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β-1,3-GLUCANASE ISOZYMES FROM THE LATEX OF *HEVEA BRASILIENSIS*

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Key Word Index—*Hevea brasiliensis*; rubber latex; β-1,3-glucanase; isozyme; purification.

Abstract—Two β-1,3-glucanase isozymes (EC 3.2.1.39) GI and GII were purified from fresh *Hevea* latex using ion-exchange and affinity chromatography. The two isozymes, as determined by SDS–PAGE, are monomeric proteins of M_r *ca* 32 000 and 35 000, respectively. Specificity studies indicate that the two isozymes require relatively long runs of contiguous β-1,3-D-glucosidic linkages with a low degree of glucosyl substitution, such as in CM-pachyman and laminarin. The enzymes hydrolyse neither the β-1,4-D-glucosidic linkages of lichenin and barley glucan nor the β-1,6-D-glucosidic linkages of yeast glucan and pustulan. Kinetic analyses with laminarin as substrate indicate apparent K_m values of 1.25 mg ml^{-1} (GI) and 1.33 mg ml^{-1} (GII), and V_{max} of 2.86 nkat (GI) and 2.65 nkat (GII). The pH optima of the two isozymes are 4.5 (GI) and 5.0 (GII). Both isozymes are relatively heat-stable and retain full activity up to 60°. GII may be the glucanase isoform which plays an important role in response to fungal infection by rubber trees.

INTRODUCTION

Plant β-1,3-glucanases are 'pathogenesis-related' proteins that are expressed in response to microbial invasion [1]. High activities of chitinase and β-1,3-glucanase are frequently found in higher plants [2], but chitinase has no known substrate in the plant itself, and the substrate for β-1,3-glucanase is usually present only in small quantities [3]. However, chitin and β-1,3-glucans are important components of the cell walls of many fungi [4]. Chitinase and β-1,3-glucanase are coordinately induced by wounding, ethylene, pathogen infection [1, 5] and by elicitors [6–8] in various tissues. Mauch *et al.* [9] have shown that chitinase and β-1,3-glucanase act synergistically to inhibit fungal growth.

Latex is the cytoplasm of cells known as laticifers. *Hevea brasiliensis* latex can be fractionated by ultracentrifugation into a top layer of rubber particles, a clear serum and a pellet that contains vacuoles known as lutoids [10]. The transcripts of several defence genes including chitinase, pathogenesis-related proteins, phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase are expressed at 10- to 50-fold higher levels in *Hevea* laticifers than in the leaves [11]. *Hevea* latex has been reported to contain high levels of chitinase and chitinase/lysozymes [13]. Hevein, a chitin-binding protein from the lutoid particles of *Hevea* latex, exhibits antifungal properties [13]. In this paper, we report on the isolation and purification of two isozymes of β-1,3-glucanase from the lutoid particles. The significant amount of these isozymes found in the latex may be due to the frequent tapping of the trees to obtain rubber. When the laticifers are wounded, infection by pathogens may cause elevation of these enzymes. Also, since the level of the glucanase can be enhanced by ethylene in other plants [5], and ethylene (applied as the ethylene generating compound 2-chloroethylphosphonic acid) is widely used to stimulate rubber production, it is possible that this gas acts synergistically with infection to raise the concentration of the enzymes in the latex.

RESULTS AND DISCUSSION

Purification of β-1,3-glucanases from Hevea *latex*

The collected latex was separated into rubber particles, C-serum and bottom fraction by ultracentrifugation [10]. The lutoid particles in the bottom fraction were disrupted by freezing and thawing to give the B-serum. One litre of *Hevea* latex gave about 85–95 ml of B-serum which contained about $1.3–1.4 \times 10^4$ kats of β-1,3-glucanases. Twenty-five millilitres of B-serum was used for the purification procedure described below. Enzyme GI was purified 18-fold and GII, 13-fold, with 70 and 17% recoveries, respectively (Table 1).

CM cellulose chromatography

Proteins in B-serum including β-1,3-glucanases were bound to a CM-cellulose column (2.5 × 12 cm) equilibrated with 20 mM Na-acetate buffer, pH 6, and a linear gradient of 0–1.0 M NaCl in the same buffer (250 ml). Most of the proteins came off the column at low concentrations of NaCl (0–0.4 M), the two β-1,3-glucanase isozymes were eluted at higher concentrations (0.6–1.0 M).

Table 1. Enzyme yield during purification of β-1,3-glucanases from *Hevea* latex, 25 ml of B-serum was used

Purification step	Protein (mg)	Specific activity (kat mg^{-1})	Recovery (%)	Purification (fold)
B-Serum	325	12	100	—
CM-cellulose	37	97	93	8
Con A agarose (GI)	13	217	70	18
(GII)	4	159	17	13

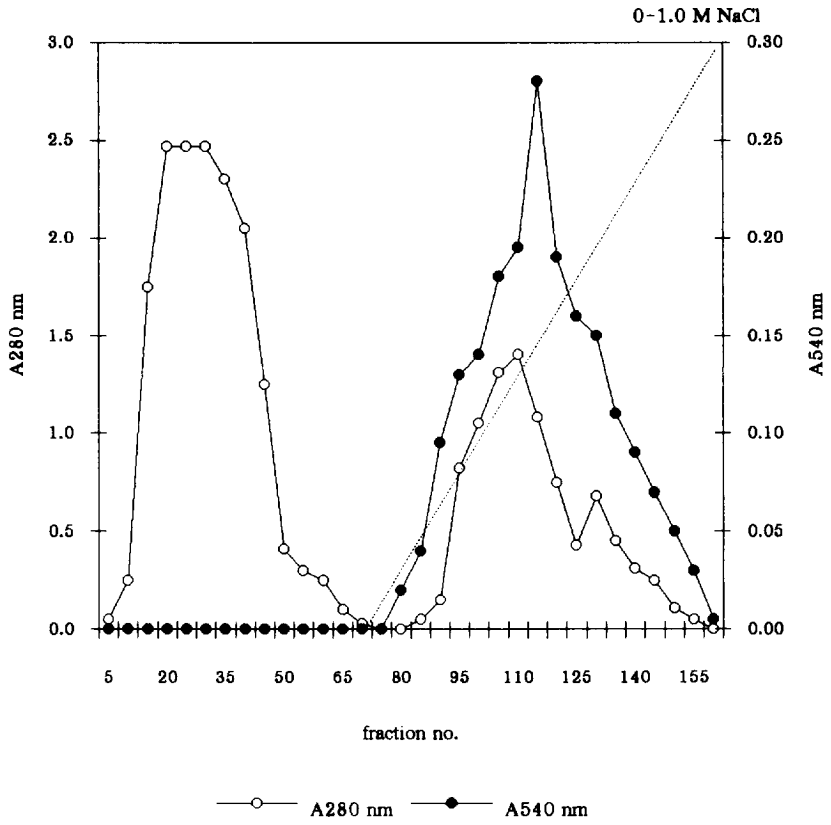

Fig. 1. Ion-exchange chromatography on CM-cellulose. Fractions (*ca* 1.5 ml each) were assayed for β-1,3-glucanase activity (●) and protein (○). ....., NaCl. For full details see text.

This result suggests that the isozymes have higher p*I*s than the other proteins in the B-serum (Fig. 1). Both isozymes were basic proteins because they ran from anode to cathode at pH 8.3, on a non-denaturing gel (data not shown).

Con A agarose chromatography

The active fractions from the CM-cellulose column were applied to a Con A agarose column (2.5 × 3 cm) equilibrated with 0.1 M Na-acetate buffer pH 6, containing 1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. The purified GI isozyme was eluted on washing with three column volumes of the same buffer. The GII enzyme was eluted with 0.2 M α-D-methylmannoside (Fig. 2). These findings suggested that the GI isozyme bound non-specifically to the Con A agarose column. However, it bound to the column more strongly than other proteins in the B-serum. The GII isozyme is a glycoprotein containing glucose or/and mannose, because it bound strongly to the Con A column. The result reported here is similar to that found with β-1,3-glucanase isozyme purified from *Saccharomyces cerevisiae* which was effectively precipitated by concanavalin A, unlike the other forms which failed to precipitate [14].

Purity of isozymes GI and GII

SDS–PAGE under reducing conditions showed that the purified isozymes GI and GII contained single protein

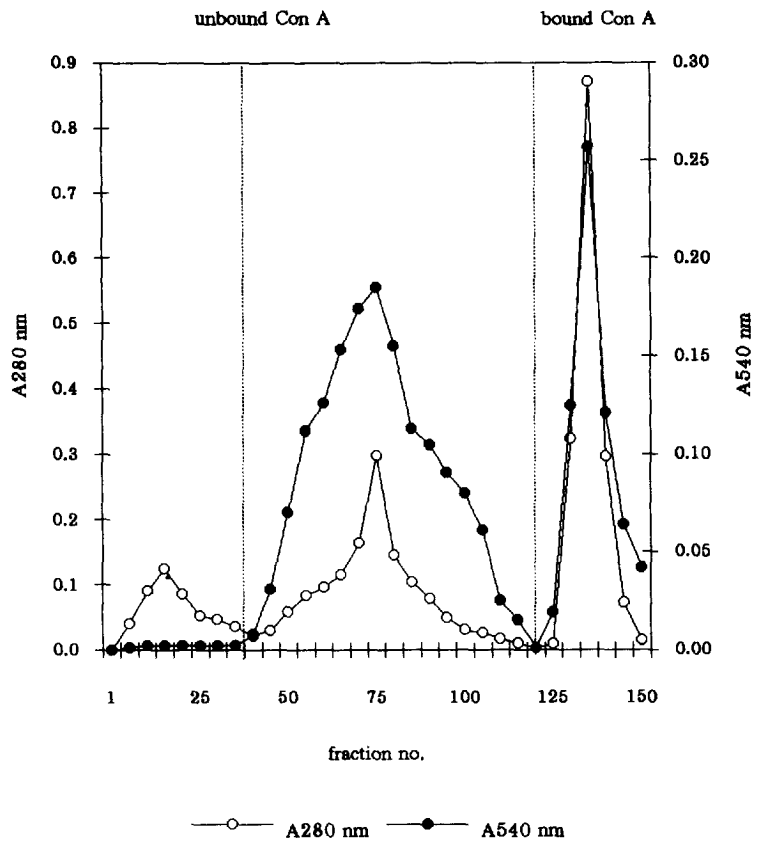

Fig. 2. Affinity chromatography. The pooled active fractions (120 ml) from the CM-cellulose column, were made 1 mM with respect to $MgCl_2$, $MnCl_2$ and $CaCl_2$ and the solution applied to a Con A agarose column. Each fraction from 1–40 contained 3 ml, from 41–124 contained 2 ml and 125–150 contained 1 ml. Fractions were assayed for β-1,3-glucanase activity (●) and protein (○). See text for full details.

bands with M_rs of about 32000 and 35000, respectively (Fig. 3). SDS–PAGE under non-reducing conditions gave the same results (data not shown). Therefore, GI and GII were monomeric. The M_rs found using Bio-gel P-150 were 30000 and 33000, respectively.

Substrate specificities

Six β-D-glucans were tested as substitutes. CM-pachyman (*Poria cocos*) which contains only β-1,3-D-glucosidic linkages [15]; pustulan (*Umbilicaria pustulata*): only β-1,6-D-glucosidic linkages [16]; laminarin (*Laminaria digitata*) and yeast glucan (*Saccharomyces cerevisiae*): β-1,3-D- and β-1,6-D-glucosidic linkages in a ratio of 7:1 and 4:1, respectively [17, 18]; lichinin (*Cetraria islandica*) and barley glucan (*Hordeum vulgare*): β-1,3-D- and β-1,4-D-glucosidic linkages in a ratio of 1:2 and 1:2.5, respectively [19, 20]. Only two substrates were hydrolysed by the *Hevea* glucanases. In the case of GI, laminarin from *L. digitata* was hydrolysed at the highest rate, and CM-pachyman was also hydrolysed at a significant rate (the relative rate of hydrolysis of GI on laminarin was arbitrarily set to 100%, and the relative rate on CM-pachyman was 66.5%). By contrast, GII hydrolysed CM-pachyman much faster than laminarin (the rates of hydrolysis of CM-pachyman and laminarin were 100 and 9.4%, respectively). Both isozymes had no activity against pustulan (β-1,6-D-glucan), and yeast glucan, lichenin and barley glucan, which contain some β-1,3-D-glucosidic linkages. The findings suggest that the enzymes require a long run of contiguous β-1,3-D-glucosidic linkages where the glucosyl units are neither substituted nor branched. In the case of lichenin and barley glucan, which also contain some β-1,4-D-glucosidic linkages, the enzymes had no activity suggesting that *Hevea* glucanases do not hydrolyse β-1,4-D-glucosidic linkages.

Characterization

Kinetic analyses were performed at 35° by incubating 10 and 15 μg enzymes GI and GII in 0.1 M Na-acetate buffer, pH 5, containing laminarin over the concentration range of 0.2–1.4 mg ml^{-1}. Data were analysed using Michaelis–Menten kinetics. The K_m values for GI and GII were 1.25 and 1.33 mg ml^{-1}, and the V_{max} values, 2.86 and 2.65 nkat, respectively. The pH optimum of GI isozyme was 4.5 and the GII, 5.0. Both isozymes retained full activity up to 60°. At 70° the activity was only some 3% of that at 60° while at 80° the enzyme was inactive.

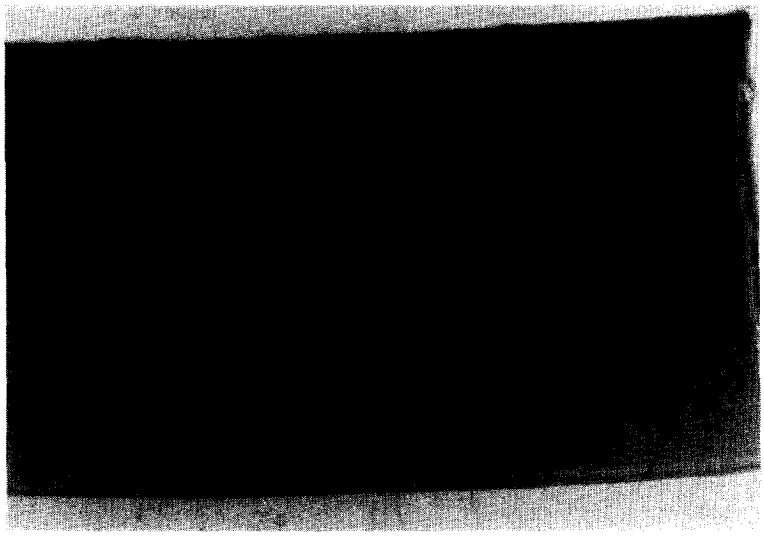

Fig. 3. SDS–PAGE of purified β-1,3-glucanase isozymes GI and GII. Lane 1: B-serum (200 μg); lane 2: unbound on CM cellulose (20 μg); lane 3: bound to CM cellulose (10 μg); lane 4: unbound on Con A agarose (10 μg); lane 5: purified GI (5 μg); lane 6: purified GII (5 μg); lane 7: standard protein markers.

Both isozymes are endoglucanases based on the observation that they failed to hydrolyse *p*-nitrophenyl-β-D-glucoside. The *Hevea* endoglucanases were similar to β-1,3-glucanases from other plants, sharing similar properties such as their M_rs (30 000–35 000), acidic optimum (pH 4.5–5), basic nature (p*I* 8.5–10), and relatively high-temperature stability (50–60°) [3, 21–24]. From the CM-cellulose column, GI and GII co-eluted at similar ionic strength. However, they gave differing results in the substrate specificity studies. Their affinities to Con A were also different. Mauch *et al.* [5] discovered that only one of two isozymes purified from pea was induced by wounding and fungal infection, the other isoform accumulated during the course of maturation. In the experiments reported here, the two glucanase isozymes were purified from the latex of the rubber tree clone, RRIM 600, which is widely grown in the southern part of Thailand. This is a high yielding clone, but is considered to have low disease resistance. B-Serum from this clone contained a higher level of the GI isozyme (Table 1). On the other hand, GII is the major isozyme in B-serum of clone GT1, which is the most disease resistant clone grown in Thailand; GI isozyme was hardly detected in this clone. In addition the GT1 clone contained two to three times the level of glucanase activity found in RRIM 600; i.e. 35 kat ml^{-1} of latex (GT1) vs 13 kat ml^{-1} of latex (RRIM 600). The numbers presented here were measured in B-serum of each clone and adjusted to kat ml^{-1} of latex. There are other β-1,3-glucanase isozymes in the C-serum of centrifuged latex, but the relative level of these glucanases are the same in both clones. Thus GII may be the only isoform which is induced by fungal infection.

EXPERIMENTAL

Latex. Fresh *Hevea* latex of rubber clone RRIM 600 was obtained from the Rubber Research Center, Songkla, Thailand.

Chemicals. Laminarin (*Laminaria digitata*), *p*-nitrophenyl-β-D-glucoside, lichenin, yeast glucan, barley glucan, concanavalin A (Con A) agarose and α-D-methylmannoside were purchased from Sigma. CM-cellulose was from Whatman Ltd (U.K.). Bio-gel P-150 was from Bio-Rad (U.S.A.). 3,5-Dinitrosalicylic acid was from BDH Chemicals Ltd. LMW electrophoresis calibration kit and gel filtration standard proteins were from Pharmacia. Pustulan and CM-pachyman were generously provided by Professor B.A. Stone (La Trobe University, Victoria, Australia).

Enzyme isolation. The latex was collected into a container chilled in ice, filtered through cheese-cloth and centrifuged at 59 000 *g* (ave.) for 45 min at 4°. It separated into: a top layer of rubber particles, a clear serum (C-serum), and a pellet (bottom fraction), containing mainly lutoids [10]. The bottom fraction was frozen and thawed several times to obtain the soluble proteins contained in the lutoids. Lutoid membranes were removed by centrifuging the B-serum at 15 000 g (ave.) for 15 min at 4°, and the resulting soln used for purification of the β-1,3-glucanases.

Enzyme purification. B-Serum was loaded onto a CM-cellulose column (2.5 × 12 cm) equilibrated with 20 mM NaOAc buffer, pH 6. After washing, the bound proteins were eluted with a 0–1 M gradient of NaCl in the same buffer. Fractions with enzyme activity were pooled and loaded onto a Con A agarose column equilibrated with 0.1 M NaOAc buffer, pH 6.0, containing 1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. The purified GI and GII glucanases were found in the unbound fractions and in the bound fractions, respectively. The purified enzymes were dialysed and kept frozen at − 20° before use in the experiments described below. Protein was determined by the Lowry method.

Gel filtration. Purified GI and GII enzymes from the Con A column were dialysed and concentrated by freeze-drying then applied to a 0.9 × 67 cm column of Bio-gel

P-150. The column was equilibrated with 20 mM NaOAc buffer, pH 6, containing 0.85% NaCl. Blue Dextran was used as a void vol. marker and BSA (67 000), pepsin (36 000), carbonic anhydrase (29 000) and lactalbumin (14 000) were used as standard proteins.

SDS–PAGE. Performed according to ref. [25] using a 7.5–15% gradient of polyacrylamide. Proteins were detected by staining with Coomassie Brilliant Blue R-250.

Enzyme assays. Laminarin (2 mg ml^{-1}) in 0.1 M NaOAc buffer, pH 5, was added to 50 μl enzyme soln and the mixture incubated at 35° for 10 min. The reaction was stopped by heating in boiling water for a few minutes. The increase in reducing sugars was measured by adding 1 ml 3,5-dinitrosalicylic acid reagent [26] and boiling for 5 min. Glucose was used as a standard and the absorbance was read at 540 nm. One kat of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 mol of reducing sugars per sec under the given conditions.

Assays with nitrophenyl substrates. Reaction mixtures contained: 250 μl 4 mM *p*-nitrophenyl β-D-glucoside, 50 μl 0.5 M NaOAc buffer, pH 5.0, and 200 μl enzyme. The reaction mixtures were incubated for 30 min at 35°, and then 400 μl 0.3 M Na_2CO_3 was added for colour development, and the absorbance read at 420 nm.

Substrate specificities. Solns of six β-D-glucans differing in their linkage types were prepared at a concentration of 2 mg ml^{-1} in 0.1 M NaOAc buffer, pH 5. These substrates were incubated with 10 μg GI and 15 μg GII for activity measurements.

Enzyme properties. The pH optima of the β-1,3-glucanases were determined over the pH range of 4–6.5 using laminarin at 2 mg ml^{-1} in 0.1 M NaOAc buffer, with 50 μg GI and 70 μg GII. Heat stabilities were determined by measuring residual activity after incubating 50 μg GI and 70 μg GII in 0.1 M NaOAc buffer, pH 5, at various temps for 20 min.

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