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Note

Rapid fluorescamine based protein assay usable in the presence of interfering substances

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Fluorescamine (4-phenylspiro-[furan-2-(3H), 1'-phthalan]-3-3'-dione) is a sensitive reagent for the detection and quantitation of proteins[1–4]. Its widespread use is somewhat hampered because the reagent is subject to both fluorescent and non-fluorescent interference[2,5] and the reaction conditions can affect the observed fluorescence intensities[2,6,7]. In addition it is often tedious to process large numbers of samples such as would be required in monitoring column effluents.

We have developed a rapid, simple and accurate protein assay which involves spotting samples on a solid support such as paper and developing with fluorescamine. A chromatographic step can be used before the fluorogenic reagent to remove interfering substances and to provide a common solvent environment for reaction.

MATERIALS AND METHODS

Materials

Fluorescamine was from Hoffman-LaRoche; crystallized and lyophilized ovalbumin was purchased from ICN-Nutritional Biochemicals. All other reagents were the best commercially available.

Method

Individual samples (5 μl each) were spotted on a piece of Whatman 3MM chromatography paper along a line at 2.54-cm intervals marked lightly with a lead pencil. The line of application was 3 cm from the bottom edge of the paper. When chromatography was desired the paper was run in an ascending manner after drying the sample spots. The chromatography solvent was ethanol–0.2 *M* sodium acetate (55:45, v/v), pH 4.5. In this solvent ovalbumin remained at the origin. After chromatography the paper was thoroughly dried. Fluorescent derivatives of the proteins were formed by either spraying or spotting the developing reagents on the paper. Fluorescamine was used as an 18-mg/ml solution in dry acetone. The fluorescamine solution was sprayed on the paper at a level of *ca.* 0.05 ml/cm^2; when the reagent was spotted on the protein region a total of 6 μl of reagent were used in 2-μl aliquots with drying after each application. To optimize the pH for reaction a 0.2 *M* borate buffer, pH 9.0, was applied after the fluorescamine using the same methods and volumes for the fluorescamine. The paper was thoroughly dried before visualization.

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Visualization, recording and quantitation

Direct visualization was carried out by illumination of the paper with a hand-held Model UVS-11 Mineralight Lamp (Ultra-Violet Products, San Gabriel, CA, U.S.A.).

Photographic recording was done using Kodak Tri-X 4 × 5 sheet film, using a yellow KV500 optical filter and a KV370 optical filter (Schott Optical Glass, Duryea, PA, U.S.A.) between the fluorescent spots and the camera lens. Photographs were also taken using a Wratten No. 8 filter in place of the KV500 filter. The paper was illuminated from the side using a Model C-62 long wavelength Chromato-Vue Transilluminator (Ultra-Violet Products). Under these conditions a typical exposure was 1 sec at *f* 4.7 when the film was developed by standard methods.

Scanning of the fluorescent pattern was done on a Quick Scan R & D electrophoresis thin-layer chromatography (TLC) densitometer (Helena Laboratories, Beaumont, TX, U.S.A.) equipped with front surface UV illuminator and optically filtered fluorescence detection to permit front surface fluorescence densitometry. Peak areas were quantitated using the strip chart record either with the built-in rolling integrator or by drawing a suitable baseline on the chart and cutting out and weighing the peak of interest. The concentrations of protein samples were quantitated by comparison with standard proteins of known concentration which were treated in an identical manner.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram run developed as described above. Each of the ten tracks contained 0.5 μM of tris(hydroxymethyl)aminomethane (Tris) (5 μl of 100 mM Tris–HCl, pH 8.0). In addition, the first five tracks contained increasing amounts of ovalbumin (from the left: 0.312, 0.625, 1.250, 2.500 and 5.000 μg, respectively). These five tracks were not chromatographed, and fluoresced blue with apparent equal intensity when viewed directly. The next five tracks (6–10) initially contained identical samples as in tracks 1–5 but were chromatographed as described above before reaction with fluorescamine. The protein remained at the origin and showed a greenish fluorescence under direct illumination. The interfering Tris moved almost at the solvent front and showed a blue fluorescence.

Fig. 2 shows the areas measured by weighing peaks cut from strip chart scans of fluorescent spots produced using a standard solution of ovalbumin. The residual standard deviation for the assay shown is 2.01 units.

The procedure reported here obviates some of the difficulties associated with a practical use of fluorescamine based protein assays. Fluorescamine is subject to several types of interference[2,6]: (a) some compounds such as Tris used here produce fluorescent products; (b) some compounds such as thiols produce non-fluorescent products but compete for the fluorescamine and potentially reduce the extent of reaction with the protein; (c) compounds such as alcohols affect the rate of the fluorescamine reaction with proteins and potentially alter the apparent fluorescence yield in assays run for fixed times; (d) pH can affect both the protein reactivity and the fluorescent yield of the product with fluorescamine. These difficulties are limited in the present procedure since interfering substances are removed and common reaction conditions are introduced by the chromatographic step.

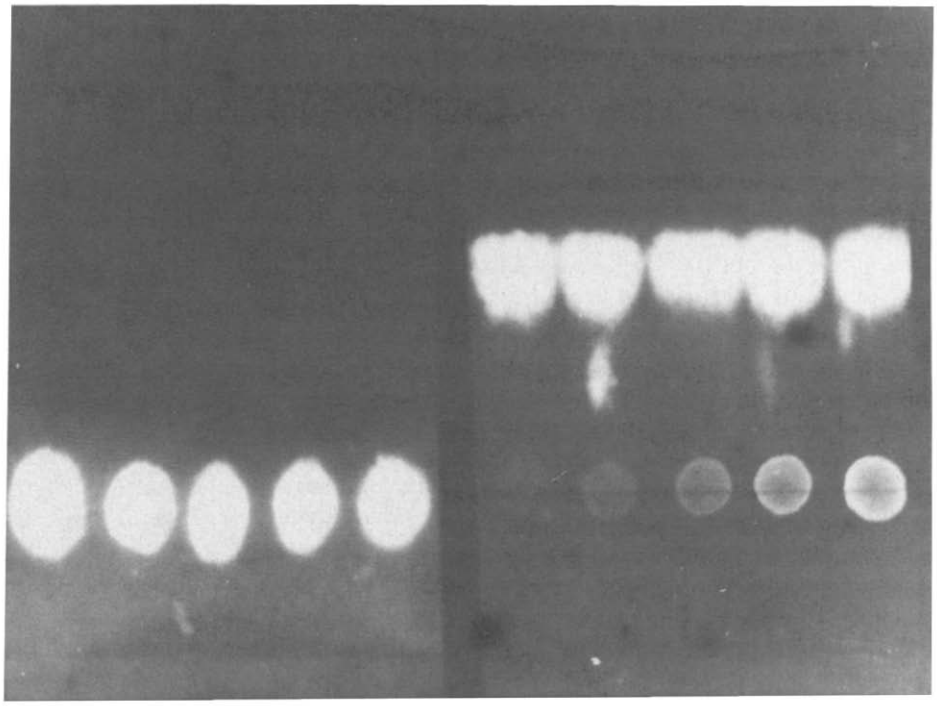

Fig. 1. Paper chromatogram of ovalbumin serially diluted with 100 m*M* Tris buffer, pH 8.0. Each of the ten tracks contained 0.5 μM of tris. The first five tracks from the left were not chromatographed and contained increasing amounts of ovalbumin (from the left: 0.312, 0.625, 1.25, 2.50 and 5.00 μg, respectively). The next five tracks (6–10) contained samples identical to those in tracks 1–5 but were chromatographed and reacted with fluorescamine by spraying as described in Materials and Methods.

The conditions reported here are routinely used in this laboratory for a variety of proteins. However, modifications can easily be introduced to satisfy individual requirements: other solid supports like silica gel can be used, other chromatographic solvent systems can be chosen to maximize the separation of interferences, and other

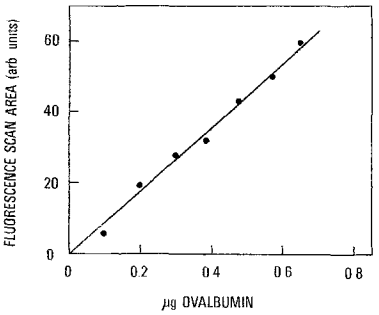

Fig. 2. Standard curve for the assay of ovalbumin with fluorescamine. The indicated amounts of ovalbumin were spotted as 1-μl samples. The protein was reacted with fluorescamine by spotting and scanned as described in Materials and Methods. The relative areas were determined by weighing the appropriate regions of the strip chart record.

reagents like triethylamine can be used in place of borate to optimize the pH of the reaction[8–10].

There are a number of additional practical advantages to the procedures described here. Among these are: sensitivity can be increased by applying multiple spots to the same area with drying between each application, each sample can be spotted a number of time, and large numbers of samples can be routinely handled and roughly quantitated by visual comparison with standards without using very specialized equipment.

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