[Chem. Pharm. Bull. 28(12)3696—3698(1980)]

A Simple Fluorescent Post-labeling Technique with *o*-Phthalaldehyde for the Analysis of Proteins by Polyacrylamide Gel Electrophoresis

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(Received June 28, 1980)

A new polyacrylamide gel electrophoretic system with post-labeling of proteins with *o*-phthalaldehyde (OPT) has been developed. In the present system, the widely used tris, glycine and ammonium persulfate were replaced by triethanolamine, L-hydroxyproline and sodium persulfate, respectively. Proteins were visualized by dipping the gels in the OPT reagent for several minutes immediately after electrophoresis, and the gels were then scanned in a fluorescence densitometer. Fifty nanograms of bovine serum albumin could be detected by this technique.

Keywords——proteins; polyacrylamide gel electrophoresis; fluorometry; post-labeling; *o*-phthalaldehyde; triethanolamine; L-hydroxyproline

Polyacrylamide gel electrophoresis has been widely used for the separation and determination of proteins since its introduction into the field of biochemistry by Ornstein[2)] and Davis.[3)] Various dyes and fluorescent compounds have been employed for the visualization of proteins in the gels. In the pre-labeling method, the physico-chemical proterties of proteins are altered by fluorescence labeling, generally by means of reactions of the free amino, carboxyl or hydroxyl groups of the proteins. Thus, it remains desirable to develop post-labeling methods in order to determine intact proteins. Some very sensitive methods have already been developed for pre-labeling of proteins using fluorescamine,[4)] dansyl chloride[5)] or *o*-phthalaldehyde (OPT).[6)] On the other hand, post-labeling methods using silver-staining[7)] or fluorescamine[8)] include time-consuming procedures in spite of their high sensitivity. A simple method using anilinonaphthalene sulfonate[9)] provides insufficient sensitivity.

In this communication, a post-labeling method using OPT is described that is faster and more sensitive than the existing methods.

Materials and Methods

Acrylamide, N,N′-methylenebisacrylamide (BIS), sodium persulfate, and Coomassie brilliant blue R-250 were obtained from Nakarai Chemicals, Ltd., Kyoto. L-Hydroxyproline and β-mercaptoethanol were purchased from Wako Pure Chemical Industries, Ltd., Osaka. *o*-Phthalaldehyde (OPT) was a product of Tokyo Chemical Industry Co., Ltd., Tokyo. Bovine serum albumin (BSA, Cohn Fraction V) was obtained from Daiichi Pure Chemicals Co., Ltd., Tokyo. Triethanolamine (Koso Chemical Co., Ltd., Tokyo) was distilled twice under reduced pressure under nitrogen, and kept in a sealed tube under nitrogen until use.

Densitometric scanning was performed with a Helena FLUR-VIS auto scanner (Helena Laboratories, Beaumont, Texas, U.S.A.).

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Preparation of Gels——Stock solutions used are listed in Table I. Gels were prepared in glass tubes (5 mm × 70 mm) by mixing the stock solutions in the proportions shown in Table II. Other general procedures for the preparation of gels were conventional ones such as those described by Shuster.[10] Gelation times for running gels and spacer gels were about 50 and 55 min, respectively, at room temperature.

Table I. Stock Solutions

A	1 M HCl	48.0 ml
	Triethanolamine	50.0 ml
	Water	to 100.0 ml
	pH 8.9	
B	1 M HCl	48.0 ml
	Triethanolamine	8.0 ml
	Water	to 100.0 ml
	pH 6.8	
C	Acrylamide	30.0 g
	BIS	0.8 g
	Potassium ferricyanide	70.0 mg
	Water	to 100.0 ml
D	Acrylamide	10.0 g
	BIS	2.5 g
	Water	to 100.0 ml
E	Sodium persulfate	146.5 mg
	Water	to 100.0 ml

Table II. Working Solutions

Running gel		Spacer gel		Electrode buffer	
A	1 part	B	1 part	1 M Triethanolamne	5 ml
C	2 parts	D	2 parts	1 M L-Hydroxyproline	116 ml
E	4 part	E	5 parts	Water	to 1000 ml
Water	1 part			pH 8.1	

Preparation of OPT Reagent——The OPT reagent developed for liquid chromatography[11] was used with slight modifications. OPT (40 mg) was dissolved in 1 ml of 95% ethanol, then 0.2 ml of β-mercaptoethanol and an appropriate amount of 0.4 M borate buffer (pH 9.7) to make a final volume of 25 ml were added. This reagent should be prepared just prior to use.

Electrophoresis, Staining and Densitometry——Electrophoresis was carried out at 1 mA per tube for about 70 min. The gels were removed from the glass tubes as soon as electrophoresis was over, and immersed in the OPT reagent for 8 min at room temperature. The stained gels were scanned immediately with a fluorescence densitometer.

Results and Discussion

Since OPT fluoresces by reacting with primary amines, the reagents used in the Davis system,[3] tris, glycine and ammonium persulfate, had to be replaced by non-primary amino compounds. The reagents chosen for this purpose were triethanolamine, L-hydroxyproline and sodium persulfate. Potassium ferricyanide was added to running gels to adjust the gelation time, as triethanolamine, in the presence of persulfate, accelerates the polymerization of acrylamide too much.

Running gels prepared in the presence of potassium ferricyanide (5.3 mM, final concentration) were gelated in about 50 min at room temperature. On the other hand, a similar gelation time (about 55 min) was obtained for spacer gels even in the absence of riboflavin and

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potassium ferricyanide, since the concentration of triethanolamine in the spacer gels was lower.

It was found that the higher the concentration of OPT, the faster the fluorescence reaction of proteins in the gel, and that β-mercaptoethanol increased the sensitivity considerably. However, higher concentrations of these reagents increased the background fluorescence as well. Thus, the concentrations of OPT and β-mercaptoethanol chosen for the present system were 2 and 4 times those in the OPT reagent used for liquid chromatography,[11] respectively.

The time course of the fluorescence reaction between proteins in the gel and the OPT reagent is shown in Fig. 1. Fluorescence was measured with a densitometer after immersing the gels in the OPT reagent for various times. It was found that the relative fluorescence was highest at around 8 min. The decrease in relative fluorescence after the peak was due to a gradual increase in the background fluorescence. The reason for this increase in the background is unknown at present. Thus, it was decided that for routine analyses the gels would be reacted with the OPT reagent for 8 min and subjected to densitometry immediately after staining.

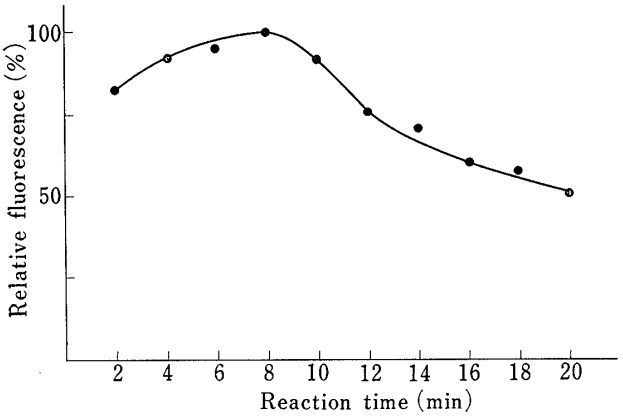

Fig. 1. Time Course of the Fluorescence Reaction between *o*-Phthaladehyde and Proteins in the Gel

One μg of BSA was electrophoresed and gels were immersed in the OPT reagent immediately after electrophoresis. After the indicated periods, the fluorescence at the position corresponding to BSA monomer (the highest peak) was measured with a densitometer. The results are expressed as relative intensities of fluorescence with intensity obtained at 8 min as 100%.

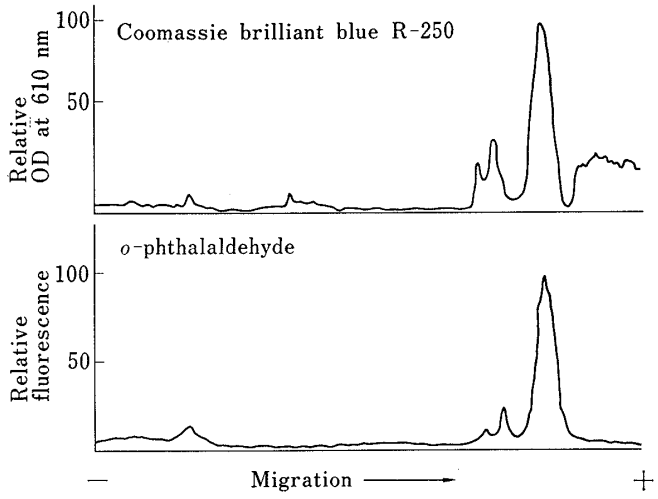

Fig. 2. A Comparison of OPT and Coomassie Brilliant Blue R-250 Staining of BSA

One μg of BSA was electrophoresed as described in "Materials and Methods." Proteins in the gel were first stained with OPT and scanned with a fluorescence densitometer (lower), and they were then stained with Coomassie brilliant blue R-250 and scanned at 610 nm (upper). Both densitograms were obtained by using the auto gain mode, which automatically adjusts the intensity of the highest peak (BSA monomer) to 95% of the full scale.

The resolution of electrophoresis by the proposed system was examined with 1 μg of BSA as a reference protein. The gels after electrophoresis were first examined by the fluorometric procedure as described above and then by a conventional Coomassie brilliant blue staining method. As shown in Fig. 2, the results with the OPT method were comparable to those with the Coomassie blue method. These results also indicate that the resolving power of the proposed system is comparable to that of Davis' original system (data not shown) as judged from the resolution between the monomer and oligomers of BSA.

The limit of detection by this new fluorometric method was found to be 50 ng of BSA per gel (this means that less than 50 ng of proteins could be detected, because the BSA preparation used in the above experiments was a mixture of monomer and oligomers).