Proteomics and purification methods of the target peak

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Abstract

Proteomics was performed to identify key factors involved in the development of symptoms. For surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF MS) analysis, we centrifuged the collected human serum (200 µL) at 21,040 × *g* for 10 min to obtain a supernatant. A volume of 1.8 mL of 50 mM Tris buffer (pH 9.0) was added, followed by cooling on ice for 20 min and centrifugation at 21,040 × g for 10 min. The supernatant was applied to an anion-exchange column Q Sepharose Fast Flow (C/N 17-0510-01; GE Healthcare, Little Chalfont, UK) and washed four times with four column volumes of buffer (pH 9.0) for equilibration. Fractions were eluted at pH 7.0, collected, and then analyzed by SELDI with a cation exchange chip (CM10) to detect the target peaks in the non-adsorbed (pH 9.0) and eluted (pH 7.0) fractions. The purified crude fraction was subjected to chromatographic fractionation. For refinement and identification of target peaks, protein samples of the fractions obtained through purification by reverse-phase high-performance liquid chromatography (HPLC) were dried, digestion buffer was added, and the mixture was incubated at 70 °C for 3 min. Modified trypsin (Promega, Madison, WI, USA) was added, followed by incubation at 35 °C for 5 h. The digested product was subjected to SELDI analysis (NP20 chip). External calibration was conducted for Arg-8-Vasopressin (1,084.25 Da), porcine dynorphin A 209–225 (2,147.5 Da), human ACTH 1-24 (29,335 Da), and bovine insulin b-chain (3,495.94 Da). The trypsin-digested solution was analyzed using liquid chromatography (LC)–tandem mass spectrometry (MS) (Q-TOF Ultima API LC/MS/MS; Waters Micromass, Manchester, UK), followed by a Mascot search. Non-adsorbed (pH 9.0) and elution (pH 7.0) fractions, obtained with the anion exchange column, were adjusted to pH 6.0 with 10 % acetic acid, followed by fractionation using cation-exchange column Q Sepharose Fast Flow (C/N 17-0510-01, GE Healthcare), and washing (equilibration) three times with five-column volumes of buffer (pH 6.0 + OG). The 0.2 and 0.3 M NaCl elution fractions obtained with a cation-exchange column were diluted five times with 0.1 % TFA, then subjected to reverse-phase HPLC (2-mm column), followed by elution using an acetonitrile concentration gradient under the following conditions: column: TSK-GEL, SuperODS (Tosoh Corporation, Tokyo, Japan) (2 × 100 mm); flow rate: 200 µL/min; detection: 210 nm; solvent A: 0.1 % TFA; solvent B: 90 % acetonitrile/0.1 % TFA; gradient: 10–50 % B/5-40 min; fraction: 200 µL/1 min/Fr and 100 µL/1 min/Fr (second chromatography). Fractions obtained by reverse-phase HPLC were diluted five times with acetonitrile and applied to normal-phase HPLC, followed by elution using an acetonitrile concentration gradient under the following conditions: column: TSK-GEL Amide-80 (Tosoh) (2 × 150 mm); flow rate: 200 µL/min; detection: 210 nm; solvent A: 0.1 % TFA; solvent B: 90 % acetonitrile/0.1 % TFA; gradient: 100 %–85 %–65 % B/5-40 min; fraction: 100 µL/0.5 min/Fr. Fractions obtained by normal-phase HPLC were diluted five times with 0.1 % TFA and subjected to micro-reverse-phase HPLC (2-mm column), followed by elution using an acetonitrile concentration gradient under the following conditions: column: TSK-GEL SuperODS (Tosoh) (1 × 50 mm); flow rate: 50 µL/min; detection: 210 nm; solvent A: 0.1 % TFA; solvent B: 90 % acetonitrile/0.1 % TFA; gradient: 10–50 % B/5-40 min; fraction: 50 µL/1 min/Fr. Nine µL of denaturation buffer was added to 1 µL of serum, then mixed and incubated on ice for 10 min. A total of 90 µL protein chip experiment buffer (pH 6.0) was added to the mixture and a further 150 µL of protein chip experiment buffer was separately added to a cation-exchange protein chip

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(CM10), followed by equilibration at 25 °C for 5 min with stirring. These procedures were repeated twice. One hundred µL of each of the above serum samples was added to the protein chip, followed by incubation at room temperature for 30 min with stirring. Then, 150 µL of protein chip experiment buffer (pH 6.0) was added to the protein chip, followed by equilibration at room temperature for 5 min with stirring. These procedures were repeated three times. For rinsing and desalting, 200 µL of Milli-Q water was added to each protein chip, and the procedure was repeated twice. The protein chips were air-dried, and 0.5 µL of saturation energy-absorbing molecule (sinapinic acid) solution was added, followed by air-drying. This was repeated twice, followed by measurement with a protein chip reader. Baseline correction, molecular weight calibration, and normalization were conducted.

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