Derivation of Hydroxamic Acid from Pectin and Its Applications in Colorimetric Determination

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A hydroxamic acid (HX) derivative of pectin was prepared, and its potential application to simple colorimetric determination of polysaccharides was investigated. The coupling reaction between pectin and hydroxylamine (HA) progresses in the presence of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMEC). The calibration curve for pectin showed good agreement and the lower limit of detection was 0.5 mg. This is a very simple and rapid determination method, which does not require tedious pre-treatment, for polysaccharides containing carboxyl groups.

Key words pectin; colorimetric determination; hydroxylamine; water-soluble carbodiimide

Pectin has long been used as a food additive and consists primarily of galacturonic acid residues. Pectin is found in apples and citrus fruit, and is an anionic polymer, which forms gel matrix in the presence of calcium ions.[1] It has also been studied as a material for oral drug delivery because it is a safe and abundant polysaccharide.[2,3] Pectin and the oligosaccharides have also been noted as dietary supplements because of their functions in the intestinal tract, and as a result, a sensitive assay for these compounds was developed.[4,5] Hydroxamic acid (HX) is a compound formed by condensation between carboxylic acid and hydroxylamine (HA). HX is known to develop color in the presence of Fe^{3+} ions in HCl solution and this reaction has been employed determining carboxylic acids.[6] We previously reported the condensation of polyuronic acid and 2-nitrophenylhydrazine using water-soluble carbodiimide as a coupling reagent.[7] In the present paper, an HX derivative of pectin was prepared in aqueous medium containing carbodiimide, and its application to simple colorimetric determination of polysaccharides was investigated.

Experimental

Materials Pectin, chondroitin 6-sulfate, polyacrylic acid 5000, HA HCl salt and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Pullulan was obtained from Hayashibara Biochem. Lab. (Okayama, Japan), and sodium hyaluronate obtained from Kibun Food Chem. (Tokyo, Japan), and sodium alginate purchased from Nacalai Tesque (Kyoto, Japan). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMEC) was purchased from Aldrich Chem. Co. (WI, U.S.A.). Other chemicals used were high-purity materials obtained from commercial sources. And all materials were used without further purification.

Method Reagent solutions were 20 mM HA in distilled water and 0.1 M CMEC in 2% pyridine–HCl buffer (pH 5.0). Aliquots (1 ml) of HA and CMEC reagents were added to an aqueous solution (1 ml) of the test compound. Each mixture was incubated at 40 °C for 20 min, and 20 mM $FeCl_3$ in 0.1 M HCl (3 ml) was added. Absorbance at 480 nm was measured against the reagent blank in a quarts cell with 1-cm light path using a Hitachi U-1500 or Model 200-20 Spectrophotometer.

Results and Discussion

The reaction between carboxylic acids contained in the polysaccharide and HA progressed in the presence of CMEC, a coupling reagent. Coloration was recognized after adding $FeCl_3$, and the λ_{max} was found to be 480—490 nm (Fig. 1). As shown in Fig. 2, the coupling reaction was affected by pH, with an optimum pH of 4.8—5.0. However, color development was hardly observed in the case of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl salt (EDC), which is widely used in peptide synthesis. Pullulan, a natural polysaccharide containing no carboxylic acid, did not generate color under the reaction conditions. The polymer-HX derived from the coupling reaction must remain water soluble for colorimetric determination because polyacrylic acid, an artificial polymer, formed white precipitate upon addition of HA and CMEC. D-Glucose, sucrose and lactose also generated little or no color.

Figure 3 shows the effects of reagent concentration on the coupling reaction. As concentrations for determination of pectin, 20 mM HA and 0.1 M CMEC were selected. The reaction progressed rapidly at 30—50 °C and coloration plateaued at 15 min, as shown in Fig. 4. $FeCl_3$ (20 mM) was used for

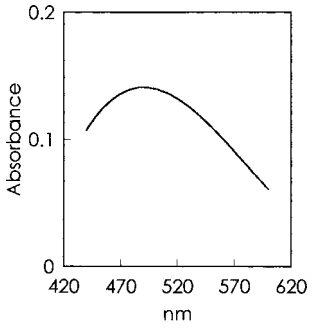

Fig. 1. Spectrum of Chlomophore
Sample: 2 mg pectin.

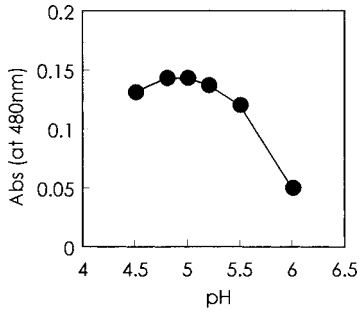

Fig. 2. Effect of pH (2% Pyridine–HCl) on Coupling Reaction

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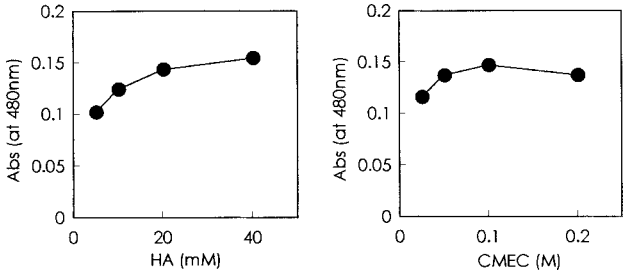

Fig. 3. Effect of Concentration of HA or CMEC on Coupling Reaction

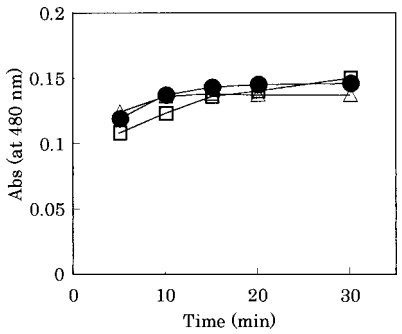

Fig. 4. Effect of Incubation Time on Coupling Reaction
□: 30 °C, ●: 40 °C, △: 50 °C.

ferric hydroxamate formation.

The calibration curve for pectin showed good agreement ($r^2=0.9995$), in the range of 0.5 to 10 mg. The coefficient of variation for determination of 2 mg pectin was 1.3% ($n=7$) and color was stable for at least 1 h, even when exposed to light at 480 nm.

The present method can also be used to determine other polyuronic acid concentrations. In the case of the calibration curve for alginate, chondroitin sulfate or hyaluronate, linearity was observed if the high viscosity of the solution did not interfere with the reaction. The coloration obtained with alginate was larger than that obtained with the other polysaccharides. Therefore, the degree of coloration may be dependent on the number of carboxyl groups within the polysaccharide.

In this study, we prepared an HX derivative of pectin using CMEC as a coupling reagent and investigated its application for colorimetric determination of the compound. This is a very simple and rapid determination method, which does not include tedious pre-treatment, for polysaccharides containing carboxyl groups. However, a separation process is required for measurement of pectin in samples containing other carboxylic acids because the reaction does not differentiate between polysaccharides, but reacts with carboxyl groups.

It has recently been reported that pectin affected intestinal microflora.[8] HX was also studied as an inhibitor of bacterial urease, which produces ammonia from urea in the gastrointestinal tract.[9] Therefore, we are now engaged in work to investigate not only utilization of this colorimetric detection method but also the effects of the HX derivative itself on intestinal flora.

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