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Note

Assay of endo-β-D-xylanase activity with a soluble *O*-(carboxymethyl) derivative of larch-wood D-xylan

SUBHABRATA SENGUPTA, SUMAN KHOWALA, AND PRODYUT K. GOSWAMI
Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta-700032 (India)
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Endo-xylanase, (1→4)-β-D-xylan xylanohydrolase (EC 3.2.1.8), from various micro-organisms[1,2] that hydrolyzes β-D-(1→4) linkages of hemicellulose D-xylan has been studied. The enzyme activity is usually assayed by colorimetric estimation of the reducing sugars liberated from the D-xylan[3–5]. Commercially available D-xylan (from larch wood, Sigma Chemical Company, USA) remains in suspension in the assay mixture, and offers some difficulties because of its uneven distribution during the assay, and so an additional step (centrifugation) is needed for removal of suspended particles after development of color. In addition, the assay procedure does not permit addition of any oxidizing or reducing agent and is nonspecific for determining any endo-hydrolytic activity of the enzyme. It appeared feasible that a soluble xylan derivative, instead of xylan itself, for exposing xylan to this enzyme activity might be developed and used as a substrate for determination of the specific endohydrolytic activity of xylanase by viscometric assay.

Soluble *O*-(carboxymethyl) derivatives of oat-rice bran[6] and wheat-straw[7] hemicelluloses have been prepared and used as a substrate for K_m determination of xylanases, but none of the preparations was compared with xylan as a substrate for the enzyme action[8,9]. Hrazdina and Neukom[7] reported a carboxymethyl derivative of xylan which was three times as susceptible as underivatized xylan to xylanase action, but the xylans used in the preparation of the derivative were not well characterized. Hemicellulose obtained from different sources, although possessing a common (1→4)-β-D-xylan backbone, are substituted to different extents by non-D-xylo-oligosaccharide branch-chains, and thus they vary widely in composition[10]. It has also been reported that these xylans are not equally susceptible to endo-xylanase action[11]. The xylan from larch wood, available commercially[12] (Sigma Chemical Company, USA), is a well characterized hemicellulosic preparation and its carboxymethyl derivative may be prepared and evaluated as an alternative substrate in the colorimetric and viscometric assay of endo-β-D-xylanase activity.

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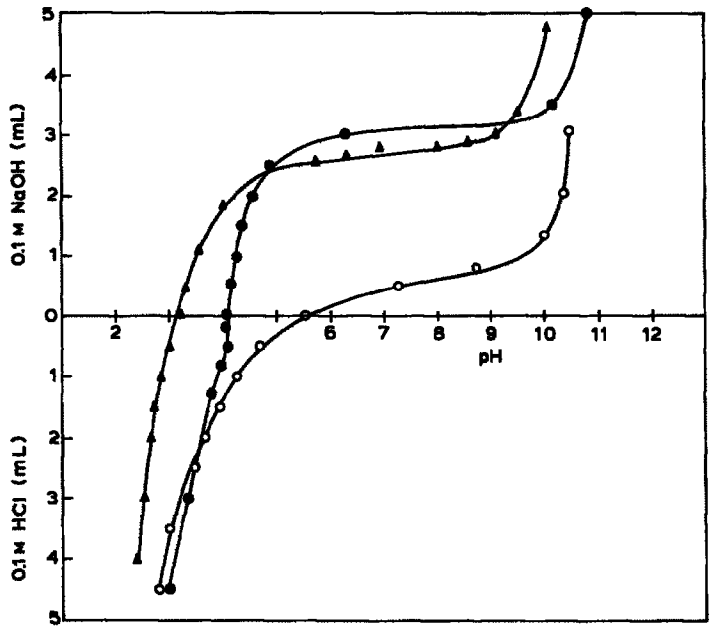

Fig. 1. Titration curves for xylan, *O*-(carboxymethyl)xylan, and glucuronic acid in aqueous phase. Key: —○—○—, xylan; —●—●—, *O*-(carboxymethyl)xylan; and —▲—▲—, glucuronic acid. Method: 500 mg each of xylan and CMXyl, and 60 mg of glucuronic acid were taken separately in 20 mL of de-ionized water. Each sample was separately titrated either with 0.1M HCl or 0.1M NaOH solution in a Radiometer pH-Stat.

RESULTS AND DISCUSSION

A soluble *O*-(carboxymethyl) derivative (CMXyl) of larch-wood xylan was prepared by treating xylan with monochloroacetic acid at room temperature; yield 50–60%; $[\alpha]_{589}^{25}$ −0.275° (*c* 0.5, in water); molecular weight, as calculated from Sephadex G-75 gel-filtration, ~26,000, which indicated that no degradation of hemicellulose had taken place. (It should be mentioned that a single-step carboxymethylation of xylan with higher concentrations of monochloroacetic acid, or at higher temperatures, led to the production of a derivative that could not be precipitated with any of the solvents tested, and it was assumed that the hemicellulose had probably been degraded under these conditions.) The titration curve of the CMXyl (see Fig. 1) had two inflection points, one in common with xylan or glucuronic acid, and the other at a lower pH, indicating the presence of a second ionizable group *i.e.*, the carboxymethyl group. The glucuronic acid content of the xylan preparation, as determined from Fig. 1, was 0.1 mmol/g of xylan. This value (~2%) is slightly lower than the proportion of glucuronic acid (3%) assayed by the carbazole–H_2SO_4 method[13]. It appeared that 0.54 mequiv. of H^+/g of the derivative was available due to the carboxymethyl group, which corresponds to an

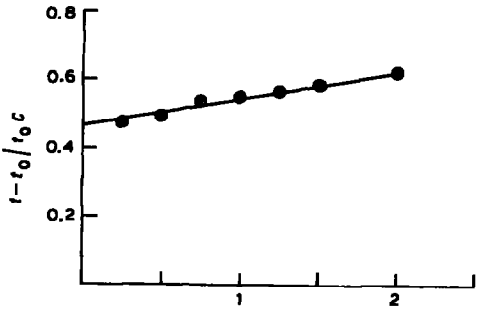

Fig. 2. Intrinsic viscosity of aqueous solution of *O*-(carboxymethyl)xylan. Viscosity was measured at 25°, using an Ostwald capillary viscometer. t_0 = efflux time of water, t = efflux time of aqueous *O*-carboxymethyl)xylan solutions at different concentrations.

average substitution of a single carboxymethyl group per twelve pentose residues. The intrinsic viscosity of an aqueous solution of CMXyl was found to be 0.47 (see Fig. 2).

Soluble CMXyl was compared with xylan as a substrate in the colorimetric assay of endo-xylanase activity present in the culture filtrate of the mushroom *Termitomyces clypeatus*[14]. The kinetics of enzyme action on both substrates were found to be identical. The K_m value as determined by a Lineweaver–Burk plot was found to be 4 mg/mL for both substrates, indicating that the enzyme has the same affinity for xylan and CMXyl. In the colorimetric assay, a CMXyl solution gives a lower blank color than a xylan suspension having the same concentration.

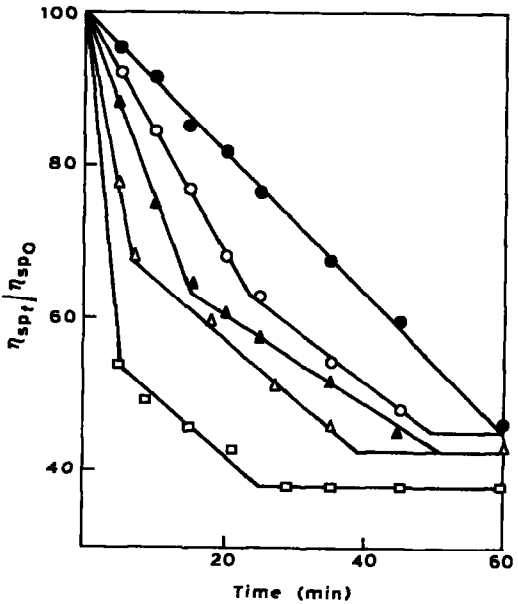

Fig. 3. Percent fall in relative viscosity (η_{sp_t}/η_{sp_0}) of *O*-(carboxymethyl)xylan incubated with various amounts of endo-xylanase. Method of assay described in text. Units of enzyme added per incubation mixtures were: —□—□—, 0.7 U/mL; —△—△—, 0.35 U/mL; —▲—▲—, 0.175 U/mL; —○—○—, 0.88 U/mL; —●—●—, 0.044 U/mL.

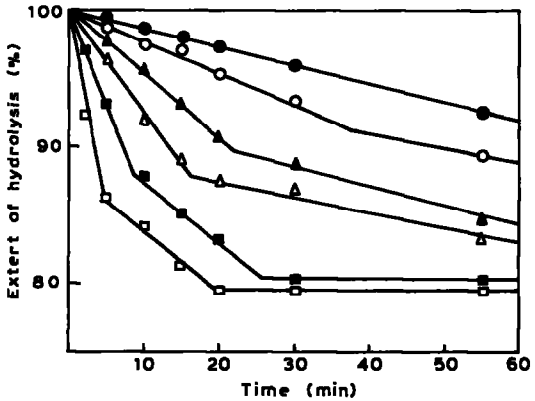

Fig. 4. Extent (%) of hydrolysis of CM-xylan in incubation mixtures containing various amounts of endo-xylanase. Methods for assay of liberated reducing groups and determination of percent glycosidic bond hydrolyzed, described in text. Enzymes added were: —□—□—, 0.7 U/mL; —■—■—, 0.35 U/mL; —△—△—, 0.175 U/mL; —▲—▲—, 0.088 U/mL; —○—○—, 0.044 U/mL; and —●—●—, 0.022 U/mL.

In the viscometric assay for endo-xylanase activity, loss in relative viscosity over a period of 60 min was determined for reaction mixtures containing 0.70 U to 0.022 U of enzyme/mL of incubation mixture (see Fig. 3). The extent of hydrolysis in the identical incubation mixtures was assayed by the Nelson[3] and the Somogyi[4] method (see Fig. 4). Linearity of assay to 1 h of incubation was observed for 22 mU of enzyme for the colorimetric assay and for 44 mU/mL for the viscometric assay, respectively; at higher concentrations, both rate curves exhibited more or less identical features, almost triphasic in the viscometric, and both bi- and tri-phasic in the reducing-group, assay. However, the initial reaction-rate, determined either by viscometry or by reducing-group liberation, was proportional to all of the enzyme concentrations used. Both the viscometric and the colorimetric assay could be made for up to 0.175 unit of enzyme/mL of the incubation mixture when the incubation period was restricted to up to 10 minutes. At lower concentrations of enzyme, the viscometric assay becomes less sensitive (as change in relative viscosity was small) compared to reducing-group liberation.

The rate of loss in relative viscosity represented the extent of hydrolysis of CMXyl the enzyme. An arbitrary unit of enzyme activity, determined by viscometric assay, was considered as being the amount of enzyme which lowered the relative viscosity of the reaction mixture by 10%/min in the initial phase of the reaction (~10 min). This enzyme unit corresponds to ~3% hydrolysis of CMXyl/min. CMXyl prepared from larch-wood xylan has some advantages in the colorimetric and viscometric assays of endo-xylanase activity, the latter method being the more specific as it is not interfered with by any oxidizing or reducing agent.

EXPERIMENTAL

Xylan (larch wood; Sigma lot No. 116C-0104-1) was analyzed for its neutral-sugar contents by g.l.c. of the alditol acetate derivatives of the xylan hydrolyzate (column 3% of EC NSS-M, at 170° at a flow rate of 40 mL of carrier gas/min). The glucuronic acid content of the xylan preparation was estimated by the carbazole–H_2SO_4 method[13]. The composition of the xylan was found to be xylose, 65; arabinose, 18; glucose, 5.0; galactose, 8.0; rhamnose, 1.0; and glucuronic acid, 3%.

O-(Carboxymethyl)ation of D-*xylan.* — A 60% (w/v) solution of monochloroacetic acid (16.6 mL) was slowly added with stirring to 100 mL of a 2% (w/v) solution of D-xylan in 2.5M NaOH, the temperature of the mixture being maintained at 20–30° by external cooling; the reaction was allowed to proceed for 1 h. The solution was acidified with HCl, and the (carboxymethyl)xylan was precipitated with ethanol at 50% (w/v) concentration. The precipitate was filtered off, washed with ethanol, and air-dried. Carboxymethylation of the dried residue was repeated once. Finally, the dried material was dissolved in water and centrifuged at 10,000*g* to remove a slight turbidity. CMXyl was precipitated from the clear supernatant liquor by ethanol, the precipitate redissolved in water, and the solution dialyzed and lyophilized. CMXyl was further purified by chromatography on Sephadex (G-75) using water as the eluant. Carbohydrate elution from the column was monitored by the orcinol–H_2SO_4 reagent[15]. CMXyl was eluted as a sharp single peak. On lyophilization, CMXyl appeared as a slightly yellowish fluffy powder, soluble in water and in buffers of pH 2–9. The molecular weight of the polysaccharide was determined from the same gel-chromatography by using suitable dextrans of mol. wt. (range 10,000 to 70,000) as standards[16].

Source of enzyme. — Lyophilized culture-filtrate of the mushroom *Termitomyces clypeatus,* grown under submerged conditions on 1% (w/v) xylan as the carbon source, was used as the source of the enzyme[14]. Enzyme solutions of different activities were prepared by dilution with 0.1M acetate buffer (pH 5.0).

Assay of xylanase activity. — Colorimetric assay of xylanase activity was carried out by measuring the amount of reducing groups liberated from xylan or CMXyl according to the method of Nelson[3] and Somogyi[4], as previously described[2]. One unit of enzyme activity was expressed as the amount of enzyme which liberated 1 μmol of xylose equivalent per min under the assay conditions.

In the viscometric method of assay, diminution in the relative viscosity (η_{sp_t}/η_{sp_0}) of the reaction mixture by the action of enzyme was measured in an Ostwald capillary viscometer mounted vertically in a water-bath at 40°. Initially, all of the solutions were equilibrated at 40°. The incubation mixture was prepared by rapidly mixing 2.0 mL of 1% CMXyl in 0.1M acetate buffer (pH 5.0) with 0.5 mL of enzyme; this was immediately pipetted into the viscometer, and the efflux time of the solution was noted immediately and at regular time-intervals.

The relative viscosity of the incubation mixture at different time intervals was calculated from the following relationship.

$$\eta_{sp_t}/\eta_{sp_0} = [t_2 \text{ (complex)} - t_1]/[t_2 \text{ (control)} - t_1],$$

where η_{sp_0} and η_{sp_t} are the specific viscosities of the incubation mixtures at time 0 and t, respectively, and t_2 (control) and t_2 (complex) are their corresponding efflux times. The value of t_1 was the same for 0.1M acetate buffer (pH 5.0) under the same conditions. The extent (%) of hydrolysis was considered to be the percent ratio of μmol of xylose liberated to the equivalents present in the xylan or CMXyl added in the incubation mixture.

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