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An Efficient Identification Method of a Specific Binding Protein for a Bioactive Compound Using On-Bead Digestion and Mass Spectrometry

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Affinity chromatography is widely utilized as a tool in biomedical research and biotechnology. This purification method provides increased sensitivity for various kinds of analyses, such as mass spectrometry. This report describes a protocol to identify a specific binding protein for a bioactive compound (BAC) using BAC-affinity beads and nanoflow liquid chromatography tandem mass spectrometry (nano-LC/MS/MS). After the target protein was digested directly on the affinity beads, the obtained peptide fragments were determined by MS analysis. The binding-specificity was shown by a competition experiment using beads lacking bound BAC. In this study, we describe two examples for benzenesulfonamide- and methotrexate-binding proteins.

Ever since the declaration that the whole human genome had been deciphered, proteomics has become one of the most important projects in post-genomic research. Therefore, methods for the determination of each protein expressed in cells are very important to evaluate the basis for understanding the molecular origins of diseases and for drug design. A conventionally used method for separating a particular protein is affinity chromatography.[1–13] On the other hand, mass spectrometry (MS) is generally used for the analysis of protein.[14–16] In particular, nanoflow liquid chromatography tandem mass analysis (nano-LC/MS/MS) is very useful for the analysis of enzyme-digested peptide fragments, low amounts of protein, or protein mixtures. The competition experiment is now widely used in order to find specific binding proteins for bioactive compounds (BACs; drugs, natural products, toxins, etc.). In this method, proteins bound to the resin in binding and competition assays are checked by SDS-PAGE and the intensity of both bands compared visually. After that, changed protein bands on the gel are cut out, MS measurement samples are prepared by in-gel digestion, and MS measurement and analysis are performed.[12,17,18] However, this method is impossible in cases where bands cannot be observed clearly because of abundant non-specific protein bound to affinity beads. Moreover, this method requires multiple steps, long preparation time, and sample recovery is reduced. Thus, a more efficient method is needed. The present paper reports that the identification of BAC-binding protein is possible by using on-bead digestion, in which the protein is digested with trypsin directly on the affinity beads, and nano-LC/MS/MS. Previous studies have simply focused on an on-bead digestion method.[19–21] However, this method is limited to specific applications because only the specific protein is immobilized with antibody or chelate columns. An on-bead digestion using BAC-affinity beads has not been reported. It seems that a lot of non-specific proteins are bound to the affinity beads, due to weak BAC–protein interaction. A study was conducted demonstrating the identification of proteins bound to carboxybenzenesulfonamide (=4-sulfamoylbenzoic acid, CBA) or methotrexate (=*N*-(4-[(2,4-diamino-6-pteridinylmethyl)methylamino]benzoyl)-L-glutamic acid, MTX) as models. The authors project that this alternative method will find application in drug discovery, post-genomic, proteomic, chemical genomic, and chemical proteomic research.

Experimental

Materials. Acetonitrile and water for nano-LC were purchased from Merck. Methanol was from Kanto Chemical. Water for non-LC/MS was obtained form a Millipore Milli-Q system. CBA, DMF, formic acid, acetic anhydride, skim milk, DTT, and iodoacetamide were from Wako. MTX was from Aldrich. Rats were from Charles River. THP-1 cells were from ATCC. TOYOPEARL was from Tosoh. HOBt was from Watanabe Chemical. EDC/HCl was from Kokusan Chemical. Gel for SDS-PAGE and nitrocellulose membrane were from BioRad. Trypsin was from Promega. Rabbit anti-DHFR antibody and CAII were from Sigma. Anti-rabbit IgG, alkaline phosphatase conjugate was from BioSource. NBT/BCIP was from Pierce. All other chemicals used for the experiments were from Wako.

Synthesis of CBA-Affinity Beads. CBA-affinity beads were based on a previous report.[22] A mixture of 4-sulfamoylbenzoic acid (24 mg, 120 μmol), TOYOPEARL AF-Amino-650 (400 μL, 40 μmol of terminal NH_2), EDC/HCl (23 mg, 120 μmol), and HOBt (16 mg, 120 μmol) in DMF (2 mL) were stirred at room

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temperature for 14 h. Progression of the reaction was confirmed by measuring the residual amino group by the ninhydrin reaction.[11] The yield was about 70%. After completion of the reaction, the resin was washed five times with DMF. The resulting resin was added to 20% (v/v) acetic anhydride/DMF (2 mL), and the mixture was stirred for 1 h at room temperature. Then, the resin was washed adequately with DMF and stored in 20% (v/v) ethanol/water until use.

Synthesis of MTX-Affinity Beads. MTX-affinity beads were based on a previous report.[23] A mixture of MTX (19 mg, 42 μmol), TOYOPEARL AF-Amino-650 (500 μL, 50 μmol of terminal NH_2), EDC/HCl (8 mg, 42 μmol), and HOBt (7 mg, 52 μmol) in DMF (2 mL) were stirred at room temperature for 14 h. Progression of the reaction was confirmed by measuring the residual amino group by the ninhydrin reaction. The yield was about 80%. After completion of the reaction, the resin was washed five times with DMF. The resulting resin was added to 20% (v/v) acetic anhydride/DMF (2 mL) and the mixture was stirred for 1 h at room temperature. Then, the resin was washed adequately with DMF and stored in 20% (v/v) ethanol/water until use.

Preparation of Carbonic Anhydrase II Protein Solution. Commercially available carbonic anhydrase II (2 mg) was dissolved in Buffer A (0.25 M sucrose, 25 mM Tris-HCl, pH 7.4) (1 mL), and a 2 mg mL^{-1} stock solution was prepared. Stock solution (15 μL) and Buffer A (985 μL) were mixed to prepare the carbonic anhydrase II protein solution.

Preparation of Rat Brain Lysate. Buffer A (22 mL) was added to rat brain (2.2 g) and a homogenate was prepared; it was then separated by centrifugation for 20 min at 9500 rpm. The supernatant was recovered and separated by centrifugation again for 30 min at 50000 rpm. The supernatant was used as the lysate. All of the experiments were conducted at 4 °C or on ice.

Preparation of THP-1 Lysate. THP-1 cells (1 × 107) were freeze-thawed in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA). This solution was centrifuged at 10000 rpm for 10 min. The supernatant was used as the lysate and kept at −80 °C before use. All of the experiments were conducted at 4 °C or on ice.

Binding Assay. CBA- or MTX-affinity beads (10 μL) and lysate (1 mL) diluted 2-fold with Buffer A or standard solution (1 mL) was gently shaken for 1 h at 4 °C. The supernatant was then removed, and the beads were washed adequately five times with Buffer A. To check binding proteins, resulting eluates from beads were subjected to SDS-PAGE, followed by silver or CBB staining as needed.

Competition Assay. A quantity (5 μL) of a solution of CBA (7.5 μg, 38 nmol) or MTX (0.28 mg, 0.625 μmol) in DMSO was added to the lysate (1 mL) diluted 2-fold with Buffer A and stirred gently for 1 h at 4 °C. CBA- or MTX-affinity beads (10 μL) and lysate (1 mL) treated as described were gently shaken for 1 h at 4 °C. The supernatant was then removed and the remaining affinity beads were washed adequately five times with Buffer A. To check binding proteins, resulting eluates from beads were subjected to SDS-PAGE, followed by silver or CBB staining as needed.

On-Bead Digestion. To each of the affinity beads (10 μL) obtained in the binding assay or competition assay, 13 μL of denaturant Buffer B (mixture of 195 mg urea, 250 μL 1 M Tris-HCl, pH 8.5 and 50 μL 0.1 M EDTA-2Na) was added. A quantity (2 μL) of reducing solution (2 mg DTT dissolved in 50 μL of Buffer B) was added and gently shaken for 1 h at 60 °C. A quantity (6 μL) of cysteine-blocking solution (2 mg of iodoacetic acid dissolved in 50 μL Buffer B) was added and gently shaken in the dark for 30 min at 37 °C. After addition of 50 μL of water, 30 μL of trypsin solution (20 μg of trypsin dissolved in 1 mL of Buffer B) was added and the mixture gently shaken for 16 h at 37 °C. Then 30 μL of formic acid/acetonitrile/water: 0.1/50/50 (v/v/v) was added and after stirring vigorously at room temperature the supernatant as recovered. Then the measurements and analyses were performed using the recovered solution evaporated to about 50 μL as the sample for MS measurements.

Western Blot Analysis. The proteins, obtained in binding and competition assay on MTX-affinity beads, were applied to SDS-PAGE followed by electroblotting onto nitrocellulose membrane. After blocking with skim milk at room temperature for 30 min, the membrane was incubated with a rabbit anti-DHFR antibody as the primary antibody at room temperature for 1 h and washed three times with PBS. Then the membrane was incubated with anti-rabbit IgG, alkaline phosphatase conjugate as the secondary antibody at room temperature for 1 h and washed again. Color development was achieved using NBT/BCIP as a substrate.

LC/MS Measurement. A KYA TECH Dina system was used for nano-LC. The mass spectrometer used was a Waters Micromass Q-Tof Premier and MassLynx software. A HiQ-SiL C18 (0.75 mm ID × 50 mm L) was used as the analytical column. A HiQ-SiL C18 (0.5 mm ID × 1 mm L) was used as a trapping column. The mobile phase was Solvent A (formic acid/acetonitrile/water: 0.1/2/98 (v/v/v)) and Solvent B (formic acid/acetonitrile/water: 0.1/70/30 (v/v/v)). The flow rate for analysis was 300 nL min^{-1}. Samples were cleaned for 10 min at 5 μL min^{-1}, with Solvent A. The peptides were eluted using a linear gradient of 0% Solvent B to 50% Solvent B in 30 min. The injection volume was 1 μL. For MS measurements, a nano-ion spray probe was used, which was set in the positive mode. Q-Tof Premier was operated in Data Directed Analysis mode with an MS survey integration time of 1 s. The mass spectrometer was programmed to switch into the MS/MS mode on a single doubly- or triply-charged precursor ion. Protein identification was done with the Matrix Science Mascot search engine (ver.2.2.03) using the SWISS-PROT protein database.

Results and Discussion

Identification of CBA-Binding Protein Using On-Bead Digestion. CBA is a known substrate of carbonic anhydrase II (CAII).[22,24] First, to confirm whether CAII can be captured using our CBA-affinity beads (Figure 1) in which CBA is immobilized directly on the beads (An alkyl linker was introduced between CBA and beads in the previous report.[12]) binding assay was carried out using a CAII protein solution. Protein bound to the CBA-affinity beads was checked with SDS-PAGE and demonstrated that CAII can be accurately captured (Figure 2).

Next, using our CBA-affinity beads, binding and competition assays were performed with rat brain lysate. CAII was clearly observed in the binding assay. On the other hand, the

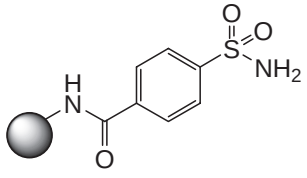

Figure 1. CBA-affinity beads.

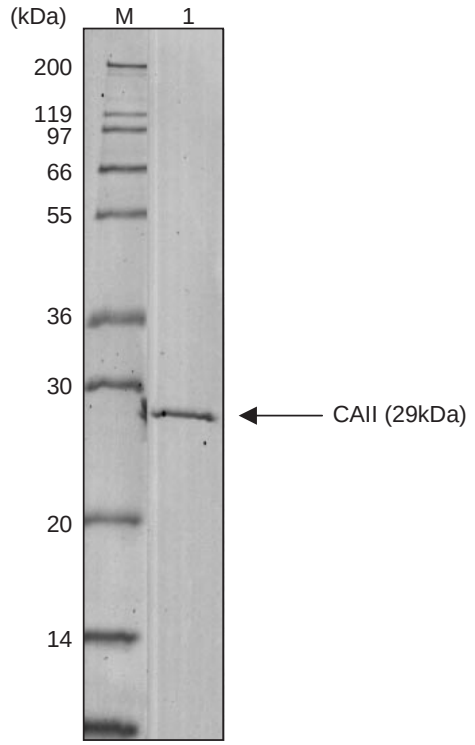

Figure 2. Binding protein on CBA-affinity beads using CAII protein solution. (Lane 1) Result of loading sample in the binding assay.

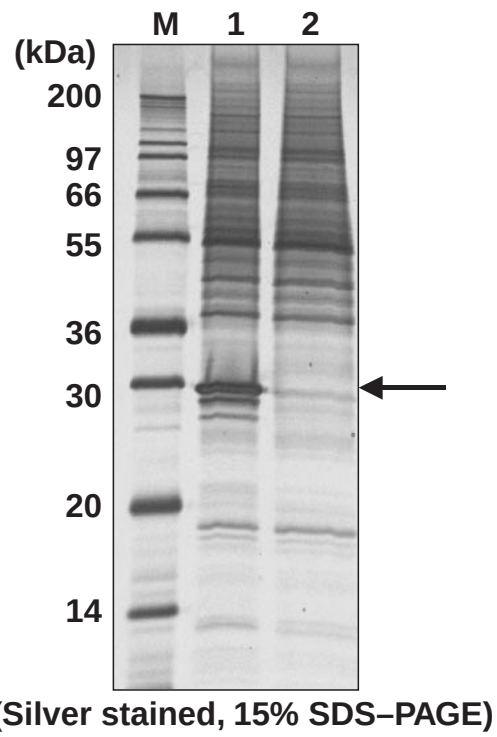

Figure 3. Binding proteins on CBA-affinity beads with rat brain lysate. (Lane 1) Result of loading sample in the binding assay. (Lane 2) Result of loading sample in the competition assay.

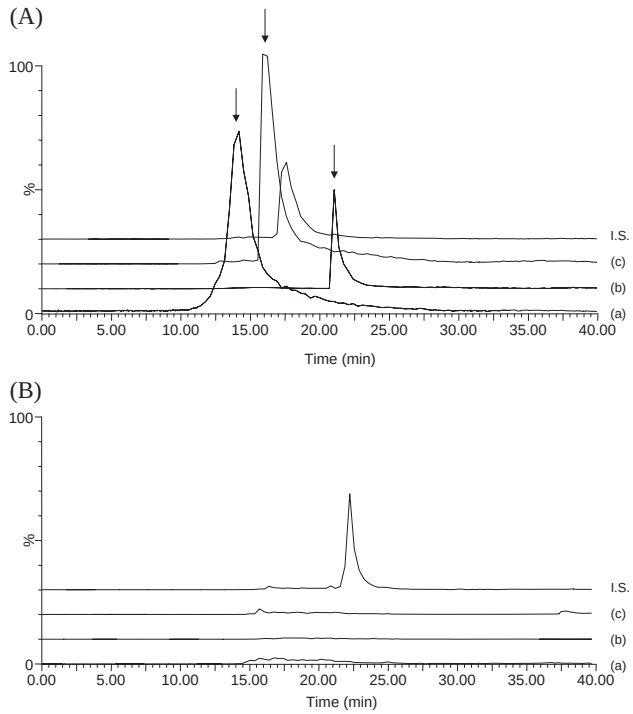

Figure 4. MS analyses of CAII digested peptide fragments. (A) MS chromatogram in the binding assay sample. (B) MS chromatogram in the competition assay sample. (a) m/z 483.2 (doubly-charged), amino acid sequence EGPLSGSYR (residue number 81–89). (b) m/z 971.4 (triply-charged), QSPVDIDTGTAQHDPSLQPLLICYDK (28–53). (c) m/z 549.9 (triply-charged), EPITVSSEQMSHFR (213–226). The digested peptide of endogenous myelin basic protein, which is a non-specific protein, was selected as an internal standard (IS). (I.S.) m/z 730.8 (doubly-charged), TQDENPVVHFFK (104–115). The scale of the y axis was set such that 100% is equivalent to an intensity of 8000 counts.

CAII band became very faint in the competition assay on SDS-PAGE (Figure 3). To confirm whether on-bead tryptic digestion could be applied, on-bead tryptic digestion was performed using CBA-affinity beads with binding proteins used in the binding assay, and the peptide fragments obtained were analyzed with MS. As a result, CAII was identified (coverage 46%, Mascot score 1020) only in the binding assay sample. On the MS chromatogram, the chromatographic peaks of digested peptides with CAII were compared in the binding and competition assay samples. As a result, the peaks were detected only in the binding assay sample (Figures 4A and 4B). Therefore, it was revealed that BAC-binding protein could be simply and efficiently identified by on-bead digestion using BAC affinity beads.

Identification of MTX-Binding Protein Using On-Bead Digestion. MTX is a known substrate of dihydrofolate reductase (DHFR).[23,25] Binding and competition assays were conducted using MTX-affinity beads (Figure 5) and THP-1 cell lysate. In a comparison of both SDS-PAGE lanes, the competition band could not be observed (Figure 6A). Then, on-bead tryptic digestion was performed using the MTX-affinity beads with binding proteins from the binding and competition assays.

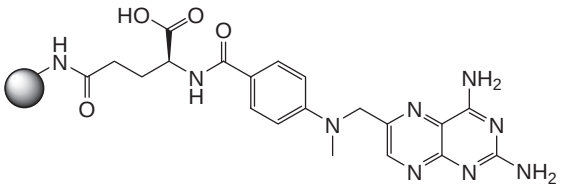

Figure 5. MTX-affinity beads.

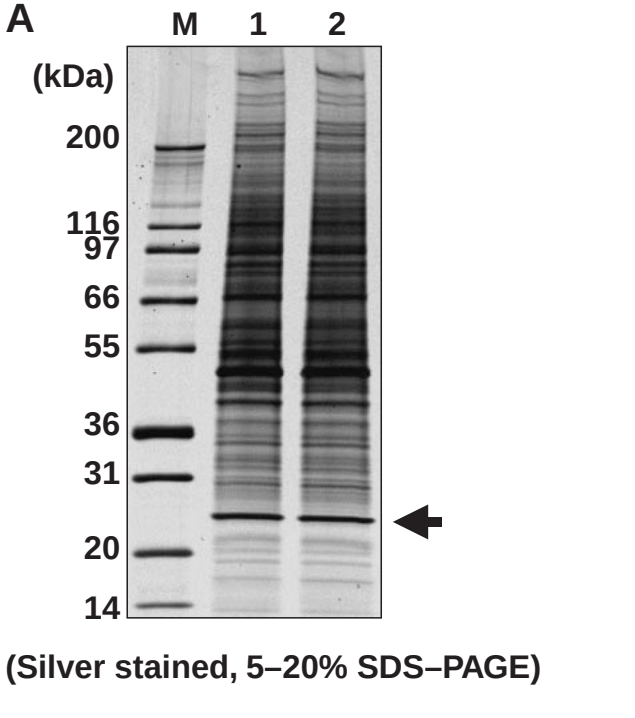

Figure 6. Binding proteins on MTX-affinity beads with THP-1 cell lysate. (A) Silver stained analyses. (B) Western blot analyses. (Lane 1) Result of loading sample in the binding assay. (Lane 2) Result of loading sample in the competition assay.

A comparison of the differentials in both protein identification lists obtained was subsequently performed. The results showed that dihydrofolate reductase (DHFR) was present predominantly only in the binding assay (coverage 22.5%, Mascot score 87). On the MS chromatogram, the chromatographic peaks of digested peptides with DHFR were also detected only in the binding assay sample (Figures 7A and 7B). In addition, DHFR was present in the binding assay sample only by Western blot (Figure 6B). It may be inferred from this that DHFR was most certainly contained only in the binding assay sample. From the results of the differential analysis, peroxiredoxin (molecular weight similar to that of DHFR) was identified at a high score in the binding and competition assay samples.

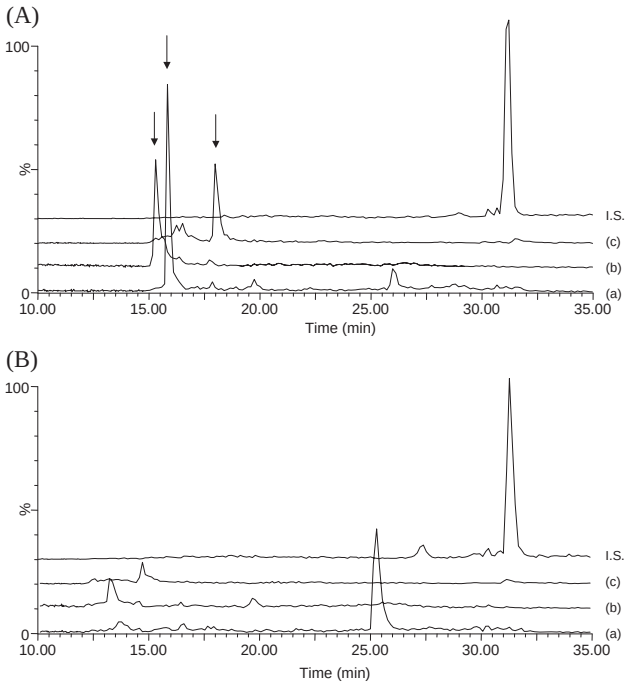

Figure 7. MS analyses of DHFR digested peptide fragments. (A) MS chromatogram in the binding assay sample. (B) MS chromatogram in the competition assay sample. (a) m/z 571.8 (doubly-charged), amino acid sequence LTEQPELANK (residue number 100–109). (b) m/z 520.7 (doubly-charged), MTTTSSVEGK (38–47). (c) m/z 407.7 (doubly-charged), INLVLSR (72–78). The self-digested peptide of trypsin was selected as IS. (I.S.) m/z 762.0 (triply-charged), IITHPNFNGNTLDNDIMLIK (78–97). The scale of the y axis was set such that 100% is equivalent to an intensity of 2000 counts.

From MS analysis, large amounts of digested peptides with peroxiredoxin I were observed (Figures 8A and 8B). It was indicated that peroxiredoxin is contained in both samples at the same level. Therefore, the competition bands could not be identified on SDS-PAGE because the DHFR bands were hidden by the peroxiredoxin bands. Currently, in the identification of BAC-binding proteins, proteins bound to the beads in binding and competition assays are checked by SDS-PAGE and the intensity of the both bands compared visually. However, this method is impossible in cases where bands cannot be observed clearly. These problems can all be solved using our proposed on-bead digestion method.

Conclusion

An on-bead digestion protocol using BAC-affinity beads was established that simultaneously treats proteins bound to affinity beads while also covalently coupling BACs. Simple identification of BAC-binding protein is possible using MS analysis. The identification method in this study is usually for the discovery of proteins binding with BACs. In this study CBA and MTX were used as models, which equilibrium dissociation constant (K_i) has been described as 151 nM (for CAII) and 3.4 pM (for DHFR), respectively.[26,27] Calculating the dissociation constant (K_d) for the immobilized BAC binding to the protein is impossible in this study method, but it was re-

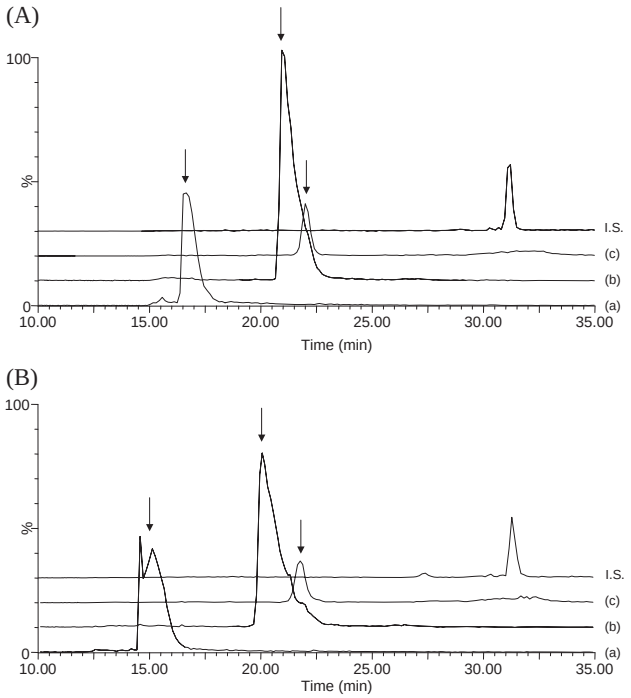

Figure 8. MS analyses of peroxiredoxin I digested peptide fragments. (A) MS chromatogram in the binding assay sample. (B) MS chromatogram in the competition assay sample. (a) m/z 447.7 (doubly-charged), amino acid sequence ADEGISFR (residue number 121–128). (b) m/z 460.7 (doubly-charged), GLFIIDDK (129–136). (c) m/z 819.8 (doubly-charged), QGGLGPMNIPLVSDPK (94–109). The self-digested peptide of trypsin was selected as an IS. (I.S.) m/z 762.0 (triply-charged), IITHPNFNGNTLDNDIMLIK (78–97). The scale of the y axis was set such that 100% is equivalent to an intensity of 6000 counts.

ported that the K_d for immobilized CBA and CAII was 0.32 mM in a previous similar study using surface plasmon resonance (SPR) technique.[22] Therefore the BAC-binding protein with an affinity equal to or greater than that is expected to be identified by our method. This method makes the same handling possible in cases of covalent coupling of BACs, peptides, or proteins with the beads, while studying interactions with the affinity beads. It should also be possible to perform high precision assays of the peptide fragments obtained by on-bead digestion, using isotope-labeled reagents such as iTRAQ® in MS differential analysis. These results have widespread application potential in drug discovery, postgenomic, proteomic, chemical genomic, and chemical proteomic research.

Abbreviations

EDC/HCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Tris-HCl, trishydroxymethylaminomethane hydrochloride; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CBB, coomassie brilliant blue; DTT, dithiothreitol; PBS, phosphate buffered saline.

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Supporting Information

MS analyses of CAII peptide fragments (Figures S1–S3), DHFR peptide fragments (Figures S4–S6), and peroxiredoxin I peptide fragments (Figures S7–S9). This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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