

P4: Development of ELIZA, enzyme-linked immunosorbent assay and BNPP

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Bacillus subtilis natto is well known to secrete several types of proteases outside the cells. In recent years, there have been many reports on studies of the characteristics of *Bacillus subtilis natto*-produced proteases, and the physiological functions, including antithrombotic activity, have attracted attention. A variety of natto and *Bacillus subtilis natto* culture extract food products have been developed. However, there has been no technique of quantifying the ingredients responsible for the physiological functions of these food products (content determination), which is essential for food analysis. A technique for determining the thrombolytic activity is available, but has disadvantages: it needs introduction of a standard enzyme and correction factor because of large variation among the lots of substrate fibrinogen and thrombin. In addition, the determination requires a very small difference of 0.04-0.08 in absorbance, and thus it is difficult to obtain an adequate standard enzyme.

We separated from the purified filtrate of *Bacillus subtilis natto* culture NKCP an ingredient that acts on blood fibrinolysis and coagulation and confirmed that the ingredient is a fragment of bacillopeptidase F. Methods using ELISA and a synthetic colorimetric substrate were developed to determine the content and bacillopeptidase activity of this functional protein produced by *Bacillus subtilis natto* (BNPP).

The determination of bacillopeptidase activity used a commercially available synthetic colorimetric substrate S-2251 for assay of plasmin-like activity (H-D-valyl-L-leucyl-L-lysyl-p-nitroanilide× dihydrochloride). After studying the reaction conditions based on the enzyme kinetics, a method for determining the activity was established: after 5-minute enzymatic reaction at 37°C, p-nitroaniline (pNA) released by bacillopeptidase was measured at a wavelength of 405 nm to determine the bacillopeptidase activity. In this activity determination, the reference standard of p-nitroaniline is easily obtainable, the time required for determination is short, and the absorbance of up to 1.0 at 405 nm linearly correlates with the enzyme concentration.

The quantification of *Bacillus subtilis natto*-produced protein (BNPP) used a sandwich ELISA using rabbit anti-BNPP polyclonal antibody. The absorbance of standard antigen solutions at different concentrations (0-100 ng/ml) was measured at 490 and 620 nm using a micro-plate reader to produce a calibration curve. The content of BNPP was determined from

the calibration curve. These reagents are available as a kit, allowing easy quantification. Validation of this quantification is now under consideration.