Note

Spectrophotometric Determination of Protein by the Stopped-flow Time Difference Analysis Technique to Enhance the Sensitivity of the Coomassie Brilliant Blue-binding Method

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The dye-binding assay for proteins by Bradford¹⁾ using Coomassie brilliant blue G250 is advantageous over other methods with respects to its simplicity, sensitivity and lack of interference by impurities. On the other hand, one of the disadvantages of this method is the large background interference (about 0.5 OD) coming from the dye, which is often apt to cause serious errors and to restrict the precision and sensitivity in ordinary spectrophotometry.

Stopped-flow apparatus, which is useful for monitoring fast reactions, has been widely used in various fields including analytical chemistry.^{2,3)} Recently, we have developed a novel, fast and sensitive analytical technique using this apparatus, and called it the "stopped-flow time difference analysis" technique (abbreviated to SFTDA technique).⁴⁻⁸⁾ This is based on the analysis of reaction curves monitored by the stopped-flow method, and has the important advantages of speed (less than a few seconds per measurement), high sensitivity and insusceptibility to background interference. Thus, an absorbance change as small as 0.002 OD can be precisely determined even under a heavy background up to about 2.0 OD. An improvement on the biuret method has successfully been achieved by this technique.⁸⁾

In this paper, the potential of the SFTDA technique for enhancing the sensitivity of the Coomassie brilliant blue-binding method, one of spectrophotometric determination methods for protein, is demonstrated by developing a precise and sensitive method for the determinations of bovine serum albumin and urinary total protein, using a combination of the dye-binding method and the SFTDA technique.

A stopped-flow spectrophotometer (Union Giken SF-70) was employed and the light path of the cell used was 10 mm. The dead time of the flow-system was about 2 msec.

Bovine serum albumin (abbreviated to BSA) was purchased from Sigma Chemical Co., and Coomassie brilliant blue G250 was obtained from Aldrich Chemical Co., Inc. All other chemicals were commercially available, and of analytical-reagent grade. Human urine was obtained from a healthy male adult, and was diluted with water up to 10 times its volume immediately before the determination.

To 4 ml of a BSA standard solution or the diluted urine, 1 ml of a mixed solution of 85% H_3PO_4 : ethanol: water = 6:5:9 was added. The resultant solution was kept at 25°C for 30 min before it was transferred into one of the reservoirs of the stopped-flow apparatus. Into the other reservoir, 5 ml of a color-developing reagent (the filtrate obtained through a membrane filter from a mixture of 5 ml of 10 g/l dye-ethanol solution, 6 ml of 85% H_3PO_4 and 89 ml of water) was transferred. The two solutions were rapidly mixed in a 1:1 ratio with the stopped-flow apparatus, and the absorbance change at 595 nm was monitored for 2 sec as a function of time.

Typical examples of the reaction curves obtained for BSA are shown in Fig. 1. BSA concentration could be determined by using the magnitude of absorbance change (ΔA), which was obtained from the reaction curve as the

FIG. 1. Typical Examples of Reaction Curves. BSA concentrations in the initial standard solutions (see the text): A, 2.0; B, 1.0; C, 0.5; D, 0.0 $\mu\text{g/ml}$.

FIG. 2. Typical Examples of Calibration Curves.

Each point represents the mean of three determinations of ΔA by the method recommended in the text. The vertical bars indicate the standard deviations ($\pm$ S.D.).

TABLE I. COMPARISON OF DETECTION LIMITS

Method	Detection limit ^a for BSA ($\mu\text{g/ml}$)
Present	0.06
Bradford ^b	0.4
Lowry ^c	1
Biuret ^d	100
Micro-biuret ^e	15
UV (280 nm) ^f	9
UV (215:225 nm) ^f	1

^a Estimated to be the concentration of BSA in an initial standard solution (see the text), which corresponds to three times the standard deviation in the procedural blank determination.

^b A commercially available kit, "Bio-Rad Protein Assay," was used.

^c Miller's procedure¹⁰⁾ was used.

^d See ref. 11.

^e The method by Itzhaki *et al.*¹²⁾ was used.

^f Ultraviolet absorption method using a single wavelength at 280 nm or dual wavelengths at 215 and 225 nm.¹³⁾

difference in absorbance between the starting time of the reaction (zero sec) and the fixed time of 2 sec after the mixing. The determination and subtraction of a procedural blank (ΔA_0) was not necessary. It was recognized under the recommended conditions that the calibration curve was rectilinear from zero to 10 $\mu\text{g/ml}$ of BSA concentration, as shown in Fig. 2.

In spite of the heavy interfering background, the standard deviation (SD, $n=20$) of the procedural blank was only about 0.0002 OD, which corresponded to about 0.02 $\mu\text{g/ml}$ of BSA. The detection limit for BSA was estimated to be 0.06 $\mu\text{g/ml}$ ($\approx 3 \times \text{SD}$). As shown in Table I, this detection limit is about 1/7 and 1/20 of that of the conventional dye-binding method (the Bradford method¹⁾)

and the widely used Lowry method,⁹⁾ respectively. The coefficients of variation (CV, $n=20$) for 1.0 $\mu\text{g/ml}$ and 5.0 $\mu\text{g/ml}$ of BSA were 2.8% and 0.7%, respectively. The present method was applied to the determination of total protein in urine. When BSA was used as the standard protein, the total protein content in the urine determined by the present method was $129 \pm 0.6 \mu\text{g/ml}$ (CV=0.5%, $n=5$), while that by the conventional dye-binding method, using the commercially available "Bio-Rad Protein Assay" kit, was $132 \pm 5.7 \mu\text{g/ml}$ (CV=4.3%, $n=5$).

Thus, the SFTDA technique has been demonstrated to have great potential for improving the precision and sensitivity of the Coomassie brilliant blue-binding method.

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