and the residue was dissolved in MeOH (50 ml). Treatment of the solution with ammonia for 1 hr under ice cooling, followed by concentration gave the barium salt of sucrose 1′,6′,6-trisulfate (1.8 g) as an amorphous powder from EtOH. Treatment of the barium salt on a column of Amberlite IR-120 (Na^+) as described above gave the sodium salt of sucrose 1′,6′,6-trisulfate (1.38 g) as an amorphous powder from EtOH; the product contained a small amount of disulfate, detectable by paper electrophoretic analysis.[6] The analytical values are given in Table I.

[Chem. Pharm. Bull. 28(2) 641—644 (1980)]

A Novel Method for the Fluorometric Assay of Proteins using Hypochlorite-Thiamine Reagent[1]

Toshio Kinoshita,[2a] Jun-ichiro Murayama,[2b] Kazuyo Murayama[2c] and Akio Tsuji[2b]

School of Pharmaceutical Sciences, Kitasato University,[2a] School of Pharmaceutical Sciences, Showa University,[2b] and Yuki gosei Kogyo Company Ltd.[2c]

(Received May 18, 1979)

A fluorometric assay method for proteins based on the reaction of peptide groups is described. The N-H groups of peptides are chlorinated with sodium hypochlorite, and the resulting N-chloropeptides are allowed to react with thiamine, giving fluorescent thiochrome. The present method is highly sensitive and is applicable to 1—10 μg of protein. Little variability of fluorescence intensity was observed among proteins.

Keywords——protein, fluorometric determination; peptide, N-chlorination; fluorometric determination, protein; thiamine; thiochrome; sodium hypochlorite; determination of protein, fluorescence

Recent progress in fluorometry has made possible the ultramicroanalysis of proteins by the fluorescamine method,[3] the O-phthaldialdehyde method[4] and the dansyl chloride method.[5] This method are sensitive and simple, but they are based on the reaction of amino groups and are therefore affected by the content of amino groups in proteins.

In 1962, Mazur *et al.*[6] proposed a method for the detection of peptides through N-chlorination, followed by the oxidation of benzidine by the N-chloropeptides to give a blue color. This method was modified by Sandford *et al.*[7] for the determination of amides and peptides using amylose-iodide reagent, which is less hazardous than benzidine.[8] Since this method is based on the reaction of N–H groups, the fluorescence intensity reflects the amount of peptide groups. Thus, Sandford's method is less influenced by various amino acid residues,

1) This paper constitutes part VIII of a series entitled "Microanalysis of Proteins and Peptides." Preceding paper, Part V: Ref. 10. A preliminary account of this work has been presented: T. Kinoshita, J. Murayama, and A. Tsuji, *Chem. Pharm. Bull.*, **24**, 2901 (1976).

2) Location: *a) 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan; b) 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142, Japan; c) 3-37-1, Sakashita, Itabashi-ku, Tokyo 174, Japan.*

3) *a)* S. Udenfriend, S. Stein, P. Böhlen, W. Leimgruber, W. Dairman, and M. Weigele, *Science*, **178**, 871 (1972); *b)* S. Stein, P. Böhlen, W. Dairman, and S. Udenfriend, *Arch. Biochem. Biophys.*, **155**, 202 (1973); *c) Idem, ibid.*, **155**, 213 (1973).

4) M. Roth, *Anal. Chem.*, **42**, 880 (1971).

5) T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.*, **22**, 2413 (1974).

6) R.H. Mazur, B. Ellis, and P.S. Cammerata, *J. Biol. Chem.*, **237**, 1619 (1962).

7) P.A. Sandford, A.J. Nafziger, and A. Jeanes, *Anal. Biochem.*, **42**, 422 (1971).

8) D.P. Griswald and J. Weisburger, *Cancer Res.*, **28**, 924 (1968).

whereas Lowry's method[9] is affected by thyrosine. However, the sensitivity was poor, because of the color reaction involved.

We have recently reported a method for the assay of proteins using thiamine, which gives intense fluorescence on oxidation by N-chloropeptides.[10] In this procedure, proteins were spotted on a thin layer plate, which was sprayed with sodium hypochlorite solution. Excess hypochlorite was then removed by aeration, and thiamine solution was sprayed to develop fluorescence. Interfering materials could be readily removed by developing the plate with NaCl solution prior to the assay. Although 0.1 μg/sample of protein could be determined by this method, this amount should be contained in a volume of 1 μl, and dilute sample solutions must be concentrated.

In the present study, this fluorescence reaction was applied for the estimation of proteins in aqueous solution, since there is a growing demand for the accurate measurement of extremely dilute protein solutions in the fields of biochemistry and clinical chemistry.

Experimental

Materials——Aqueous NaOCl (Antiformine^R) was obtained from Koso Chemical Co. The concentration of available chlorine in this solution was accurately determined by iodometric titration. Isopropanol, thiamine hydrochloride, and $NaHCO_3$ of reagent grade were purchased from Wako Pure Chemical Industries. Thiamine hydrochloride was recrystallized from acetone and water before use. Thiochrome was prepared according to the procedure of Risinger and Parker.[11] Bovine serum albumin (BSA, fraction V), bovine chymotrypsin, egg white lysozyme (6 times recrystallized) and ovalbumin were purchased from Wako Pure Chemical Industries, Miles Laboratries Ltd., Seikagaku Kogyo Co., and Nutritional Biochemical Corporation, respectively. Glass-distilled water was used throughout the experiments.

Instrument——Fluorescence spectra and fluorescence intensity were measured with a Hitachi MPF-2A fluorescence spectrophotometer. Quinine sulfate solution (100 mM in 0.1 N H_2SO_4) was used as a standard of fluorescence intensity.

Hypochlorite Reagent——Commercial NaOCl solution was diluted with 125 mM $NaHCO_3$ and made up to 0.1 w/v % with respect to available chlorine. This reagent should be prepared fresh daily.

Nitrite Reagent——$NaNO_2$ (2 g) was dissolved in 250 mM $NaHCO_3$ to make 100 ml.

Thiamine Reagent——Aqueous thiamine hydrochloride (20 μg/ml) was used.

Assay Procedure——Immediately after each addition of reagent, the solution was mixed vigorously with a TB-1 automatic mixer.

The hypochlorite reagent (0.4 ml) was added to 0.5 ml of the sample solution containing 1—10 μg/ml of protein, and the mixture was left to stand for 30 min. In order to destroy excess hypochlorite, 0.2 ml of nitrite reagent was added and the mixture was again left to stand for 30 min. Next, 0.2 ml of thiamine reagent was added, and the reaction mixture was allowed to stand for 10 min for the development of fluorescence. Finally, 2 ml of isopropanol was added, and the fluorescence intensity was measured at excitation and emission wavelengths of 370 nm and 430 nm, respectively, against a reagent blank.

Results

The reaction conditions were examined using 781 ng/ml (final concentration) of BSA. Figure 1 shows the excitation and emission spectra of the fluorophor produced from BSA by this assay procedure. The excitation and emission maxima were at 370 nm and 430 nm, respectively. These are identical with those of thiochrome. The fluorescence intensity showed a maximum in the available chlorine range of 0.1 to 0.3% in the hypochlorite reagent. Table I summarizes the effect of temperature on the N-chlorination. The fluorescence reached its maximum 30 min after N-chlorination at room temperature. The excess available chlorine was completly destroyed by addition of 2 w/v% $NaNO_2$ in 250 mM $NaHCO_3$. More concentrated $NaNO_2$ decomposed N-chloropeptides, and more dilute reagent resulted in a higher blank value. The reaction conditions for N-chlorination in the assay were selected on the

9) O.H. Lowry, N.J. Rosengrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) T. Kinoshita, F. Iinuma, K. Atsumi, and A. Tsuji, *Anal. Biochem.*, **77**, 371 (1977).
11) G.E. Risinger and P.N. Parker, *Experientia*, **21**, 305 (1965).

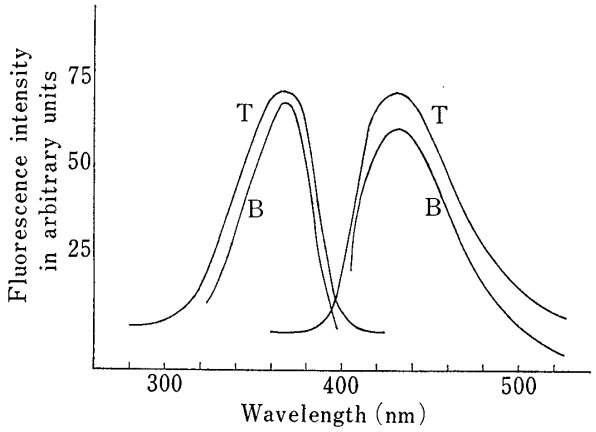

Fig. 1. Fluorescence Excitation and Emission Spectra of BSA (B) Reacted with the Reagents and of Thiochrome (T) Mixed with the Reagent Blank

Fluorescence intensities were adjusted for convenience in comparing the shapes of the spectra.

Table I. Effect of Temperature of Reaction of BSA (781 ng/ml) with Hypochlorite Reagent on the Fluorescence Intensity

Temperature (°C)	Relative fluorescence intensity
2	46.2
20	49.0
37	42.5
70	5.0

basis of the above results. The fluorescence intensity reached a plateau at thiamine concentrations between 10 μg and 30 μg/ml. When a 20 μg/ml thiamine solution was used, the maximum fluorescence was reached in 5 min. Although the fluorescence intensity decreased from 15 min after the reaction, addition of isopropanol stabilized it. The fluorescence was constant for 24 hr after the addition of isopropanol. Isopropanol was also found to enhance the fluorescence intensity by a factor of 6.0. The procedure for the development of fluorescence was therefore chosen on the basis of the above results.

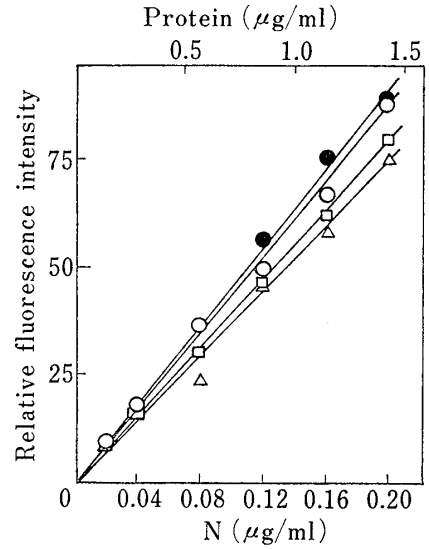

Fig. 2. Standard Curves for Ovalbumin (●), BSA (○), Lysozyme (□), and α-Chymotrypsin (△)

The numbers on the abscissa indicate the final concentration of protein (top) or nitrogen (bottom) as determined by the kjeldahl method.

Table II. Relative Fluorescence Intensity of BSA (final concentration; 781 ng/ml) in the Presence of Various Compounds

Compound	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	50.0
	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

Figure 2 shows the standard plots for BSA, lysozyme, chymotrypsin, and ovalbumin. The concentration of the proteins (abissa) was determined by the Kjeldahl procedure. All the plots were linear in the range of final protein concentration of 0.14—1.4 μg/ml, and passed through the origin. The four lines showed similar slopes. The coefficients of variation for 0.5 μg/ml of BSA, chymotrypsin, lysozyme, and ovalbumin were 1.7%, 2.6%, 1.0% and 1.4%, respectively ($n=9$).

Interference by various compounds was examined by measuring fluorescence intensity (Table II). Sodium chloride, guanidine-HCl, tris (hydroxymethyl)-aminomethane, or glucose at a concentration of 750 μg/ml had practically no effect on the intensity. Urea gave weak fluorescence at 75 μg/ml. Amino acids, peptides, barbital, creatinine, histamine and ATP interfered with this method. These compounds may be chlorinated under the present reaction conditions.

Discussion

Thiamine was converted into highly fluorescent thiochrome upon oxidation with N-chlorinated proteins. This was confirmed by the identity of the excitation and emission spectra of the oxidation product with those of synthetic thiochrome (Fig. 1).

In the present method (the thiamine method), the sensitivity was considerably improved and the reaction time was reduced compared with Sandford's method.

Table III. The Contents of Protein Determined by the Kjeldahl, Lowry, and Thiamine Methods, together with the Lowry/Kjeldahl and Thiamine/Kjeldahl Ratios

	The content of protein in μg per mg of sample determined by			Ratio of the results of different methods	
	Kjeldahl method	Lowry method[a)]	Thiamine method[a)]	Lowry Kjeldahl	Thiamine Kjeldahl
BSA	903	9.3	903	1.00	1.00
Lysozyme	944	1291	840	1.37	0.89
Chymotrypsin	858	1165	786	1.36	0.92
Ovalbumin	854	876	912	1.03	1.07

a) BSA (781 ng/ml) was used as a standard.

Table III summarizes the protein values obtained by the thiamine method and Lowry's method, in comparison with those by the Kjeldahl method. The results indicate that the thiamine method gives protein values in good agreement with the results of the Kjeldahl-method, whereas Lowry's method gives rather different values. This may be because the thiamine method is based on the reaction of peptides and is less affected by particular amino acid residues, whereas Lowry's method is influenced by the tyrosine content of proteins. In our previous report,[10] the estimation of proteins on a thin layer plate showed some variability even though its principle is essentially the same as that of the present method. This may be due to differences in the mode of adsorption of proteins on the thin layer. This shortcoming was overcome in the present method.

Although thiochrome is further oxidized by N-chloride to give a non-fluorescent product, the addition of isopropanol greatly stabilized the thiochrome. This solvent also enhanced the fluorescence intensity of thiochrome.

The present method should be useful for the accurate estimation of proteins during their purification.