Gel free proteomics

(1) Trypsin/Lysyl endopeptidase C digestion

Proteins (100 μg) were cleaned up with chloroform/methanol to remove lipids and detergents [1]. The samples were adjusted to a volume of 100 μL with water, 400 μL of methanol was added to the sample, and the resulting solution was mixed. Subsequently, 100 μL of chloroform was added and mixed. For phase separation, 300 μL of water was added and vortexed again. The resulting mixture was centrifuged at 20,000g for 10 min to enhance phase separation. The upper aqueous phase was discarded, and 300 μL of methanol was added slowly to the organic phase. The samples were mixed gently and then centrifuged at 20,000g for 5 min. The resulting supernatants were discarded, and the pellets were dried. The dried pellets were resuspended in 50 mM NH_4HCO_3, and the proteins were reduced with 25 mM dithiothreitol, alkylated with 30 mM iodoacetamide and digested using 1 μg of trypsin and 1 μg of lysyl endopeptidase C at 37°C for 16 h. The resulting peptide solutions were acidified with formic acid and centrifuged. The supernatants were filtered through Pierce detergent removal Spin column and then subjected to LC-MS/MS analysis.

(2) LC-MS/MS analysis

The obtained peptides were separated using an Ultimate 3000 nanoLC (Thermo Fisher Scientific). The peptides were injected to a C18 PepMap trap column (300 μm ID × 5 mm, Thermo Fisher Scientific) with 0.1% formic acid, and eluted using a linear gradient of acetonitrile containing 0.1% formic acid at a flow rate of 200 nL/min. The eluted peptides were further separated on a C18 Tip column (75 μm ID × 120 mm, NANO HPLC CAPILLARY COLUMN, NTTC-360/75-3, Nikkyo Technos, Tokyo, Japan), which is a sprayer integrated analytical column. The separated peptides were sprayed at the end of the C18 Tip column with a spray voltage of 1.5 kV. The peptide ions in the spray were detected in the LTQ-Orbitrap discovery mass spectrometer (Thermo Fisher Scientific) using data-dependent acquisition mode with XCalibur software (ver. 2.0.7, Thermo Fisher Scientific). Fullscan mass spectra were acquired in the Orbitrap over 400-1,500 m/z with a resolution of 30,000. In the fullscan with orbitrap mass analyzer, the lock mass function was enabled. The top ten most intense precursor ions at the fullscan were selected for collision-induced fragmentation in the linear ion trap mass analyzer at normalized collision energy of 35%. Dynamic exclusion was employed within 90 s.

(3) Protein identification using Mascot

(a) Protein identification using obtained MS/MS data was performed by Mascot software (ver. 2.4.1, Matrix Science, London, UK) through Proteome Discoverer software (ver. 1.4, Thermo Fisher Scientific). In Mascot search, a soybean peptide database (73,320 sequences) obtained from the soybean genome database (Phytozome ver. 9.0, http://www.phytozome.net/soybean) [2] was used for the protein identification. Parameters used in the Mascot searches were as follows: carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da, and peptide charge was set at +2, +3, and +4. The percolator function in the Proteome Discoverer software was enabled.

(b) Protein identification using obtained MS/MS data was performed by Mascot software (ver. 2.4.1, Matrix Science, London, UK). In Mascot search, a soybean peptide database (73,320 sequences) obtained from the soybean genome database (Phytozome ver. 9.0, http://www.phytozome.net/soybean) [2] was used for the protein identification. Parameters used in the Mascot searches were as follows: carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da, and peptide charge was set at +2, +3, and +4. The percolator function was enabled.

(4) Calculation of protein contents in samples

Protein contents in samples were calculated based on emPAI [3] values calculated by Mascot software in the protein identification. The obtained emPAI value for each protein was used for calculation of protein content (mol%) using a formula: emPAI /Σ(emPAI) × 100 [4].

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