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1,3-β-GLUCAN HYDROLASE FROM *CITRUS*

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Key Word Index—*Citrus aurantifolia*; Rutaceae; Mexican lime; polysaccharide degradation; 1,3-β-glucan hydrolase.

Abstract—A 1,3-β-glucan hydrolase has been isolated and partially purified, using affinity chromatography, from soluble fractions from both bark and leaf extracts of Mexican lime. The enzyme has a MW of 12 000, an optimum pH of 5 and a K_m of 2.1 mg laminarin/ml at 23°. Soluble laminarin, insoluble laminarin, pachyman and yeast cell-wall glucan were hydrolysed by the enzyme. However, compounds with 1,3-β-glycosidic linkages like 4-nitrophenyl-β-D-glucopyranose and salicin are not substrates. Glucose, a product of *exo* action, could not be detected by means of PC or enzymic techniques, suggesting an *endo* mechanism of action of the 1,3-β-glucan hydrolase. Ca^{2+} or Mg^{2+} ions were activators.

INTRODUCTION

1,3-β-Glucans are widely distributed in plants. Although they are not as abundant as starch or cellulose, they often occur in parts of plants with structural, storage or more specialized functions [1]. The enzymic degradation of 1,3-β-glucans has been reported many times in higher plants [2]. The enzymes seem to be closely related to different processes, including cell-wall solubilization in germinating seeds [2–4], coleoptile development [5, 6] and the regulation of transport through vascular tissues [7, 8]. In this regard, it has been proposed that 1,3-β-glucan hydrolases and 1,3-β-glucan synthases maintain a dynamic level of callose deposition in the phloem [8–10]. Moreover, it has been suggested that 1,3-β-glucan hydrolases can act as transglucosylases with a 1,3-β-glucan (callose) as a direct glucosyl donor for cellulose biosynthesis [11,12].

We described previously the biosynthesis of a 1,3-β-glucan in citrus phloem [13, 14]. In this paper we report the isolation of an enzyme from bark and leaf tissues of Mexican lime which is able to hydrolyse laminarin from *Laminaria hyperborea*. Some properties of the 1,3-β-glucan hydrolase (EC 3.2.1.39) as well as its mode of action are studied.

RESULTS AND DISCUSSION

The fractionation of crude extracts of leaves of Mexican limes with ammonium sulphate followed by gel filtration in Bio-gel P-100 allowed a 48-fold purification of the 1,3-β-glucan hydrolase. Using the same procedure with crude extracts of bark tissue, a 13-fold purification of the enzyme was achieved. Alternatively, a specific activity 200-fold higher than in crude extracts was obtained when the extracts were chromatographed on a laminarin column. This affinity chromatography, which takes advantage of the insolubility of laminarin from *Laminaria hyperborea* at 4°, may be used when the main objective is the purification of 1,3-β-glucan hydrolases. However, due to the small percentage of activity recovered (only 30%) and the relative expense of laminarin, we used the first purification procedure for most of our purposes.

The apparent MW of the enzyme, determined by gel chromatography in Bio-gel P-100, is 12 000. This value is similar to those obtained for 1,3-β-glucan hydrolases from other higher plants such as *Phaseolus vulgaris* [15] and *Hordeum vulgare* [16] but lower than the *Secale cereale* [17] and *Nicotiana glutinosa* [18] enzymes.

When tested in a pH range within 3.0 and 8.6, the enzyme showed a maximum at pH 5. Half-maximal activities were obtained at pH 3.6 and 6.1. The K_m values were 1.6, 2.1 and 3.7 mg of insoluble laminarin/ml at 18, 23 and 37°, respectively.

Bark and leaf extracts showed enzymic activity on substrates with 1,3-β-linkages, like soluble laminarin (*Laminaria digitata*), insoluble laminarin (*Laminaria hyperborea*), pachyman and yeast cell-wall glucan (*Saccharomyces cerevisiae*). The extracts did not hydrolyse lichenin (1,3-β- and 1,4-β-linkages) or chemical compounds with 1,3-β-glycosidic bonds, such as 4-nitrophenyl-β-D-glucopyranose and salicin, which are typical substrates for the 1,3-β-glucan *exo*-hydrolases from yeast [19]. The *endo* mechanism of action of the 1,3-β-glucan hydrolase from Mexican lime was proved by descending PC of the products from enzymic hydrolysates; R_G values of these products were identical to those obtained with the 1,3-β-oligoglucoside series from a laminarin hydrolysate [14]; no glucose was detected in the chromatograms. Moreover, free glucose could not be detected by the hexokinase/glucose-6-phosphate dehydrogenase test [20] after the enzymic assays.

Some authors [16, 21] have proposed that 1,3-β-glucan hydrolases are Ca^{2+}-dependent enzymes, as are some

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other polysaccharide hydrolases. However, a general salt effect is suggested by others [2] in the activation process of 1,3-β-glucan hydrolases from higher plants. We have observed that in the presence of 50 mM calcium chloride the activity of 1,3-β-glucan hydrolase in crude extracts of Mexican limes was 3-fold higher than in the absence of Ca^{2+}. Assay mixtures containing either EDTA (Ca^{2+} and Mg^{2+} complexing agent) or EGTA (Ca^{2+} complexing agent only) showed that Mg^{2+} could replace Ca^{2+} as an activator (Figs. 1 and 2). This observation may be of importance in respect to the regulation of the enzyme *in vivo*, since Ca^{2+} cannot be translocated in the phloem vessels (and in addition its concentration is very low), whereas Mg^{2+} is translocated through the phloem at concentrations of around 10 mM. We have reported previously [13] that 1,3-β-glucan synthase is the enzyme which regulates 1,3-β-glucan biosynthesis in the phloem of Mexican lime. As this enzyme is also Mg^{2+}-dependent [14], the possibility exists that the Mg^{2+} concentration may play an important role in the dynamic level of callose deposition in the phloem.

Finally, the similarities found in all the enzymic properties studied, with extracts obtained from bark and leaf tissues, indicate that they are the same enzyme.

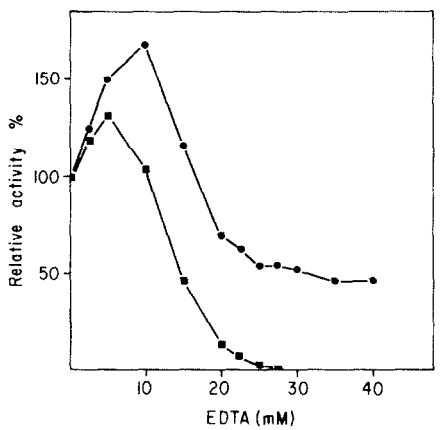

Fig. 1. Effect of EDTA concentration on the activity of 1,3-β-glucan hydrolase in the presence (●) or absence (■) of Mg^{2+} (10 mM). Extraction and enzymic assay were as indicated in Experimental.

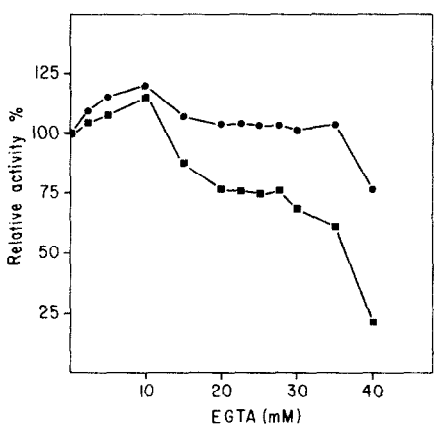

Fig. 2. Effect of EGTA concentration on the activity of 1,3-β-glucan hydrolase in the presence (●) or absence (■) of Mg^{2+} (10 mM).

EXPERIMENTAL

Mexican limes [*Citrus aurantifolia* (Christm.) Swing.] (4 years old) were grown in a greenhouse.

Extraction. Bark or leaf tissue was homogenized in a cold mortar with 9 ml/g (bark) or 5 ml/g (leaves) of 50 mM Tris–HCl, pH 7.4, containing 10 mM 2-mercaptoethanol, 50 mM $CaCl_2$ and 0.2 g insoluble PVP/g of fr. wt. Homogenates were centrifuged at 39 000 *g* for 40 min. The pellets were discarded and the supernatants taken for enzyme purification.

Purification. Supernatants were fractionated with $(NH_4)_2SO_4$. The fraction which precipitated between 50 and 65% of saturation was resuspended in 1 ml 50 mM Tris–HCl, pH 7.4, containing 50 mM $CaCl_2$ and chromatographed on a Bio-gel P-100 column (2 × 38 cm). Elution of the column was carried out with the same buffer and the fractions showing 1,3-β-glucan hydrolase activity were collected. In a second purification procedure, gel chromatography was substituted by affinity chromatography in a laminarin column (1 × 4 cm). The other purification steps were the same as those described for the first procedure. All operations were performed at 4°.

The MW of the enzyme was determined by means of a Bio-gel P-100 calibrated previously with standard proteins: aldolase (158 000), bovine serum albumin (68 000), ovalbumin (45 000), α-chymotrypsinogen (25 000) and cytochrome *c* (12 500).

Protein determinations. The protein content of the samples was determined by the method of ref. [22].

Enzyme assays. These were carried out with the active fractions eluted from the Bio-gel P-100 column. The routine reaction mixtures contained a soln of laminarin (20 mg/ml) in 0.1 M NaOAc buffer, pH 5, and the eluate in the proportion 1:1 (v/v). The incubation time was 30 min and the reactions were stopped by heating the mixture at 100° for 10 min. Reducing sugars in the reaction mixtures were determined by the method of ref. [23].

In the determination of optimum pH, the buffers and the range of pH covered were: 50 mM glycine–Tris, pH 2.4–3.1; 100 mM Na_2PO_4–50 mM citric acid, pH 2.7–7.0; 50 mM Na citrate, pH 2.9–5.6; 50 mM NaOAc, pH 3.7–5.6; 12.5 mM maleate–NaOH, pH 5.8–7.8; and 37.5 mM Tris–HCl, pH 6.9–9.2. In the determination of K_m the concns of insoluble laminarin used ranged from 0.75 to 60 mg/ml.

The substrate specificity assays were carried out with the following compounds and concns: 24 mM 4-nitrophenyl-β-D-glucopyranose, 24 mM 2-nitrophenyl-β-D-galactopyranose, 24 mM 4-nitrophenyl-α-D-glucopyranose, 24 mM salicin, 0.2% cellobiose, 0.2% gentiobiose, 0.1% mannan, 0.1% dextran, 0.1% starch, 0.1% pustulan, 0.2% lichenin, 2% laminarin (*Laminaria hyperborea*), 2% laminarin (*Laminaria digitata*), 1% pachyman and 1% yeast cell-wall glucan (*Saccharomyces cerevisiae*).

The reaction mixtures containing the Ca^{2+} complexing agents EDTA or EGTA (ethyleneglycol bis-(β-amino-ethyl ether)-*N*,*N*'-tetra-acetic acid) were carried out at concn ranges between 0 and 40 mM in the presence or absence of 10 mM Mg^{2+}.

Chromatography of hydrolysis products. Descending PC was conducted on Whatman No. 1 filter paper [24]. The solvent system was EtOAc–pyridine–H_2O (10:4:3). Glucose and a hydrolysate of laminarin [14] were used as comparative standards. Reducing sugars on chromatograms were detected with the reagent of ref. [25].

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