Color Change of Ternary Associate by Drying on Membrane Filter for Visual Protein Detection

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A simple yet highly sensitive method has been developed for visual detection of protein in an aqueous solution. The proposed method consists of the ternary association between protein and Bromochlorophenol Blue (BCPB), the extraction on a membrane filter, and the significant color change of the dye in the associate by drying.

In the medical field it is necessary to develop a highly sensitive method for protein detection in liquid samples. Laboratory practice in protein purification also requires a rapid and sensitive method for the measurement of protein. This has led to extensive studies of protein assays. For example, dye-binding assays have become an important method for protein determination. The assays based on spectral change of coomassie brilliant blue[1,2] and pyrogallol red-molybdenum (IV)[3] are more sensitive than the Lowry method.[4] It was also reported that the binding of sulfophthalein dye to albumin causes a spectral change or enhancement of the fluorescent intensity.[5] However, most of these methods require expensive equipment such as spectrophotometer or spectrofluorometer.

In our previous work we have shown that visual measurement may serve as a simple and sensitive analytical method,[6-9] avoiding the practical disadvantages associated with sophisticated instrumentation and laboratory skill. Here we describe a novel visual method for protein detection by using Bromochlorophenol Blue **1** (BCPB), based on membrane filtration of the ternary associate, $HBCPB^{-}$-$BCPB^{2-}$-protein, and subsequent color change of the chromophore by drying. Human serum albumin (HSA) was used for the investigation as a protein of medical importance.

The typical procedure is as follows: 1 ml of sample solution containing 10-300 mg/l of HSA was added to 2 ml of the dye solution (2.0×10^{-4} mol dm^{-3}) adjusted to pH 3.0 with 0.1 mol dm^{-3} sodium chloroacetate-HCl buffer. The mixture was diluted to 5 ml with water. The solution was filtrated with a membrane filter (MF) of mixed cellulose ester having 0.2 μm pore size and 25 mm diameter (ADVANTEC, Tokyo, Japan) using a disposable syringe with a filter holder (ADVANTEC, Tokyo, Japan). Then, the filter was dried in an oven at 60 °C for 10 min. The protein concentration was visually measured from the resulting color of the dried filter. Reflective spectrophotometry was also carried out to evaluate visual results with a densitometer (CS-9300PC, SHIMADZU, Tokyo, Japan).

When HSA is added to the dye solution, the yellow color of the dye solution at pH 3.0 changes to blue. This indicates that $IBCPB^{-}$ is converted to $BCPB^{2-}$ because of the interaction with HSA. The absorption spectrum of the dye solution at pH 3.0 primarily shows two spectral components with centers at 435 and 590 nm (Figure l(a)). The acid dissociation constant was determined as pKa(OH) = 4.2 by spectrophotometry. Increasing the HSA concentration diminishes the yellow band

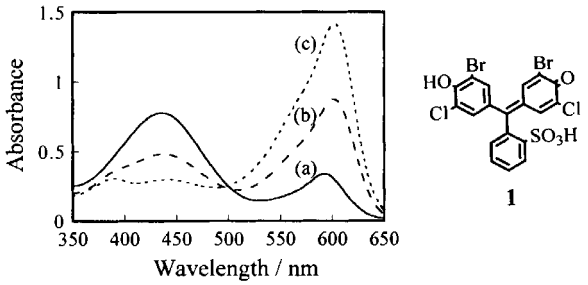

Figure 1. Absorption spectra of BCPB solution. (a) HSA free, (b) 100 mg/l HSA, (c) 300 mg/l HSA; [BCPB] : 3.5×10^{-5} mol dm^{-3}; pH 3.0.

while enhancing the blue component (Figure 1 (b), (c)). The slight shift of the maximum absorption wavelength of the blue component, from 590 nm to 600 nm as the HSA content increases, reflects the interaction of $BCPB^{2-}$ with protein. It is generally recognized that there are two kinds of dye-binding modes to albumin: specific and nonspecific. In the former, strong double-charged dye-binding occurs at a few distinct sites in albumin molecules, while in the latter, relatively weak single-charged dye-binding occurs at many sites.[10-13] Therefore, the color change from yellow to blue in a solution is due to an increase in the binding of $BCPB^{2-}$ to the specific sites.

In earlier work, S. Taguchi, et al. described the membrane filtration method for solid-phase extraction of metal chelates which was used in trace elements determination.[14,15] In our study, we found that MF displayed a yellow-greenish color immediately after filtration of a sample solution, while after drying at 60 °C for 10 min, the color changed from yellow-greenish to deep blue (Figure 2). Taking the two binding modes on protein into consideration, the yellow-greenish color if MF suggests that the single-charged dye, $HBCPB^{-}$, also associates with protein. Thus, in the presence of excess dye the

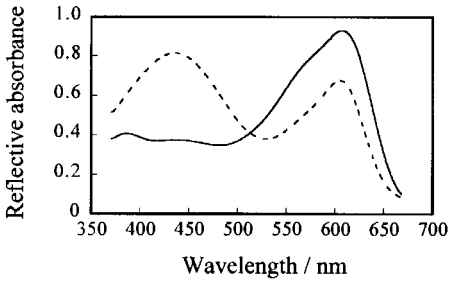

Figure 2. Color change of MF by drying. Dotted line: wet sample (visual perception: yellow-greenish); solid line : dried sample (visual perception: deep blue); [BCPB]: 1.0×10^{-3} mol dm^{-3}; [HSA]: 80 mg/l; sample volume: 10 ml; pH: 3.0; MF: mixed cellulose ester.

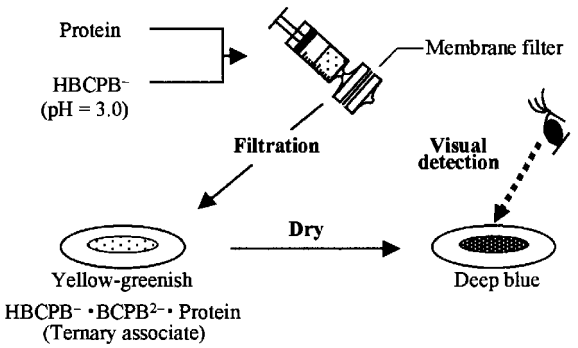

Figure 3. Outline of the proposed method.

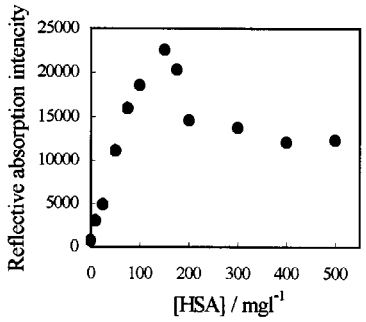

Figure 4. Relationship between reflective absorption intensity and protein concentration. BCPB: 1.75×10^{-4} mol dm^{-3}; sample volume: 5 ml; MF: mixed cellulose ester; wavelength: 590 nm.

ternary associate, $HBCPB^{-}$-$BCPB^{2-}$ protein, forms. Furthermore, the yellow dye, which collected on the membrane filter as the ternary associate, changes into the blue dye, $BCPB^{2-}$, by drying. Figure 3 shows the outline of the proposed method. The mechanism of the color change during drying still remains to be investigated .

The reagent blank was sufficiently low, indicating that the free dye was only slightly extracted onto MF. The blue color becomes darker with increased HSA content, for example until approximately 150 mg/l, when the BCPB concentration is **1**. 75×10^{-4} mol dm^{-3} (Figure 4). Then the color becomes lighter after critical HSA concentration. The color dependence on HSA indicates the difference between the extractability of the binary ($BCPB^{2-}$-protein) and the ternary ($HBCPB^{-}$-$BCPB^{2-}$-protein) associates onto MF. In the presence of excess dye, the ternary associate with a high extractability is formed, resulting in a deep blue color. In contrast, at HSA concentrations higher than the critical region, the binary associate with a low extractability forms, resulting in a light blue color. The critical HSA concentration depends on the amount of dye added. The deep blue region expands with increasing dye concentration as shown in a HSA-BCPB concentration diagram (Figure 5).

Consequently, the high extractability of the temary associate onto MF and the significant color change by drying provide a sensitive visual method for protein detection. Under analytical conditions (at 2.0×10^{-4} mol dm^{-3} BCPB), the visual detection limit is 10 mg/l when 5ml of the solution is filtrated. In a separate experiment, the same color change was observed for γ-globulin. Application of the proposed method to fluids of medical importance such as urine will be reported in detail in a future paper, as well as the investigation on other proteins.

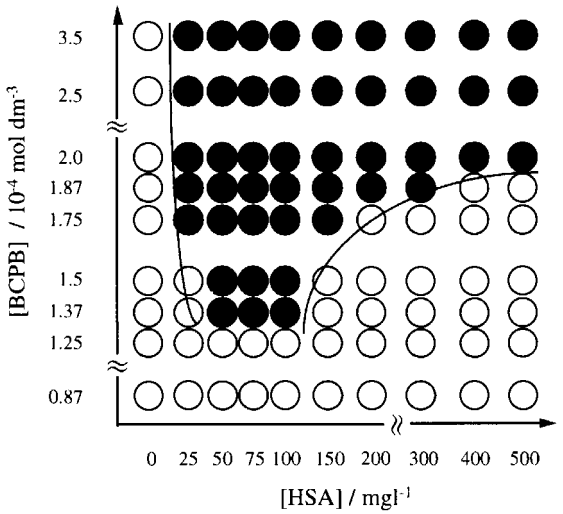

Figure 5. Color transition diagram with respect to BCPB and protein concentration. ●: Reflective absorbance is more than 15000. ○: Less than 15000. The other conditions are the same as those in Figure 4.

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