Cleveland Analysis

(1) Prepared samples are separated by 2D-PAGE. Then the gels are stained with Coomassie brilliant blue, and gel pieces containing protein spots are removed.

(2) The protein is electroeluted from the gel pieces using an electrophoretic concentrator run at 2 W constant power for 2 h. After electroelution, the protein solution is dialyzed against deionized water for 2 d and lyophilized.

a. Cut out stained protein spots from 2D gels and soak in deionized water.

b. Fill 750 μL of electroelution buffer in the 2-mL Eppendorf tube containing protein spots (5–20 gel pieces). Shake for 30 min.

c. Cut 12- to 15-cm-long seamless cellulose tubing (small size, no. 24, Wako, Osaka, Japan) as space is needed for clipping. Fill a 300-mL beaker with 250 mL deionized water, boil it for 5 min, and keep the tubing membrane in it. Wet the small pieces of cellophane film in a small beaker with deionized water.

d. Close the bottom of the small part of cup with a cellophane film, open the bottom of the large part of cup, connect and twist the tubing membrane, and close the distal end by clipping.

e. Fix the cup in the electrophoretic concentrator (Nippon Eido, Tokyo, Japan). Deposit gel pieces containing proteins on the cellophane film (the small part of the cup), and add 750 μL of the electroelution buffer from the Eppendorf tube. Fill the small part of the cup with electroelution buffer, and then fill the large part of the cup with electroelution buffer in such a way that a layer of buffer joins both the cup parts, allowing movement of protein from the small part of the cup to the tubing membrane. Fill the apparatus with electroelution buffer. The small part of cup containing protein spots should be toward the positive side.

f. Run at 2 W constant power for 2 h.

g. Remove the tubing membrane and clip to close the end. Dialyze in a cold room (4°C). Change the deionized water three times on the first day. On the next day, change the deionized water two times.

h. Transfer the protein solution to two to six 2-mL Eppendorf tubes. Freeze-dry overnight.

i. Dissolve the protein in 30 μL of SDS sample buffer (pH 6.8).

(3) The protein is dissolved in 20 μL of SDS sample buffer (pH 6.8) and applied to a sample well of an SDS-PAGE gel. The sample solution is overlaid with 20 μL of a solution containing 10 μL of *Staphylococcus aureus* V8 protease (Pierce, Rockford, IL, USA) (0.1 μg/μL) in deionized water and 10 μL of SDS sample buffer (pH 6.8). Electrophoresis is performed until the sample and protease are stacked in the stacking gel. The power is switched off for 30 min to allow digestion of the protein,

and then electrophoresis is continued.

a. Fix glasses (100 x 140 x 1 mm) with clip, keeping a 1-mm space between the plates.
b. Prepare separating gel solution in a 100-mL beaker. Mix the solutions, and fill the plates about 3 cm from top. (Caution: Pour the solutions into the plates immediately after adding 10% APS and TEMED.)
c. Overlay the separating gel solution with 1 mL water (MQ).
d. Leave the gel for 40 to 60 min at room temperature for polymerization.
e. Remove the overlaid water and pour the following stacking gel solution.
f. Prepare the stacking gel solution in a 100-mL beaker. Mix well, pour on the separating gel, and insert comb.
g. Leave the gel for 20 min at room temperature for polymerization.
h. Take out the comb, clips, and silicon tubes.
i. Clean the wells with a syringe.
j. Fix the gel plates with the apparatus. Pour SDS-PAGE running buffer.
k. Dissolve the protein in 20 µL of SDS sample buffer (pH 6.8), and apply to a sample well in SDS-PAGE. Overlay the sample with 20 µL of a solution containing10 µL of *Staphylococcus aureus* V8 protease with 1 µg/µL in deionized water and 10 µL of SDS sample buffer (pH 6.8). Add 30 µL BPB solution.
l. Electrophoresis was performed until the sample and protease were stacked in the upper gel and interrupted for 30 min to digest the protein.
m. Run the gel at 35 mA until the BPB line reaches about 5 mm near the bottom.
n. Disconnect the electricity, and take out the plates.
o. Separate two plates with a spatula.
p. Separate the stacking gel, and take out the separating gel.