Simple Spectrophotometric Method for the Estimation of Algal Polysaccharide Concentrations

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A rapid and simple spectrophotometric method is described for the estimation of microgram quantities of algal polysaccharides following the formation of soluble complexes with methylene blue dye. The binding of the dye to algal polysaccharides causes the absorption maximum (664 nm) to decrease, which is almost linear over the range of 0−30 μg for the algal polysaccharides studied. The absorbance at 664 nm can be measured immediately after the mixing of algal polysaccharides and dye solution and is stable over a period of 2 h. No heating, centrifugation, lengthy equilibration, or sophisticated instrumentation, which hamper other methods, is required. The interference due to individual monosaccharides, neutral polysaccharides, bovine serum albumin, sodium dodecyl sulfate, and high concentrations of inorganic salts is discussed.

Keywords: *Algal polysaccharides; methylene blue; spectrophotometry*

INTRODUCTION

Algae are used traditionally as human food, because they are a rich source of protein (Richmond, 1988; Shekharam et al., 1987). A considerable amount of work has been done on the characterization of the protein (Brejc et al., 1995; Sokhi et al., 1990). However, recent interest highlights polysaccharides of algae (Coombe et al., 1987; Johnston et al., 1987; Zhou and Chen, 1990; Wakunage Pharmaceutical Co. Ltd., 1984; Blumreisinger et al., 1983; Liu et al., 1998; Ou and Liu, 1996; Parish et al., 1987).

The determination of algal polysaccharide (AP) concentration is usually carried out by measuring the content of either uronic acid or hexosamine. These procedures work quite well but are neither rapid nor particularly simple. A spectrophotometric procedure for the determination of APs after electrophoretogram is staining with alcian blue (Crist et al., 1981). After destaining, the alcian blue−AP complexes are isolated, solubilized, and dissociated. The amount of dye present is then determined spectrophotometrically and used to estimate the quantity of APs in the complexes. These methods do not readily lend themselves to the simple measurement of total AP concentration.

A spectrophotometric method is described in this paper which allows the rapid and reproducible estimation of microgram quantities of APs. It is easy to use, requiring only one reagent. The procedure is based on the different absorption spectra of dye and dye−AP complexes.

MATERIALS AND METHODS

Materials. Methylene blue (MB) was purchased from Shanghai, the third reagent factory, China. Dye solution was prepared daily. Best results were obtained with solutions prepared shortly before use. MB dye was dissolved in water to produce a final dye concentration of 2.5 mg/30 mL. The dye dissolved completely, and no filtering was necessary. Due to the light sensitivity of the dye, the MB dye solution must be stored in an amber bottle.

All other compounds and reagents were of analytical or guaranteed reagent grade, and all solutions were made with distilled water.

Isolation of APs. Freeze-dried *Spirulina maxima* and *Aphanothece halophytica* were defatted with $CHCl_3$/MeOH (2:1, 900 mL, 3 × 3 h) and the free sugars extracted with hot 70% EtOH (900 mL, 3 × 3 h). Polysaccharides isolated from a blue-green alga, *spirulina maxima* (PSM), and from a blue alga, *aphanothece halophytica* (PAH), were kindly provided by Prof. Z. L. Liu and H. Chen.

AP Assay. To each tube were added 0.4 mL of the dye solution, and from 0 to 30 μg of APs in 5-μg increments in a total volume of 6.0 mL, and the contents were thoroughly mixed. After 8 min, the absorbance at 664 nm of each tube was measured against the blank in cuvettes with a 1-cm light path. All runs were thermostated at room temperature and were performed in triplicate.

Apparatus. A U-3000 spectrophotometer (Hitachi, Tokyo, Japan) was used for recording absorption spectra or measuring the absorbance at a given wavelength.

RESULTS AND DISCUSSION

Spectra of Dye and Dye−AP Complex and Linearity of the Assay. Figure 1 shows the absorption spectra of MB dye and MB−AP complexes from 400 to 800 nm. The dye alone exhibits two absorbance maxima at wavelengths of 664 and 614 nm. Adding APs decreases the absorbance of the 664 and 614 nm peaks and elevates the absorbance of the 566 nm peak. This peak, attributable to the AP−dye complex, is apparently different from the absorption peak of the dye because the wavelengths corresponding to three absorbance maxima are different.

In view of the molecular structure of MB and APs (Shekharam et al., 1987; Sokhi and Vijayaraghavan, 1990; Crist et al., 1981, 1990), it is not possible to reach a conclusion that MB combines preferentially with a particular group on APs to form a complex. A reasonable

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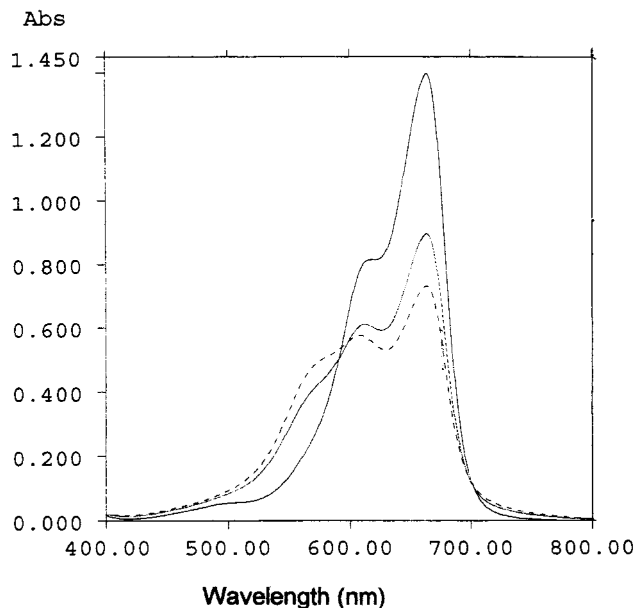

Figure 1. Absorption spectra of MB−APs mixtures: (—) 0.5 mL of MB operating solution plus 5.5 mL of distilled water; (···) 0.5 mL of MB operating solution, 5 mL of 0.01 mg/mL PAH, and 0.5 mL of water; (- - -) 0.5 mL of MB operating solution, 5 mL of 0.01 mg/mL PSM, and 0.5 mL of distilled water.

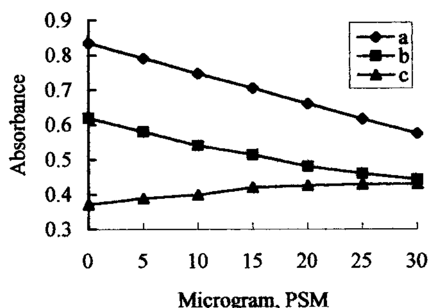

Figure 2. MB assay plots with PSM at selected wavelengths: a, at 664 nm; b, at 614 nm; c, at 556 nm.

explanation of these molecular events is that MB interacts with APs by nonspecific, electrostatic forces. Owing to the presence of the sulfate groups, the whole APs molecules are negatively charged under the conditions of Figure 1. However, the MB species have positive charges. Therefore, APs and MB species should be bound together by electrostatic forces. The metachromasia reported (Liu et al., 1998) is the property shown by certain pure dyestuffs in coloring certain tissue elements in a different color, usually of a shorter wavelength absorption maximum, than most other tissue. Dye binding requires a macromolecular form with a sulfate group.

Linearity of the assay is assessed by measurements on PSM at 664, 614, and 566 nm, as shown in Figure 2. The plot at 664 nm is consistently more linear than the plots at 614 and 566 nm in the range over 0−30 μg of total PSM. These results also show that the assay at 664 nm is about twice as sensitive as that at 566 nm. Linear plots at 664 nm are also found for PAH, as shown in Figure 3.

Sensitivity and Accuracy of the Assay. The MB dye assays of microgram quantities of two purified APs are shown in Figures 2 and 3. Each AP complex with the dye gives a linear decrease in absorbance at 664 nm over the range of 0−30 μg of APs.

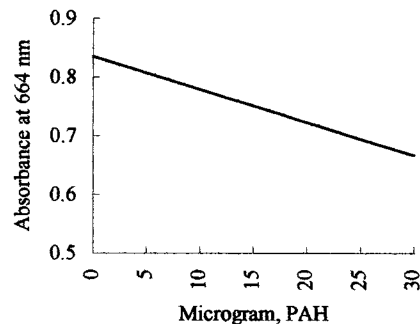

Figure 3. Response of the MB assays to increasing amounts of PAH.

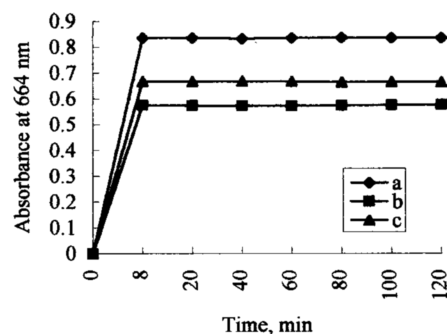

Figure 4. APs−MB complex formation rate and color stability: a, MB operation solution mixed with water; b, 30 μg of PAH with MB operation solution; c, 30 μg of PSM with MB operation solution. MB operation solution is 0.4 mL.

Stability of the Dye and the AP−Dye Complex. It is clear from Figure 4 that the formation of the MB−AP complex is complete within 8 min of mixing and remains stable for a period of 2 h, thus making the procedure very rapid and yet not requiring critical timing for the assay. After 24 h, a precipitate is not observed.

Because the AP−MB complexes have a tendency to bind to cuvettes, there is a decrease in absorbance after long standing in cuvettes. If very precise determinations are required, investigators should take precaution to read the absorbance of samples during the first 1−5 min after the MB−AP complexes are pipetted into the cuvettes. This still gives ample time to read samples. Although surface dye-APs staining could be observed with the quartz cuvette, it is apparently not as severe as with the glass cuvettes.

Interference by Other Components. A wide spectrum of reagents has been tested to determine whether they produce color by themselves or have some stimulatory or inhibitory effects on the production of color by APs when they are assayed by using the MB method (Figure 5). The monosaccharide sugar moieties glucose, *N*-acetylglucosamine, and glucuronic acid do not interact with the MB dye, nor do they have any effect on the formation of dye−AP complexes. The same is true for polysaccharides of *Schizophyllum commune* (PSC), polysaccharides of *American ginseng* (PAG), and bovine serum albumin (BSA). However, ammonium sulfate [$(NH_4)_2SO_4$] and sodium chloride produce measurable increases in absorbance at 664 nm when mixed with MB−AP complexes. The sodium dodecyl sulfate (SDS) not only binds the MB dye to decrease the absorption at 664 nm but also affects the MB−AP assay.

A high concentration of these inorganic anions could therefore interfere with AP estimation. For controlling the pH and the ion strength, buffer and salt must be

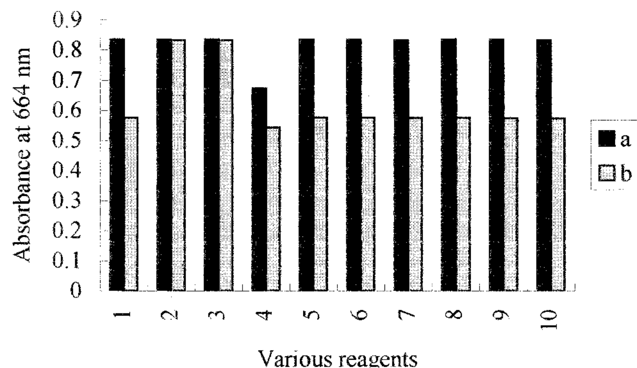

Figure 5. Effect of various reagents on MB−PSM assay and MB: a, various reagents response with MB, MB operating solution 0.4 mL; b, various reagents effect on PSM−MB assay, PSM usage 30 μg in total assay volume; 1, distilled water; 2, 0.2 M $(NH_4)_2SO_4$; 3, 0.2 M NaCl; 4, 0.05% SDS (1 mL of each reagent is assayed in the total assay volume); 5, 50 μg of BSA; 6, 50 μg of PSC; 7, 50 μg of PAG; 8, 50 μg of glucose; 9, 50 μg of *N*-acetylglucosamine; 10, 50 μg of glucuronic acid (dissolved 50 μg these reagents, respectively, in the total assay volume).

added to the solution. The concentrations of the anions are usually 100−1000-fold higher than that of the dye species, so the cationic dye species are actually surrounded by anions which prevent the dye species from binding to APs (Jiao and Liu, 1998a,b). If the concentration is known, the use of appropriate controls can substantially reduce the interference by anions. If the salt content is high, desalting of sample prior to assay may be necessary for accurate AP estimation. The difference in response between APs and inorganic anions demonstrates the importance of nonelectrostatic interaction in the metachromasia. However, the binding forces of APs to the dye seem to be weak because the metachromatic reaction of APs is easily diminished by the addition of salt or heating.

Uses of the Assay. The spectrophotometric method for AP estimation, as described in this paper, is simple, sensitive, and reproducible. It offers distinct advantages over methods previously employed and appears to be adaptable to a variety of uses. It can be used in AP purification or separation procedures for the simple location of material following various fractionation methods.

ABBREVIATIONS USED

APs, algal polysaccharides; MB, methylene blue; PSM, polysaccharides from *Spirulina maxima*; PAH, polysaccharides from *Aphanothece halophytica*; PSC, polysaccharides from *Schizophyllum commune*; PAG, polysaccharides from *American ginseng*; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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