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Note

Colorimetric determination of alginates and fragments thereof as the 2-nitrophenylhydrazides

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Alginic acid, which consists of α-L-guluronic acid and β-D-mannuronic acid residues, has been investigated widely[1–5], and alginate and/or uronic acids, obtained by hydrolysis of alginic acid, have been determined severally[6–8]. The derivatisation of carboxylic acids with 2-nitrophenylhydrazine[9–11] provides a selective and simple colorimetric assay, and we have applied this method to alginate and two kinds of homopolymeric blocks, obtained by partial hydrolysis, by reaction with 2-nitrophenylhydrazine in aqueous solution in the presence of a water-soluble carbodi-imide. The purple colour produced in basic solution after derivatisation of alginate, the G-, M-, and MG-fractions, and glucuronic acid has λ_{max} 525–530 nm. The derivatisation proceeded better at lower pH (Fig. 1). However, a fibrous precipitate separated at pH <4.5, which reflected the lower solubility of alginate in acidic conditions. Pyridine in the range 0.25–1% in the reaction mixture did not appreciably affect the derivatisation and 0.5%

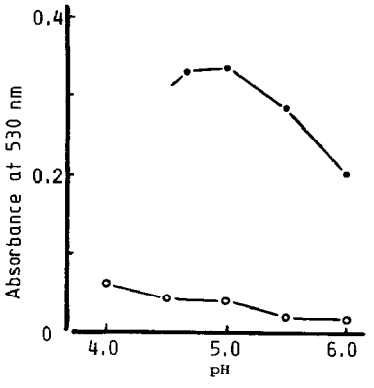

Fig. 1. Effect of pH (1% pyridine–HCl) on the coupling of 2-nitrophenylhydrazine with alginate: -●-, sample; -○-, blank.

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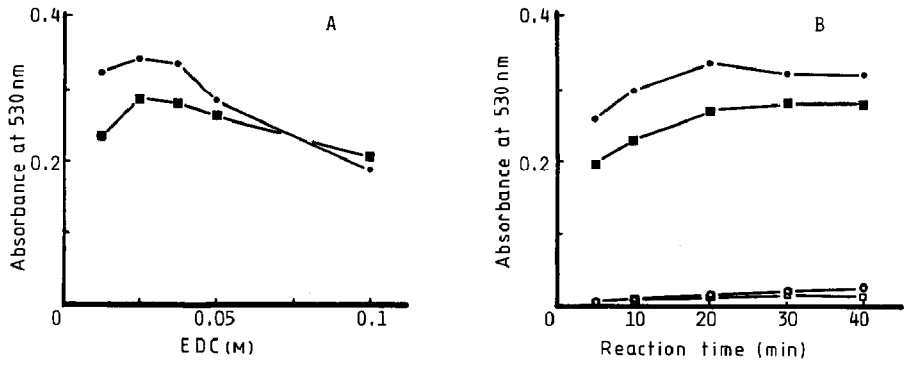

Fig. 2. Coupling of alginate with 2-nitrophenylhydrazine: *A*, effect of the concentration of EDC; *B*, rate of the reaction; -●-, 2.5mM ONPH; -■-, 1.25mM ONPH; -○- and -□-, blanks.

pyridine–hydrochloric acid buffer (pH 5.0) was employed generally. The effect of the concentration of the carbodi-imide EDC is shown in Fig. 2A and the rate of reaction under standard conditions is shown in Fig. 2B. The colour was stable for at least 100 min, even when exposed to light at 530 nm. D-Glucose, D-glucitol, D-mannitol, sucrose, and lactose generated little or no colour under the standard conditions of reaction.

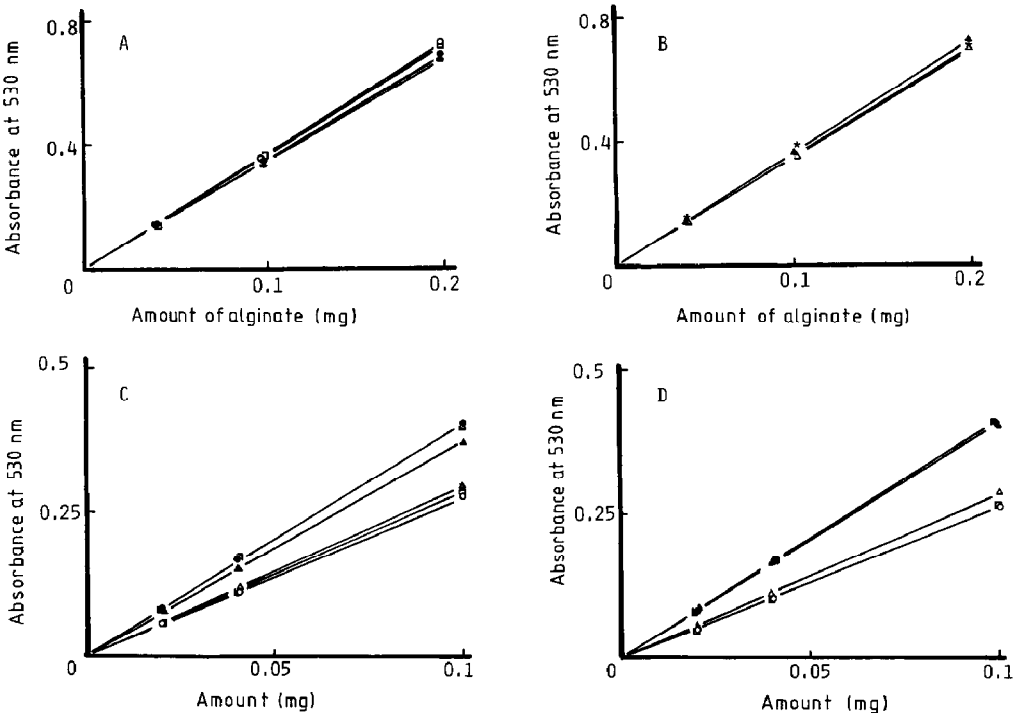

Fig. 3. Calibration curves: *A*, alginates A (●), B (○), C (■), and D (□); *B*, alginates E (▲), F (△), and G (*); *C*, G-fractions A (●), B (■), and C (▲), M-fractions A (○), B (□), and C (△); *D*, G-fractions E (●), F (■), and G (▲), M-fractions E (○), F (□), and G (△).

The calibration curves for alginate samples A–G (Figs. 3A and 3B) showed fairly good agreement, in spite of the differences in viscosity and source. The lower limit of determination was 20 μg, which corresponded to 0.001% of alginate in the solution of the sample. This method was more selective than the phenol–sulfuric acid method. The coefficient of variation for the determination of 0.1 mg of alginate (sample A) was 0.87% (n = 10).

The calibration curves (Figs. 3C and 3D) for the G- or M-fractions agreed closely, regardless of the type of alginate used, but the slopes were different; the reasons for this difference are not clear at present. The MG-fraction could be detected quantitatively in diluted hydrolysates (data not shown).

The above method is simple, selective, and rapid, and facilitates determination of the polyuronate without decomposition into mono-uronate. The method can be applied also to pectate[12], chondroitin sulfate, and hyaluronate. Contaminants which possess carboxyl groups, or polypeptides which contain acidic amino acids, interfere with the determination of alginate, *e.g.*, dextran sulfate [λ_{max} 460–500 nm (absorbance at λ_{max} when a 1-mg sample was assayed with this method; 0.052)], carrageenan [530 nm (0.048)], bovine serum albumin [545 nm (0.36)], gelatin [540 nm (0.47)]. Therefore, combination with a specific separation method such as h.p.l.c.[13] may be necessary.

EXPERIMENTAL

Materials. — Alginates A–C with low, medium, and high viscosity, respectively, were purchased from Sigma. Alginates D from WAKO (Osaka) and E–G (300, 500, and 1000 c.p.s., respectively) from Nacalai Tesque (Kyoto) were also used.

Sodium alginates were partially hydrolysed[14] (0.3M hydrochloric acid, 2 h, 100°), and the M- and G-fractions were separated by centrifugation from MG-fractions. The G- and M-fractions were separated by the method of Haug *et. al.*[15]. Each fraction was precipitated with ethanol after neutralisation, then centrifuged, and the pellet was washed with ethanol and ether, then dried *in vacuo* over P_2O_5 for 24 h.

2-Nitrophenylhydrazine hydrochloride (ONPH) was recrystallised from methanol–ether. The reagent solutions were 10mM ONPH in 0.15M hydrochloric acid and 0.1M *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride (EDC, WAKO) in aqueous 2% pyridine.

Analysis procedure. — Aliquots (1 mL) of the ONPH and EDC reagents were added to an aqueous solution (2 mL) of the test compound. Each mixture was incubated for 20 min at 40°, and M sodium hydroxide (1 mL) was added, followed by incubation for 10 min at 40°. After cooling to room temperature, the absorbance at 530 nm was measured against the reagent blank with a Hitachi 200-20 Spectrophotometer.

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