has a different affinity for immune complexes. Protein A and Protein G bind the Fc domain of antibodies. IgG3 is captured on Protein G but not Protein A, and IgM, IgA, IgD, and IgE are captured on Protein A but not Protein G. On the other hand, Proceptor™ captures ICs without binding monomeric Ig (Chauhan and Moore, 2006). Our results show that the selectivity of each bead in collecting serum CICs is clearly different, given their different affinities, and their parallel use can thus cover a wide range of CIC-antigens in immune complexome analysis.

5. Conclusion

In this study, we compared three methods to examine which most selectively elutes CIC-antigens from CICs bound on beads, allowing selective detection of CIC-antigens by nano-LC-MS/MS. In this context, Method C, in which CICs collected by the beads were eluted from the beads by papain-digestion, elution, and then tryptic digestion in solution, allows the highly sensitive detection of CIC-antigens without interference from non-specifically bound proteins and antibodies. In addition, we established that parallel use of multiple bead types can comprehensively identify CIC-antigens from pooled serum when using Method C. This method could be useful for identifying antigens in ICs in biological fluids from patients with a variety of diseases and thus antigens in ICs may aid the development of diagnostic biomarkers that could lead to specific treatments for these diseases.

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Potential conflict of interest

Nothing to report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.06.021.

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