HIGH-SENSITIVE FLOW INJECTION ANALYSIS OF PROTEINS COUPLED WITH ON-LINE ALKALINE HYDROLYSIS AND THE FLUORESCENT DERIVATIZATION METHOD

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Sub-nanogram levels of proteins were determined by a flow-injection analysis coupled with an on-line alkaline hydrolysis and o-phthalaldehyde fluorescent derivatization method.

KEYWORDS protein analysis; flow injection analysis; o-phthalaldehyde derivative; protein hydrolysis; fluorescence detection

A rapid and highly sensitive method for quantitatively analyzing proteins was developed using flow injection analysis (FIA). A protein sample was hydrolyzed under alkaline conditions, and the product amino acids were derivatized to their respective fluorescent compounds by the conventional reaction[1] with o-phthalaldehyde (OPA) and 2-mercaptoethanol. Quantitative determination of a sample was made by measuring total fluorescence intensities of the OPA-derivatives of its constituent amino acids. Thus the proposed method was highly sensitive in comparison with the conventional method based on the reaction between free amino groups of proteins and OPA, and was less dependent on the kind of proteins. As the entire process could be carried out in an on-line system, this method proved to be facile and rapid with good reproducibility.

THE FLOW SYSTEM

Conventional hydrolysis of protein has generally been carried out in a sealed tube under strong acidic or alkaline conditions.[2] In this study, the hydrolysis was effected in a flow system instead of a sealed tube under alkaline conditions in which the reaction time was not so long as under acidic conditions and the material of the system was alkaline proof but not acid proof. To produce the same high pressure as in a sealed tube at high temperature, a special device was designed as follows.

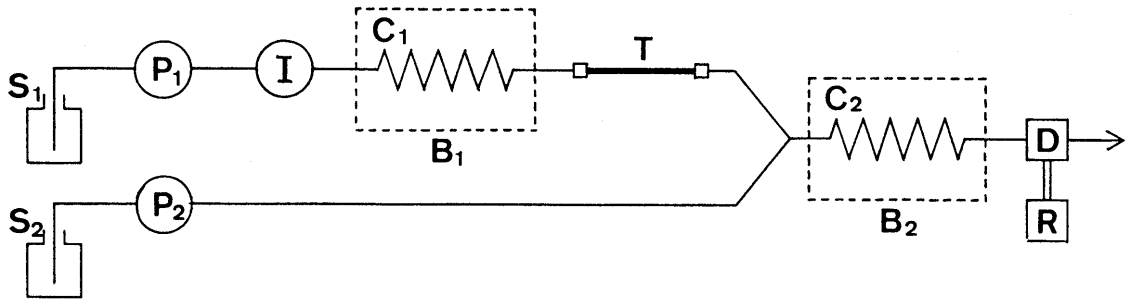

Fig. 1. Block Diagram of the Flow System

S1, 0.5 N KOH carrier solution (flow-rate, 0.2 ml/min); S2, OPA reagent solution in 0.4 M boric acid (flow-rate, 0.6 ml/min); P1, pump for carrier solution; P2, pump for OPA reagent solution; I, sample injector (20 µl); C1, titanium reaction coil for hydrolysis (0.5 mm I.D. x 10 m); B1, dry bath at 160 °C; T, pressure device (0.5 mm I.D. x 250 mm), see text; C2, stainless steel reaction coil for OPA-amino acid reaction (0.25 mm I.D. x 10 m); B2, water bath at 55 °C; D, detector (flow cell, 12 µl), ex. 340 nm, em. 450 nm; R, recorder SIC-7000A integrator (System Instruments Corporation).

A block diagram of a flow system is illustrated in Fig. 1. This system contains two key parts. One is a pressure device, which is made of an alkaline-proof polymeric tube packed with ceramic powder. Twenty-five centimeter of this device generates a pressure of ca. 200 kg/cm^2 at a flow-rate of 0.2 ml/min, and also prevents the carrier solution from boiling in the reaction coil at high temperatures. The other is a reaction coil, which is made of titanium tubing (0.5 mm I.D. x 10 m), in which hydrolysis is effected under vigorous conditions such as strong alkaline solution at high temperature and pressure. Protein samples are passed through 10 m of this coil for 10 min at a flow-rate of 0.2 ml/min.

FLOW CONDITION

The carrier solution used for the hydrolysis was 0.5 N potassium hydroxide. It is expected that the higher the concentration of the alkaline solution, the more rapidly and completely the hydrolysis is accomplished. However, if a high concentration of alkaline solution is used, it is difficult to adjust the pH to an appropriate level for the OPA derivatization reaction after hydrolysis. Also, using a concentration of alkaline that is too high might corrode the flow system. The flow-rate of the carrier solution was controlled to apply the desired pressure to the reaction coil for hydrolysis. In this study, the flow rate was fixed at 0.2 ml/min to produce a pressure of ca. 200 kg/cm^2.

The temperature is also critical in obtaining good hydrolysis efficiency. Although higher temperature is desirable for better efficiency, the carrier solution may boil explosively in a reaction coil at high temperature. This makes the base line unstable. The base-line noise was minimized by adding glycerol to the carrier solution. The glycerol increased the boiling point and the viscosity of the solution (data not shown). In this study, the best result was obtained at 160 °C.

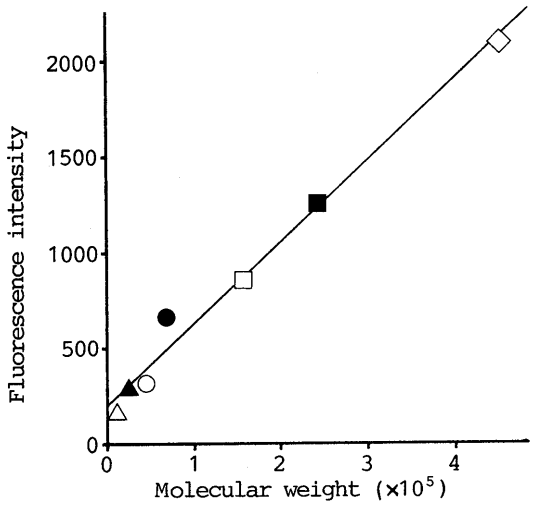

Fig. 2. Relationship between Molecular Weight of Proteins and Fluorescence Intensity

The equimolar amount (20 µl of 1 x 10^{-6} M) of proteins for the molecular weight standard was determined by the FIA method. The regression equation calculated is;

$Y \doteq 437.0\ X + 176.6$ $(r = 0.991)$.

△, cytochrome c; ▲, chymotrypsinogen A; ○, hen egg albumin; ●, bovine serum albumin (BSA); □, aldolase; ■, catalase; ◇, ferritin.

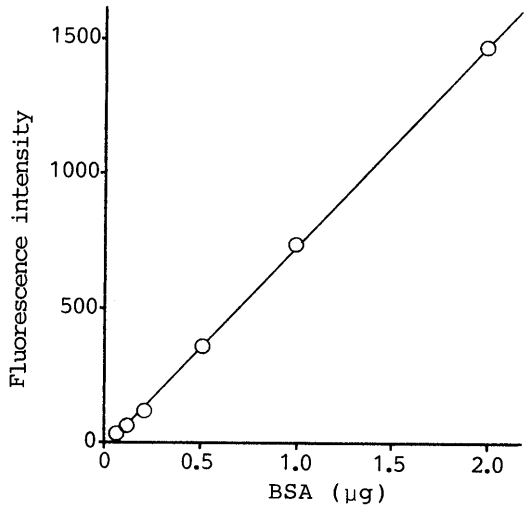

Fig. 3. Working Calibration Curve for BSA

BSA, 0.05—2.0 µg, was determined by the FIA method, each point indicates the mean of five determinations. The regression equation is;

$Y = 740.4\ X - 28.6$ $(r = 0.999)$.

The relative standard deviations are 1.3—5.5% (n = 5) at each point.

The OPA reagent solution was prepared by a conventional method[1] except that the 0.4 M boric acid solution was used without buffer solution. The flow-rate of the OPA solution was adjusted to 0.6 ml/min to get the optimum pH values of 9.5—10.5 for the reaction between OPA and amino acids.

The OPA-amino acid derivatives produced were detected at wavelengths of 340 nm and 450 nm (excitation and emission).

RESULTS AND DISCUSSION

Several standard proteins, combithek® (Boehringer Mannheim GmbH. Biochemica, W. Germany), were determined by a newly designed FIA system. These proteins were calibration proteins for determining the molecular weights of proteins in the range of 12500—450000. Their purity was checked by electrophoresis prior to use. As shown in Fig. 2, a good linear relationship ($r = 0.991$) was obtained between the molecular weight of samples of equimolar amount and the peak heights of the relative fluorescence intensities. It has been reported that the fluorescence intensities of the OPA-derivatives of amino acids vary somewhat among individual amino acids.[1] So it is expected that the fluorescence intensities obtained by this method would also vary among individual proteins. However, as shown above, the proposed method provides constant quantitative data for all kinds of proteins. Apparently the variations in fluorescence intensities associated with individual amino acids are averaged in proteins composed of a large number of amino acids.

As shown in Fig. 3, a calibration line for BSA was found to be linear ($r = 0.999$) in the range of 0.05—2.0 μg of BSA with the detection limit of 25 ng (S/N = 3). The relative standard deviations for 5 repetitive determinations of BSA were 1.3—5.5% at each measured point. A calibration output for the determination of BSA is shown in Fig. 4. The determination for each sample was completed within 18 min, and in multiple determinations, 5—6 samples could be determined per 1 h.

Thus it was shown that the system introduced here could be a potential tool for micro analyses of proteins. As this FIA system could easily be connected with an HPLC system, the combined system would allow the determination of proteins in biological fluids such as serum or urine which contain various OPA-positive substances besides proteins. Finally, the combination of pressure device and proper reaction conditions is applicable to other analytical reactions which require high temperature and pressure.

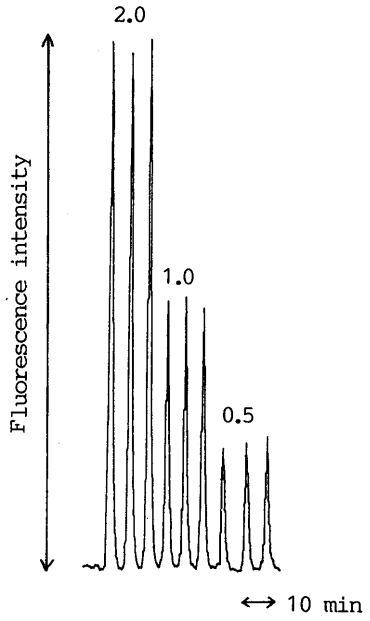

Fig. 4. Typical FIA Signals Obtained by Calibration Study for BSA

0.5—2.0 μg of BSA were determined by triplicate injection.

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