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Fluorophotometric Assay of Proteins using Hypochlorite-Thiamine Reagent

Thiamine was found to be oxdized giving thiochrome by N-chlorinated proteins and this fact was applied to the microdetermination of proteins.

Several methods have so far been reported for the fluorometry of proteins. Among these, fluorescamine method[1] and dansyl procedure[2] seem to be of practical value because of their excellent sensitivity and reproducibility. However, fluorescamine is unstable and its reaction with proteins requires rapid mixing and strict observance of reaction conditions. Although dansyl chloride is more stable than fluorescamine, fluorescent hydrolysis product of this reagent should be removed prior to the measurement of fluorescence.[2] Moreover, these two methods are based on the reaction of amino groups whose content differs among proteins to a great extent, and the fluorescence intensity is not necessarily proportional to the amount of protein.

N-Chlorination coupled with starch-iodide reagent[3] or a benzidine-dye[4] has been employed for the detection and determination of peptides and proteins. This method was based on the reaction of peptide groups whose content little deviate among proteins. However, the sensitivity was not satisfactory. Moreover, the color developed by the starch-iodide reaction was sometimes affected by the quality of starch, and benzidine derivatives were reported to be carcinogenetic.[5]

In the present study, thiamine was found to be converted into highly fluorescent thiochrome by oxidation with N-chlorinated proteins, and this finding was applied to the assay of proteins.

Hypochlorite Reagent: Commercial NaOCl solution (Antiformine®, Koso Chemical Co. Ltd.) is diluted with 125 mM $NaHCO_3$ and made to 0.1 w/v % with respect to available chlorine.

Assay Procedure: The reaction mixture is kept at room temperature throughout the procedure, and mixed vigorously with TB-1 Automatic Mixer immediately after every addition of the reagent. To 0.5 ml of a sample solution containing 1—10 μg/ml of protein is added 0.4 ml of hypochlorite reagent and the resulting mixture is left standing for 30 min. In order to destroy excess reagent, 0.2 ml of 2 w/v % $NaNO_2$ in 250 mM $NaHCO_3$ is added and the solution is again left standing for 30 min. Then 0.2 ml of aqueous 20 μg/ml solution of thiamine-HCl is added and the reaction mixture is allowed to stand for 10 min for the development of fluorescence. To the mixture is finally added 2 ml of isopropanol, and the fluorescence intensity is measured at excitation and emission wavelengths of 370 nm and 430 nm, respectively, against a reagent blank.

Table I summarizes the interferences. NaCl, guanidine, glucose, ascorbic acid did not affect the assay. Tris buffer, which affects the Lowry's method seriously, did not interfere with the thiamine method. High concentration of urea increased the fluorescence. As expected from the principle, amino acids, peptides, barbital, creatinine, histamine, adenosine triphosphate (ATP) and desoxyribonucleic acid (DNA) interfered with the present method.

Figure 1 illustrates the standard curves for ovalbumin, bovine serum albumin (BSA), lysozyme, and α-chymotrypsin. The concentration of the proteins plotted as abscissa was pre-determined by the Kjeldahl method. All the curves were linear in the range of final

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Table I. Relative Fluorescence Intensity of BSA (final concentration: 781 ng/ml) in the Presence of Several Compounds

Compounds	Concentration	Fluorescence intensity
None		50.5
+NaCl	75 μg/ml	54.0
+Guanidine-HCl	75 μg/ml	50.5
+Tris	75 μg/ml	55.5
+Glucose	75 μg/ml	50.5
+Ascorbic acid	75 μg/ml	50.5
+Urea	750 ng/ml	50.5
	7.5 μg/ml	57.0
+Glycine	750 ng/ml	51.5
	7.5 μg/ml	75.5
+L-Alanine	750 ng/ml	52.0
	7.5 μg/ml	75.5
+L-Tyrosine	750 ng/ml	60.5
	7.5 μg/ml	288.0
+Glycylglycylserine	750 ng/ml	57.4
+Barbital	750 ng/ml	79.5
	7.5 μg/ml	287.5
+Creatinine	750 ng/ml	95.0
+Histamine	750 ng/ml	308.0
+ATP	750 ng/ml	136.5
+DNA	750 ng/ml	73.0
	7.5 μg/ml	233.0

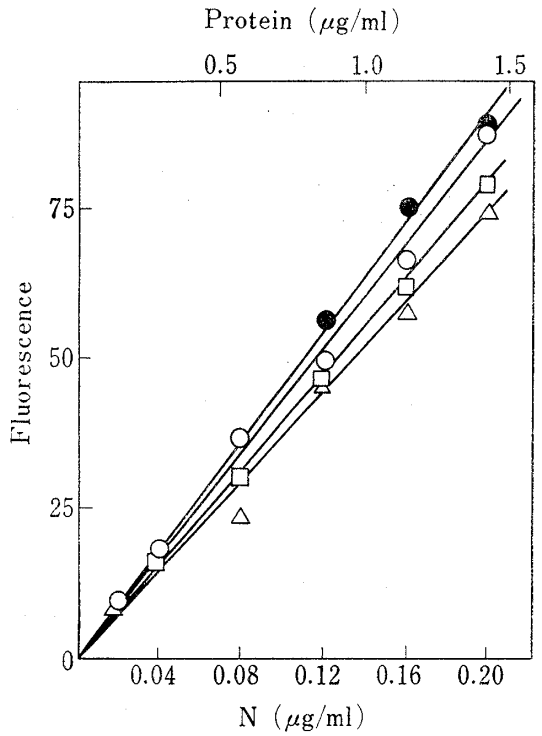

Fig. 1. Standard Curves for Ovalbumin (●), BSA (○), Lysozyme (□), and α-Chymotrypsin (△)

The numbers on the abscissa indicates the final concentration of nitrogen and protein as determined by Kjeldahl method.

concentration of 0.14 to 1.4 μg/ml as protein and passed through the origin. Coefficients of variation ($n=7$) for 0.5 μg/ml of these proteins were as follows: ovalbumin, 1.4%; BSA, 1.7%; lysozyme, 1.0%; α-chymotrypsin, 2.6%.

Figure 1 indicates that the present method is highly sensitive. This figure also demonstrates that the slopes of the standard curves for all four proteins differ only slightly each other. This may be due to the principle based on the reaction of peptide groups. Greater differences were observed among the slopes of the standard curves for these proteins by the dansyl procedure.[2] Kjeldahl method is still regarded as the standard assay procedure for proteins since it gives invariant values for most of proteins because it is based on the nitrogen content. However, the Kjeldahl method is too tedious and time consuming for the routine use. The present method is simple than the Kjeldahl method and suitable for the determination of numerous samples at one time since no special equipment is required. It is also expected to be appropriate for the determination of the specific activity of enzymes in the course of their purifications.

In addition, thiamine seems to be applicable to the analysis of wide range of amides, ureides and peptides; this is now under investigation.

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