[Regular paper]

Rapid staining of proteins in back-supported polyacrylamide gel by peroxidative action of protein-adsorbed hemin

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SUMMARY

This paper presents a new method, hemin stain, for rapid staining of proteins separated electrophoretically in polyacrylamide gel backed with a glass plate. It was found that proteins bound to hemin act as an artificial peroxidase and stain themselves by catalyzing oxidative coupling of two dye precursors in the presence of H_2O_2 . This hemin stain made the conventional destaining procedure unnecessary. The staining procedure required only 45 min with 3, 3'-diaminobenzidine and 2, 6-xyleneol used as the dye precursors. This paper compares the present method with the conventional staining method employing Coomassie brilliant blue R-250.

Key words: gel electrophoresis, rapid protein stain, hemin adsorption, artificial peroxidase.

INTRODUCTION

Proteins separated in polyacrylamide gel (PAG) by electrophoresis are usually stained with Coomassie brilliant blue R-250 (CBB). This staining is, however, a time consuming process, since it requires lengthy destaining of the background darkened with the diffusing dye molecules that aggregate in the gel matrix. Prolonged time is necessary especially for destaining of the gel backed with a supporting plate, such as one made of glass. On the other hand, the back-supporting of the gel is desirable since it prom-

ises easy electrophoretic and staining operations.

This paper presents a new method for rapid protein staining, hereafter referred to as the hemin stain, whereby proteins separated in PAG are converted into an artificial peroxidase by means of hemin adsorption. It was found that the separated proteins themselves are stained by catalyzing rapid oxidative coupling of 3,3'-diaminobenzidine (DAB) and 2,6-xyleneol in the presence of hemin and H_2O_2 . This staining method requires no destaining. A preliminary report on the hemin stain has been published previously¹⁾.

裏打ちされたポリアクリルアミドゲル中の蛋白質の、ヘミンのペルオキシダーゼ活性を利用した高速染色法。

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MATERIALS AND METHODS

The standard hemin staining procedure developed in the present study is as follows. Protein-containing PAG (thickness 0.5 mm, total PAG concentration 5 %, bis-acrylamide 0.55 %, which was backed with a siliconized glass plate according to Radola²⁾, was treated at 25 °C for 10 min with 2.5×10^{-4} M hemin (Tokyo Kasei) in a 0.07 M phosphate buffer of pH 7.5 containing 50 % methanol. The gel was then rinsed twice for 10 min each with the same buffer without hemin to remove the background and excess hemin. Following this hemin adsorption pretreatment, the gel plate was immersed for 15 min at 40 °C in a development buffer of pH 7.5 containing 1.25 mM DAB (Kantochemical), 2.5 mM 2,6-xylenol (Tokyo Kasei) and 27 mM H_2O_2 to develop blue-black color. The buffer volumes for the hemin treatment, rinsing and development were the same and were 20 times the volume of the gel matrix. Methanol was added to ensure the fixation of the proteins in the gel.

Unless otherwise noted, sample gels for staining were made not by electrophoretic separation of proteins in the gel but by polymerizing monomeric solutions of acrylamide containing ovalbumin (Sigma), bovine serum albumin (Sigma), human transferrin (Sigma), bovine thyroglobulin (Pharmacia), *Canavalia ensiformis* urease (Toyobo), horse ferritin (Miles Lab.) and ovalysozyme (Worthington) at various concentrations. The absorbance of the colored gel was measured with a Hitachi 220A spectrophotometer at 555 nm, the wavelength of maximum absorption by the dye formed by the oxidative coupling of DAB and xylenol.

Gel plates were also stained with CBB (Merck, concentration 0.1 %) for 5 hrs at 25 °C in a solution containing 7 % acetic acid and 50 % methanol. The plates were then destained at 60 °C for 7 hrs. Staining intensity of the is CBB stain was compared with that of the hemin stain.

Gel plates colored by the hemin and CBB stains were scanned with a Rhesca photodensitometer

model PPA-250A (Rhesca) with a beam size of $200 \mu\text{m} \times 200 \mu\text{m}$ to measure the local fluctuation of the absorbance value.

High-resolution two-dimensional (2D) electrophoresis without denaturing agents³⁾ was performed as previously described¹⁾ using two gel plates both of which were backed with siliconized glass plates. Isoelectric focusing for the first dimensional separation was run at 3200 volthours at 4 °C using a gel plate ($2 \times 75 \text{ mm}^2$, thickness 0.5 mm, total PAG concentration, 3 %) containing 2 % LKB ampholine. Following the focusing, the gel plate was placed in a groove made on another gel plate ($9 \times 9 \text{ cm}^2$, thickness 0.5 mm) having a PAG concentration gradient of 4 to 20 %. The second dimensional electrophoresis for the molecular sieving was then run at 950 volthours at 4 °C.

Protein samples for the 2D electrophoresis were prepared from *E. coli* C600 cells cultured in an M9CA medium for 13 hrs at 37 °C as described previously⁴⁾. The cells washed with 0.15 M NaCl were ruptured by sonicating three times for 2 min each in an ice-cold 0.1 M Tris-HCl buffer of pH 8.5. The centrifugal supernatant ($3 \mu\text{l}$) obtained from 2×10^8 cells was applied to the 2D electrophoresis.

RESULTS

The time course of the hemin stain color development is shown in Fig. 1. As is clear from the figure, the color development was very rapid and was completed within 15 min of the oxidative coupling reaction of DAB and xylenol. The sample gel for Fig. 1, which contained ovalbumin, had been pretreated for 30 min prior to the addition of H_2O_2 as already described. The total time required for the stain was thus approx. 45 min. On the other hand, the conventional CBB staining of the gel backed with a glass plate required approx. 12 hrs to produce the same absorbance value.

It was also found that the staining intensity of the hemin stain depended on the hemin concentration during the adsorption pretreatment. The intensity increased as the hemin concentration increased (Fig.

2). It should also be noted, however, that the intensity for the control gel without protein also increased with the hemin concentration. The absorbance values given in Fig. 2 and in the other figures presented in this paper were obtained by subtracting

Fig.1. Time course of the hemin stain color development.

A PAG plate with ovalbumin at 50 ng/mm² was pretreated using hemin, DAB and xylenol. Color development following addition of H₂O₂ was measured spectrophotometrically at 555 nm against time.

Fig.2. Dependency of the hemin stain on hemin concentration.

PAG plates containing ovalbumin at 50 ng/mm² were pretreated with hemin at various concentrations and were then developed with DAB+xylenol+H₂O₂.

the background from the observed total absorbance. The ratio of the signal presented in Fig. 2 to the background was 3.2 at 2.5×10^{-4} M of hemin at an ovalbumin concentration of 50 ng/mm² of gel surface.

The hemin stain intensity showed a temperature dependency. The temperature of the development buffer was varied from 20 °C to 50 °C, and the absorbance of the gel was measured following 30 min of oxidative coupling. As shown in Fig. 3, a maximal stain was obtained at 40 °C for the three proteins tested.

The pH profile of the hemin stain was examined by changing the pH value of the development buffer containing DAB, xylenol and H₂O₂. The hemin pretreatment of the gel was done at pH 7.5, and the absorbance at 555 nm was measured after 30 min of the development at 40 °C. The pH value of the development buffer was readjusted to pH 7.5 just prior to the absorbance measurement, since the absorption spectrum of the dye formed by the coupling reaction depended on the pH value of the

Fig.3. Dependency of the hemin stain on the buffer temperature during color development.

PAG plates having ovalbumin at 25 ng/mm² (curve A), bovine serum albumin at 25 ng/mm² (curve B) and thyroglobulin at 50 ng/mm² (curve C) were pretreated at 25 °C with hemin. The PAG plates were then treated with DAB+xylenol+H₂O₂ at various temperatures.

surrounding medium. In this experiment using ovalbumin at 50 ng/mm², the optimal pH value of the stain was found to be pH 7.5.

The relationship between the concentration of protein and the staining intensity of hemin stain was measured using ovalbumin. As presented in Fig. 4 (curve A), a linear relationship was observed when the ovalbumin concentration was below 50 ng/mm². A similar experiment was made using CBB, and the relationship was also given in Fig. 4 (curve B) for the purpose of comparison. It is clear that the hemin and CBB stains give similar staining intensities below 50 ng/mm² of ovalbumin. The detection limit of hemin stain was estimated from the linearity in the figure and the local fluctuation of the absorbance value observed by scanning densitometry. The scanning of the gel plate having ovalbumin at 50 ng/mm² gave an absorbance fluctuation of approx. 0.02. The detection limit was then estimated to be 5 ng/mm² which was the protein concentration corresponding to 0.04 absorbance units. The CBB stain gave a similar detection limit.

It may be deduced that the staining intensity of

Fig.4. Relationship between protein concentration and staining intensity.

PAG plates with ovalbumin of various concentrations were stained by hemin (curve A) and CBB (curve B) stains.

hemin stain depends on the molecular species of the protein to be stained, since the proteins act as chemical catalysts. To estimate this dependency, we measured the staining intensity of seven different proteins, *i. e.*, ovalbumin, bovine serum albumin, thyroglobulin, urease, transferrin, ferritin and lysozyme, and found that the coefficient of variation due to the difference of molecular species was 24 %. The coefficient was calculated from seven averaged absorbance values, which were obtained from three repeated determinations for each protein. The coefficient obtained for the CBB stain was 17 %. In the experiments, the protein concentration was determined by photometry at 280 nm, using the established molecular extinction coefficients for the proteins, and was kept constant at 50 ng/mm². The reproducibility of the hemin stain was measured with ovalbumin at 50 ng/mm², and the coefficient of variation was found to be 6 % from four repeated determinations.

Fig. 5 compares the 2D electrophoretic patterns of *E. coli* proteins visualized by the hemin (A) and CBB (B) stains. It can be seen from the figure that proteins stained by CBB were also stained by the hemin stain, and that several new spots which were not seen with CBB were detected with the hemin stain as indicated by arrows in the pattern A.

Fig.5. 2D electrophoretic patterns of *E. coli* proteins visualized by hemin (A) or CBB (B) stain.

DISCUSSION

From the results obtained in the present study, it is clear that the hemin stain is useful for rapid detection of proteins separated by electrophoresis. However, we noticed that the soybean trypsin inhibitor was not stained by the present method. We expected a high peroxidative activity in the presence of hemin for the trypsin inhibitor which has 2 histidine and 9 arginine residues in the molecule⁵⁾. This inference comes from the fact that free histidine or arginine complexed with hemin shows a high peroxidative activity⁶⁾. The protein like trypsin inhibitor that has a compact three-dimensional structure may or may not be stained by the present method. The stainability may depend on whether or not hemin can approach the effective amino acid residues.

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要 旨

平板ゲルをガラスなどで裏打ちし補強すると、これを用いた電気泳動分離ならびに染色などの操作が容易

になる。しかしながら、この裏打ちにより通常のコロイド状染料の吸着に基づく染色反応は遅くなる。本研究ではガラス基板で裏打ちしたポリアクリルアミドゲル中に泳動分離した蛋白質にヘミンを吸着させ、そのペルオキシダーゼ様の触媒活性を利用して高速に蛋白スポットを染色する方法を開発した。すなわち、このヘミン染色法と名付けた方法では、蛋白質に吸着したヘミンが過酸化水素の存在下で2,6-キシレノールと3,3'-ジアミノベンチジンの酸化重合反応を触媒する。この反応により蛋白質分子上で染料があらたに合成され染色される。本研究ではこの染色反応の反応条件の最適化を行い、45分間で蛋白質が染色されることを見出した。本論文ではヘミン染色法とクーマシーブルー染色法の比較結果についても言及する。

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