Electronic Supplementary Information

Bottom-up/cross-linking mass spectrometry via simplified sample processing on anion-exchange solid-phase extraction spin column

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Experimental

Reagents

Acetonitrile, ammonium bicarbonate (ABC), bovine serum albumin (BSA), formic acid (FA), iodoacetamide, LC-grade water, methanol, and trifluoroacetic acid (TFA) were purchased from Wako (Osaka, Japan). HEPES buffer was purchased from Nacalai Tesque (Kyoto, Japan). β-phycoerythrin, Disuccinimidyl dibutyric urea (DSBU), dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human hemoglobin (Hb) was purchased from Sigma Aldrich (St. Louis, MO, USA). Trypsin/Lys-C Mix and sequencing-grade modified trypsin were purchased from Promega (Madison, WI, USA).

Human cellular protein extract (HCPE)

Promega's MS-compatible human protein extract was subjected to reductive alkylation before use. To 100 µL of the protein extract (10 mg/mL), 1 µL of 500 mM DTT was added and incubated at 37 °C for 30 minutes, followed by alkylation with 1.5 µL of 1 M iodoacetamide for 30 minutes at 25 °C. The reductively alkylated protein extract was purified by methanol/chloroform/water precipitation as previously described.[S1] The precipitated protein pellet was reconstituted with 1 mL of 0.05% (w/v) SDS/100 mM ABC and stored at −80 °C until use.

Sample processing by AnExSP

A StageTip used for AnExSP was prepared by using a straw to punch a small circle 6 mm in diameter out of an Empore AX SPE disc (3M, St. Paul, MN, USA) and packing it into a disposable 200 µL micropipette tip. Disc fitting was done manually using an Eppendorf Combitips Plus® 10 mL piston. In order to verify that proper fitting was obtained, methanol was added to the discs in the chip, and only those that did not leak were used. The prepared StageTip was fitted with a centrifuge adapter (Catalog # 5010-21514, GL Sciences, Tokyo, Japan) and was set in a 2 mL microcentrifuge tube. For enzymatic digestion within the StageTips, Trypsin/Lys-C Mix or sequence grade trypsin with 100 mM ABC was used. Sample processing was performed as follows:

Step 1: Column conditioning

40 µL of methanol was first loaded onto the StageTip and centrifuged at 7000 rcf for 3 minutes, followed by 40 µL of elution solution (0.5% (v/v) FA/30% (v/v) acetonitrile) loaded onto the StageTip and another centrifugation at 7000 rcf for 3 minutes.

Step 2: Column equilibration

40 µL of 100 mM ABC was loaded onto the StageTip and centrifuged at 7000 rcf for 3 minutes.

Step 3: Sample loading

A protein sample was loaded onto the StageTip and centrifuged at 7000 rcf for 3 minutes.

Step 4: Sample washing

100 µL of 100 mM ABC was loaded onto the StageTip and centrifuged at 7000 rcf for 1 minute.

Step 5: Enzyme loading

A Trypsin/Lys-C Mix solution or trypsin solution was loaded onto the StageTip at an enzyme-substrate ratio of 1:20 and centrifuged at 2000 rcf for 30 seconds. After centrifugation, the StageTip was transferred to a new 1.5 mL tube set in an incubator.

Step 6: Enzymatic digestion

10 µL of 100 mM ABC was gently added onto the AX SPE disc in the StageTip and incubated at 25 °C for 1–4 hours. After incubation, the StageTip was set in the centrifuge again and centrifuged at 7000 rpm for 3 minutes.

Step 7: Peptide elution

60 µL of elution solution was loaded onto the StageTip and centrifuged at 7000 rcf for 3 minutes. The recovered solution from Steps 6 and 7 in the 1.5 mL tube was dried in a centrifugal concentrator and reconstituted in 0.1% (v/v) FA/3% (v/v) acetonitrile for LC-MS analysis.

SP3 method

The HCPE sample (1 µg or 10 µg) was subjected to cleanup and digestion with single-pot solid phase-enhanced sample preparation (SP3).[S2,S3] Briefly, two types of beads (hydrophilic and hydrophobic Sera-Mag Speed-Beads; Cytiva, Marlborough, MA, USA) were used. These beads were combined at a 1:1 (v/v) ratio, rinsed with distilled water, and reconstituted in distilled water at 20 µg solids/µL. The reconstituted beads (10 µL) were then added to the sample followed by ethanol to bring the final concentration to 75% (v/v), with mixing for 20 minutes. The beads were subsequently immobilized on a magnetic rack. The supernatant was discarded, and the pellet was rinsed with 80% (v/v) ethanol and 100% acetonitrile. The beads were then resuspended in 50 µL of 100 mM ABC with 500 ng trypsin/Lys-C Mix (Promega, Madison, WI, USA) and digested by gentle agitation at 37 °C overnight. The digested sample was acidified

with 10 µL of 5 % (v/v) TFA and then desalted using SDB StageTip (GL Sciences), followed by drying with a centrifugal evaporator. The dried peptides were redissolved in 3% (v/v) acetonitrile containing 0.1% (v/v) FA, and transferred to a hydrophilic-coated low-adsorption vial (ProteoSave vial; AMR Inc., Tokyo, Japan).

Analysis of PAGE-separated HCPE

SDS-PAGE: HCPE samples (8 µg/lane) were separated by NuPAGE 4–12% Bis-Tris gel (1 mm-thick, 15 well) with NuPAGE MES running buffer (Thermo Fisher Scientific) according to the manufacturer's instructions. The gel was stained with 40 mL of aqueous CBB solution (EzStain Aqua CBB solution, ATTO, Tokyo, Japan) for 8 minutes, and washed with 200 mL of distilled water for 30 minutes. After electrophoresis, the sample lane was divided into six fractions and the resulting gel fractions were processed by AnExSP-PEPPI or IGD.

AnExSP-PEPPI: The gel fractions were uniformly ground for 30 seconds using BioMasher disposable homogenizer tubes (Nippi, Tokyo, Japan). To extract proteins, 100 µL of 0.05% (w/v) SDS/100 mM ammonium bicarbonate were added to the macerated gels in the homogenizer tube and shaken vigorously at room temperature for 10 minutes. After filtration through a 0.45-µm cellulose acetate membrane within a Spin-X centrifuge tube filter (Corning, Corning, NY, USA), the protein filtrate was subjected to trypsin digestion (25 °C for 4 hours) by AnExSP. Digested peptides from each fraction were pooled and subjected to DIA-MS analysis.

IGD: The gel fractions were destained with 50% (v/v) acetonitrile/50 mM ABC. The gels were dehydrated by incubation in acetonitrile for 30 minutes and air-dried for another 10 minutes. The dry gels were rehydrated with 2 µL of 0.1 µg/µL trypsin, followed by incubation in 50 µL of 100 mM ABC at 37 °C for 18 hours. The digested peptides were recovered by first shaking the gel with 50 µL of 50% (v/v) acetonitrile/5% (v/v) TFA for 10 minutes and then incubating with another 50 µL of acetonitrile for 5 minutes. The recovered peptides from each fraction were pooled and purified by SDB-XC StageTips as previously described.[S4]

DIA-MS: The obtained digested peptides were analyzed using an Orbitrap Exploris 480 or a Q Exactive HF-X (Thermo Fisher Scientific) mass spectrometer coupled to an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). For LC separation, mobile phases consisted of 0.1% (v/v) FA as solvent A, and 0.1% (v/v) FA/80% (v/v) acetonitrile as solvent B. Each peptide

sample was injected onto an AURORA column (75 µm × 25 cm, 1.6 µm, Ion Opticks, Parkville, Australia) at 400 nL/min for 18 minutes using 3% B. Column temperature was set to 60 °C. Concentrated peptides were separated at a flow rate of 200 nL/min according to the following gradient schedule: 0–110 minutes, 3–36% B; 110–115 minutes, 36–65% B; hold at 65% B for 5 minutes; and re-equilibrate at 400 nL/min for 8 minutes using 3% B. For overlapping window DIA-MS [S5,S6], MS1 spectra were collected in the range of 495–745 m/z at 15,000 resolution to set an automatic gain control target of 3×10^6 and maximum injection time of "auto". MS2 spectra were collected at 200–1,800 m/z at 45,000 resolution to set an automatic gain control target of 3×10^6, maximum injection time of "auto", and stepped normalized collision energy of 22%, 26%, and 30%. The isolation width for MS2 was set to 4 m/z, and overlapping window patterns of 500–740 m/z were used for window placements optimized by Skyline v4.1.[S7]

Data analysis: The MS files were searched against human spectral libraries using Scaffold DIA (Proteome Software, Inc., Portland, OR, USA). The human spectral libraries were generated from the human protein sequence database (UniProt id UP000005640, reviewed, canonical) by Prosit.[S8,S9] The Scaffold DIA search parameters were as follows: experimental data search enzyme, trypsin; maximum missed cleavage site, 1; precursor mass tolerance, 10 ppm; fragment mass tolerance, 10 ppm; static modification, cysteine carbamidomethylation. The protein identification threshold was set to both peptide and protein false discovery rates of less than 1%. Peptide quantification was calculated by EncyclopeDIA algorithm[S10] in Scaffold DIA. For each peptide, the four highest quality fragment ions were selected for quantitation. Protein quantification was estimated from the summed peptide quantification. Pearson correlation coefficient for protein quantification between PEPPI and IGD was calculated by Perseus.[S11]

Analysis of CBB-containing HCPE

HCPE (4 µg) dissolved in 45 µL of 0.05% SDS/100 mM ABC was mixed with 5 µL of aqueous CBB solution (ATTO EzStain Aqua CBB) and subjected to trypsin digestion (25 °C for 4 hours) by AnExSP. HCPE (4 µg) dissolved in 50 µL of 0.05% SDS/100 mM ABC as CBB-free HCPE sample was also treated by AnExSP. Digested peptides were analyzed by DIA-MS as described above.

Cross-linking mass spectrometry

Sample preparation: The DSBU cross-linking reaction was performed using a sample of 33 µg of Hb or 33 µg of BSA dissolved in 50 µL of 40 mM HEPES, pH 7.5. DSBU was dissolved in dehydrated DMSO and immediately added to the protein samples at a final concentration of 0.1 mM or 1 mM. The cross-linking reaction was carried out in the dark at 25 °C for 30 minutes and quenched with 60 mM Tris-HCl, pH 8.8.

SDS-PAGE: Prior to SDS-PAGE, cross-linked samples were reduced with 65 mM DTT at 70 °C for 10 minutes and alkylated with 250 mM iodoacetamide at 25 °C for 30 minutes. The reductively alkylated samples were separated by NuPAGE 4–12% Bis-Tris gel (1 mm-thick, 10 well) with NuPAGE MES running buffer. After staining with aqueous CBB, the gel bands containing cross-linked Hb tetramer or Hb dimer (DSBU 1 mM) were excised and subjected to trypsin digestion by PEPPI-AnExSP (25 °C for 4 hours) or IGD (37 °C for 18 hours) as described above.

DDA-MS: The obtained digested peptides were analyzed using a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). For LC separation, mobile phases consisted of 0.1% (v/v) FA as solvent A, and 0.1% (v/v) FA/80% (v/v) acetonitrile as solvent B. Each peptide sample was injected onto a 75 µm × 12 cm nanoLC nano-capillary column (Nikkyo Technos Co., Ltd., Tokyo, Japan) at 750 nL/min for 10 minutes using 3% B. Column temperature was set to 40 °C. Concentrated peptides were separated at a flow rate of 200 nL/min according to the following gradient schedule: 0–22 minutes, 10–40% B; 22–30 minutes, 40–75% B; and re-equilibrate at 750 nL/min for 3 minutes using 3% B. MS1 spectra were collected in the range of 500–1,000 m/z at 120,000 resolution to set an auto gain control (AGC) target of 3×10^6. The 25 most intense ions with charge states of 3+ to 8+ that exceeded 8.0×10^3 were fragmented by collision induced dissociation with a normalized collision energy of 21%, 27%, and 33%, and MS2 spectra were collected in the range of more than 200 m/z at 45,000 resolution to set an AGC target of 2×10^5. The dynamic exclusion time was set to 30 seconds.

Data analysis: Raw MS data files were converted to .mgf files using RawConverter software.[S12] For the identification of cross-linked peptides, the converted MGF files were searched against the FASTA sequences of Hb alpha (P69905) and beta (P68871) subunits by MeroX software ver. 2.0.1.4[S13] using the following parameters: Protease sites: C-terminal at Lys and Arg (3 missed cleavages were allowed); Perform unspecific digest: On; Peptide length: 5 to 30 amino acids;

Fixed modifications: Cysteine alkylation by iodoacetamide, Variable modifications: Methionine oxidation (maximum 1); Select XL: DSBU; Cross-linker specificity: Lys, Ser, Thr, Tyr, N-terminus; Precursor mass deviation: 10 ppm; Fragment mass deviation: 10 ppm; Lower mass limit: 1000; Upper mass limit: 6000; S/N ratio: 2; Minimum charge of MS1: 3; Apply prescore: 10; FDR cut-off: 1%; Score cut-off: 50.

Data availability

All LC-MS data related to this study have been deposited to the ProteomeXchange Consortium via the jPOST partner repository with the dataset identifier PXD029444 for ProteomeXchange and JPST001370 for jPOST.

References

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Supplementary Figures

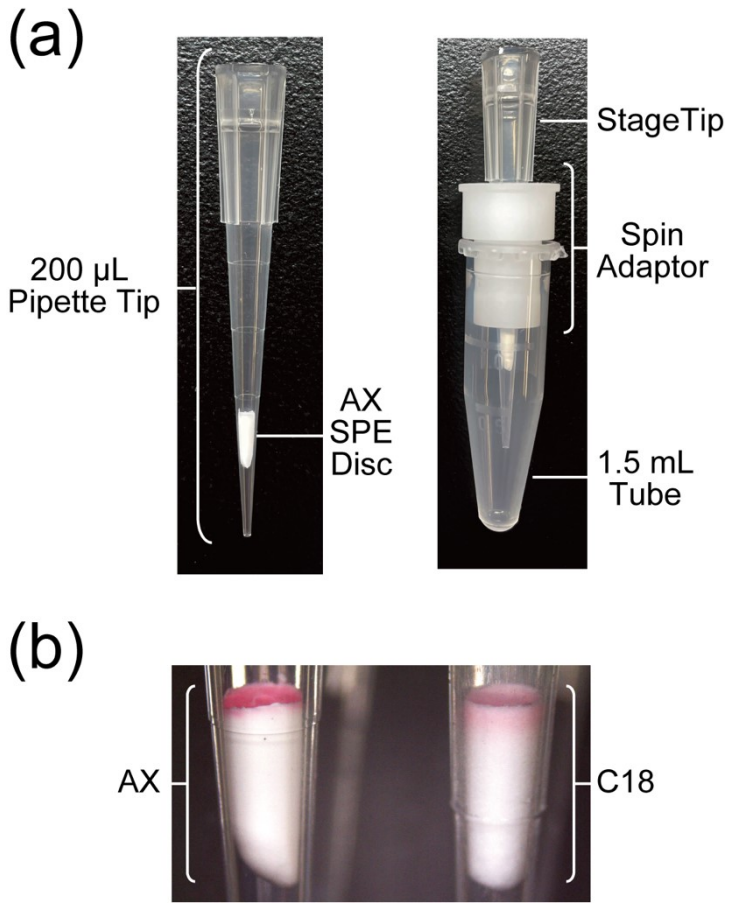

Fig. S1 (a) Self-made AX StageTip. The StageTip used in this study was prepared by packing a single Empore anion disc (6 mm in diameter) in a 200 µL size disposable pipette tip. Disc fitting was done manually using an Eppendorf Combitips Plus® 10 mL piston. (b) After loading 2 µg β-phycoerythrin: AX SPE disc (AX) and C18 RP SPE disc (C18).

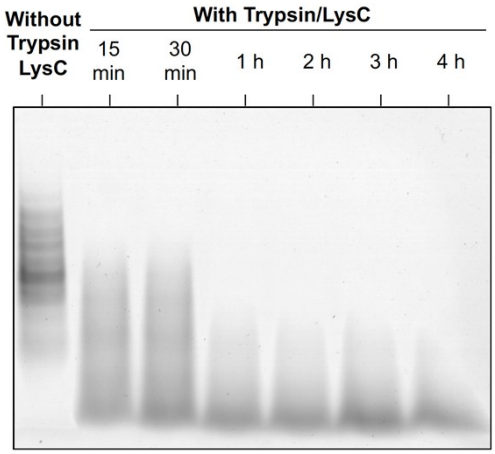

Fig. S2 An SDS-PAGE image of digested HCPE (10 µg) with Trypsin/Lys-C which recovered from AX SPE discs. The substrate enzyme ratio was set to 100:1.

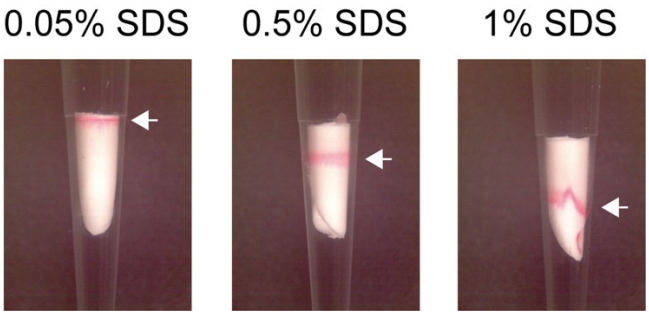

Fig. S3 SDS effect on protein localization in AX SPE discs. Protein localization in the packed AX SPE disc within an AX StageTip was examined by loading 2 µg β-phycoerythrin dissolved in 50 µL of 100 mM ABC with different SDS concentrations (0.05%, 0.5%, and 1% (w/v)).

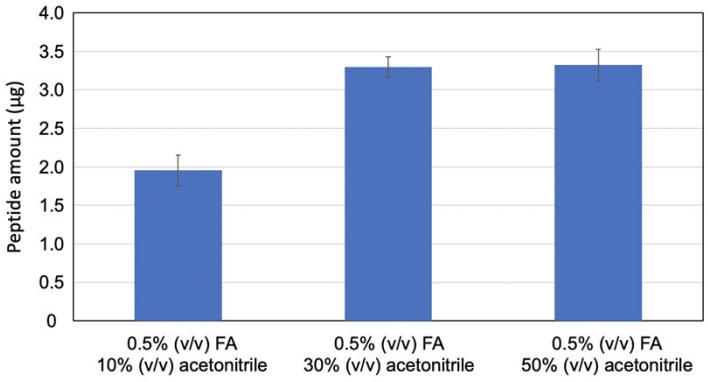

Fig. S4 Recovery of digested peptides from AX SPE discs by FA/acetonitrile solution. Five micrograms of HCPE was digested with Trypsin/Lys-C by AnExSP workflow and the resulting digested peptides were eluted from SPE discs using FA/acetonitrile solution. Sixty microliters of FA/acetonitrile solution with three different acetonitrile concentrations (10%, 30%, and 50% (v/v)) were used for peptide recovery. The amount (in µg) of digested peptides in the recovery solution was quantified using a commercially available peptide assay kit (Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay) according to the manufacturer's instructions.

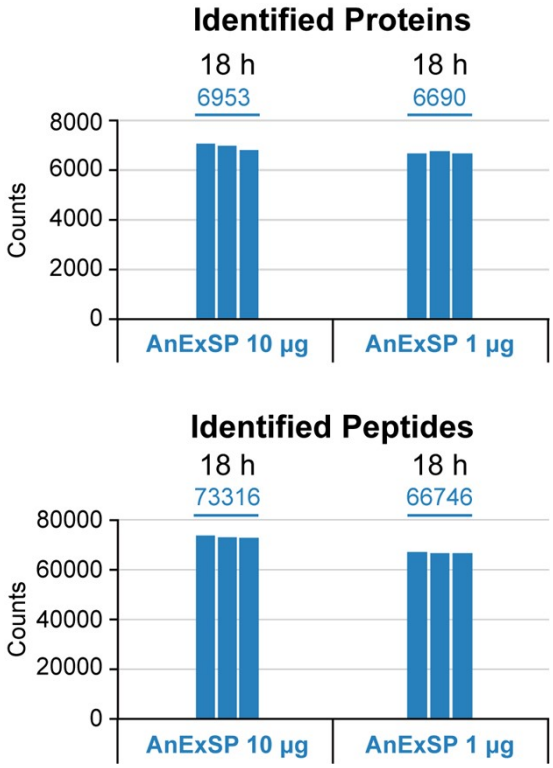

Fig. S5 DIA analysis of AnExSP-digested products. HCPE samples were digested with Trypsin/Lys-C for 18 hours.

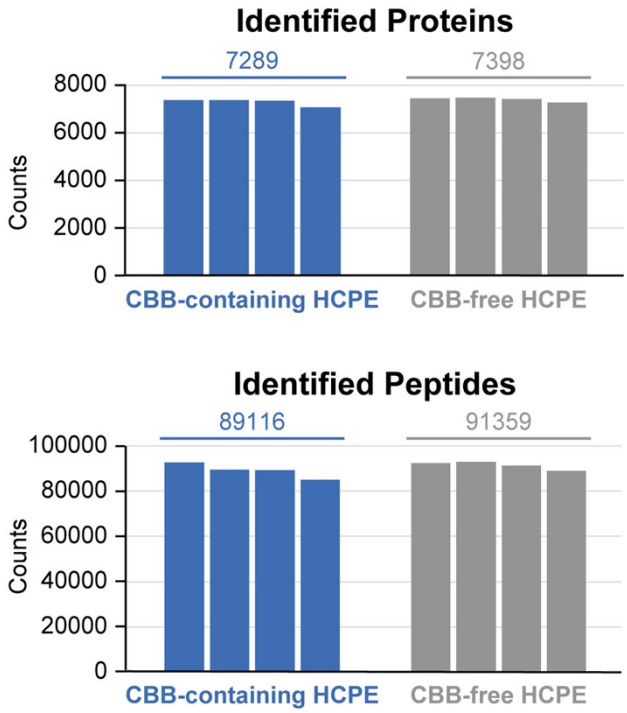

Fig. S6 Verification of trypsin digestion performance of AnExSP on CBB-containing HCPE. The number of identified peptides and proteins in DIA analysis of CBB-containing HCPE and CBB-free HCPE were compared.

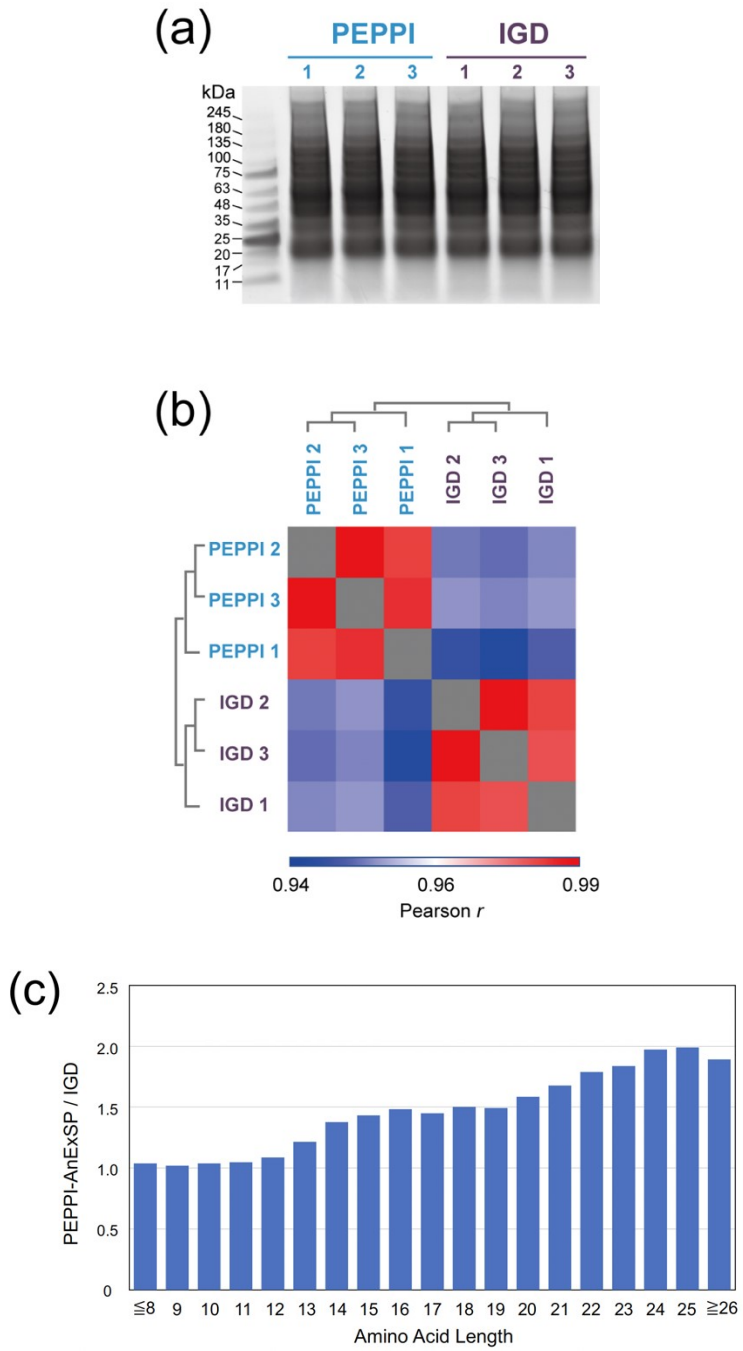

Fig. S7 Comparison of sample pretreatment performance by PEPPI-AnExSP and IGD. (a) A CBB-stained gel image of SDS-PAGE separated HCPE. The sample lane was divided into six sections and submitted to trypsin digestion by PEPPI-AnExSP or IGD. Derived peptides were subjected to DIA. (b) Reproducibility analysis of three repeated trials by Pearson correlation coefficient. (c) Comparison of the number of peptides in each amino acid length category between IGD and PEPPI-AnExSP. The PEPPI-AnExSP to IGD ratios were calculated using the number of peptides (average of three measurements) shown in Fig. 2c.

Supplementary Table

Table S1 Information of identified cross-linked Hb peptides by DDA (Excel file)