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A β-GLUCOSIDASE ASSOCIATED WITH CELL WALLS FROM CELL SUSPENSION CULTURES OF CARROT

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Key Word Index—*Daucus carota*; Umbelliferae; cell culture; cell wall-associated β-glucosidase; purification.

Abstract—The activity of β-glucosidase (EC 3.2.1.21) in the protein fraction solubilized with 3 M LiCl from cell walls of carrot cell cultures was found to be much higher than those of the other glycan-hydrolases. The cell wall-associated β-glucosidase was purified to electrophoretic homogeneity. The M_r of the purified enzyme was estimated to be 46 000 by Sephacryl S-200HR gel-permeation, and 48 000–52 000 by SDS–PAGE under denaturing conditions. The enzyme contained carbohydrate and protein in a ratio of 1:15 (w/w) and was rich in Ser, Gly, Glx and Ala. The isoelectric point was pH 8.2, the pH optimum 4.6–5.2 and the temperature optimum 50°. The activity was inhibited by Cu^{2+}, Ag^{+}, Hg^{2+}, *p*-chloromercuribenzoate, and D-glucono-1,5-lactone. The K_m and V_{max} values for *p*-nitrophenyl (PNP)-β-glucopyranoside were 0.12 mM and 0.13 mmol (mg protein)$^{-1}$ hr^{-1}, respectively. The enzyme also acted on PNP-β-cellobioside, lichenan and laminarin, but was not capable of hydrolysing the glucose-containing polymers isolated from cell walls of carrot cell cultures. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

It appears that glycan-hydrolytic enzymes in plants may play an important role in the metabolism of carbohydrate polymers in the cell walls during cell growth [1, 2]. From their notable different extractability, glycan-hydrolases of cell suspension cultures of carrot can be classified into three types [3, 4]: the first type is extracted from cell homogenates with a low ionic strength buffer, the second is solubilized from cell walls using strong saline solutions and the third is secreted by cells into the culture medium during cell growth. However, the activity of β-glucosidase solubilized from cell walls is much higher than that of the other fractions. Accordingly, most of the β-glucosidase in carrot cell cultures appears to be present ionically bound to cell wall materials. Although β-glucosidase associated with cell walls has been documented for plant species [5], the molecular properties and physiological role of the enzyme, except for the cyanogenic β-glucosidase [6] and coniferin-hydrolysing β-glucosidase [7, 8], remain obscure. The present work describes the purification and molecular properties of the wall-associated β-glucosidase from carrot cell cultures and investigates the ability of the purified enzyme to hydrolyse glucose-rich polymers and cell walls purified from carrot cell cultures.

RESULTS AND DISCUSSION

About 17% of the protein obtained by precipitation with $(NH_2)SO_4$ was retained on the CM-Sepharose CL-6B column and the enzyme activity was resolved into a major and a minor peak using a linear NaCl gradient (Fig. 1A). The presence of isoforms of the wall-associated β-glucosidase has also been reported for cell cultures of chick pea [9]. However, the data from maize roots [10] demonstrate that the minor peaks of β-glucosidase on isoelectric chromatography could be breakdown products of the major enzyme under acidic conditions; the wall-associated β-glucosidase is acid labile. Upon chromatography on CM-Sepharose, the minor peak is found at all stages of growth and changes significantly during the cell growth cycle (H. Konno, unpublished result), and so perhaps at least two isoenzymes would exist in the wall materials of carrot cell cultures. This is an intriguing result and is being further pursued. The major β-glucosidase in this experiment was bound to Con A-Sepharose and eluted with methyl-α-D-mannopyranoside and NaCl overlapping with the protein peak (Fig. 1B), suggesting a glycoprotein structure. Native PAGE on the final frac-

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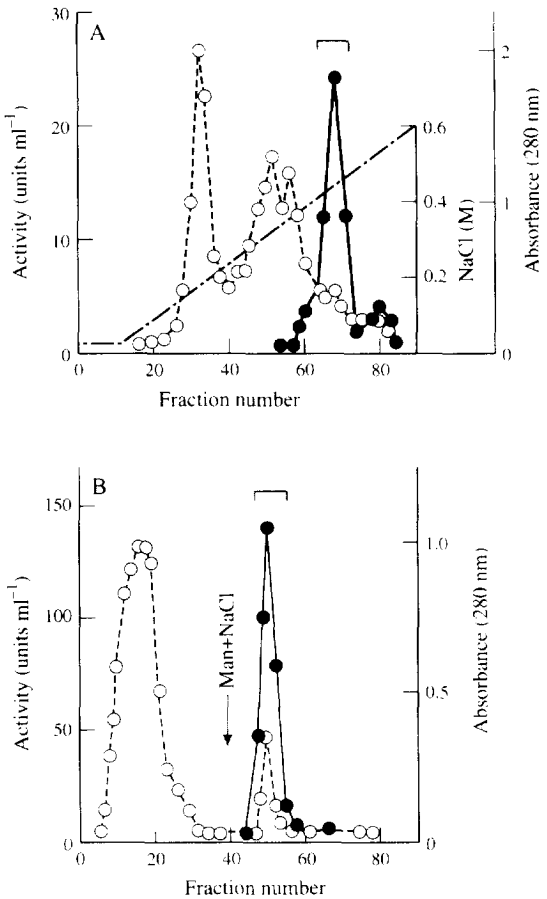

Fig. 1. Chromatography of cell wall-associated β-glucosidase from carrot cell cultures. A, CM-Sepharose CL-6B; B, Con-A Sepharose. (●) Enzyme activity; (○) A_{280}; (– – –) NaCl gradient. Bar indicates pooled fractions.

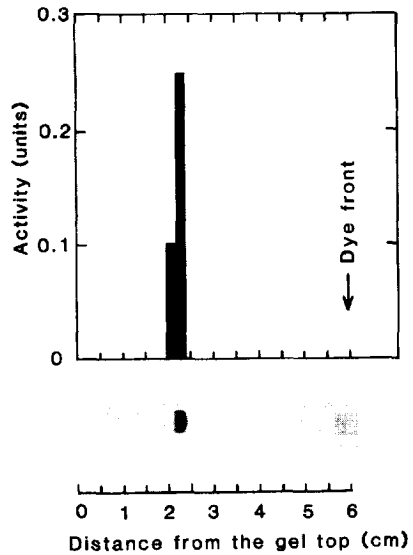

Fig. 2. Native PAGE of purified β-glucosidase. After electrophoresis of the native enzyme (*ca* 30 μg protein), part of the gel was stained for protein (bottom). The other part of the unstained gel was sliced into 2-mm segments, suspended in ice-cold 50 mM Na-acetate (pH 5.0) overnight at 2–4° and then assayed for enzyme activity (top).

tion gave a single protein band that coincided with the enzyme activity (Fig. 2). The data in Table 1, summarizing a typical purification sequence for β-glucosidase, show that a 374-fold increase in specific activity from the precipitate with $(NH_4)SO_4$ was reached with a recovery of 12%.

When analysed by SDS–PAGE under denaturing conditions, the purified β-glucosidase migrated as a band with M_r between 48 000 and 52 000. The M_r of the native enzyme, as estimated from its chromatographic mobility on the Sephacryl S-200HR column, was 46 000±3000. The native β-glucosidase thus appears to consist of a single polypeptide chain only. Some plant β-glucosidases consist of two different subunits (M_r 63000 and 43 000) [9] and two identical subunits (M_r 59 000 [11], 60 000–65 000 [12, 13], 24 000–28 000 [8]), whereas two β-glucosidases from pine xylem are large glycoproteins (M_r 90 000–110 000 [7]). The carrot enzyme also contained carbohydrate, as determined by the phenol–H_2SO_4 method [14] and protein in a ratio of 1:15 (w/w), which is compatible with the enzyme being a glycoprotein; similar properties were found for β-glucosidase from rubber leaves [15]. The p*I* of the purified enzyme was 8.2, as determined by isoelectric focusing on polyacrylamide gel. The result is consistent with the suggestion that the enzymes bound to cell walls appear to have a more alkaline isoelectric pH than cytosolic isozymes [10].

The amino acid composition was obtained by acid hydrolysis of purified the β-glucosidase (Table 2); Trp and Cys were not determined with the system used. The predominant amino acids were Ser and Gly, followed by Glx (Glu plus Gln), Ala and Thr, while the content of the acidic amino acid Asx (Asp plus Asn) was very low. The composition of carrot β-glucosidase thus differs much from those of β-glucosidases from butter bean [11] and maize [16]. Optimum activity for carrot β-glucosidase in McIlvaine's buffer was at pH 4.6–5.4, which is far removed from the alkaline pH optimum (pH 7.5) observed for the wall-associated β-glucosidase from chick-pea epicotyls [12]. The carrot enzyme displayed a half-maximal activity at pH 3.4 and 6.0, but the activity at pH values above 6.8 was very weak. Those results are of physiological interest because cell wall-loosening appears to occur only at pH 4 or below [1]. After incubation at various pH values for 20 hr at 30°, the enzyme was stable at pH 4.0–7.5 in McIlvaine's buffer, at pH 5.0–5.5 in 50 mM sodium acetate buffer and at pH 6.0–7.0 in potassium phosphate buffer, but *ca* 30% of its activity was lost at pH 7.0–8.6 in 50 mM Tris–HCl buffer. The temperature optimum for the activity was 50° when a 20-min reaction time was used. The activity of the enzyme was stable for at one month when stored at −20°. Incubation at pH 5.0 in 50 mM Na-acetate buffer without substrate for 20 min at temperatures of up to 45° had little effect on the

Table 1. Summary of purification of cell wall-associated β-glucosidase from carrot cell cultures

Purification sequence	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1})	Yield (%)	Purification (-fold)
Ammonium sulphate	3237	1384	2.34	100	1
CM-Sepharose CL-6B	2713	240	11.3	84	5
Con A-Sepharose	2152	20.4	105	66	45
Sephacryl S-200HR	1601	9.73	165	49	71
Preparative PAGE	403	0.46	876	12	374

enzyme, but 50% of the activity was destroyed when heated to 50°, and 100% when heated to 55°. The effect of 1 mM of metal ions and inhibitors on the activity was investigated after preincubation with the effectors for 20 min at 37°. Zn^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, and L-arabino-1,4-lactone had no effect on the activity. EDTA and D-galactono-1,4-lactone slightly inhibited the activity; while Cu^{2+} and Ag^{+} reduced it by 40–87%. Hg^{2+} and *p*-chloromercuribenzoate inhibited activity completely, suggesting an enzyme with sulphydryl groups at its active site. Carrot β-glucosidase and also that from sycamore cell cultures [17], was inhibited up to 80% by D-glucono-1,5-lactone a property that differs from the wall-associated β-glucosidase from chick-pea epicotyls [12].

The apparent K_m and V_{max} values for PNP-β-D-glucopyranoside determined from a Lineweaver–Burk plot were 0.12 mM and 0.13 units $(\mu g \text{ protein})^{-1}$, respectively. The K_m value was considerably lower than those of β-glucosidases from cell cultures (0.8 mM [9]) and epicotyls (2.2 mM [12]) of chick pea, butter bean (1.21 mM [11]) and maize shoots (0.58 mM [13]). Thus, carrot β-glucosidase has a higher affinity for the substrate than other β-glucosidases.

The purified β-glucosidase was tested for its ability to hydrolyse nine carbohydrate polymers and eight synthetic glycosides. Activity was found only in respect of PNP-β-D-glucopyranoside, PNP-β-D-cellobioside, lichenan and laminarin, where the amounts of product released were 79.2, 1.06, 0.30 and 0.05 (g protein)$^{-1}$ hr^{-1}, respectively. Therefore, the enzyme is capable of hydrolysing the 1,4-β-D-glucopyranosyl linkages in the polysaccharides in an exo-fashion, similar to its action on PNP-β-D-glucopyranoside used as substrate in the standard assay. It can be deduced that β-glucosidase is considered one of the key enzymes possibly responsible for cell wall metabolism, because 1,4-β-D-glucopyranosyl linkages are the main constituent of structural polysaccharides, such as cellulose, xyloglucan and β-glucan, of the cell walls [18]. To date, β-glucosidase has been detected in the cell suspension cultures of sycamore [17], chick pea [9] and cotton [19], but the physiological role in cell cultures remains unknown. Purified carrot β-glucosidase was tested for its ability to hydrolyse glucose-rich polysaccharides derived from carrot cell walls. The polysaccharides solubilized from cell walls depectinated with 1 M KOH could be fractionated on DEAE-Sepharose CL-6B into three polymers (fractions H-1, H-2 and H-3); sugar composition was determined for each polymer. Fraction H-1, which represented *ca* 77% of the yield, was rich in xylosyl (25.6% w/w) and glucosyl (17.8%) residues, indicating the presence of a significant amount of xyloglucan [20]. Fraction H-1 was further subfractionated into three fractions, H-1A, H-1B and H-1C, by gel-permeation chromatography on Bio-Gel A-1.5m. Fraction H-1C was composed of 56.9% xylosyl, 21.3% glucosyl, 7.03% galactosyl, 6.71% arabinosyl, 0.57% fucosyl, 0.19% rhamnosyl and 7.27% galacturonosyl residues, and was more enriched in glucosyl residues than other fractions. The M_r was estimated to be 36 000, as determined by comparison with the elution volume of linear dextrans of known M_r from the Bio-Gel A-1.5m column. After fraction H-1C was treated with purified β-glucosidase, the reaction products could not be detected by gel-permeation chromatography on Bio-Gel P-2 (profile not shown). Recently, it was reported that wall-bound β-glucosidase isolated from chick-pea epicotyls has hydrolytic activity on any polymers of the corresponding cell walls [12]. However, fraction H-1C used as substrate in this work is probably a xyloglucan with several short side-chains [18, 20]. It is conceivable, therefore, that the hindrance of hydrolysis by purified β-glucosidase is ascribed to the existence of some enzyme-insusceptible xylosyl linkages and several side-chains in the polymer.

Table 2. Amino acid composition of carrot β-glucosidase

Amino acid	mol %	Amino acid	mol%
Asx	1.57	Arg	2.13
Glx	12.98	Tyr	2.28
Ser	16.47	Val	4.24
Gly	15.40	Met	0.80
His	3.27	Ile	2.47
Thr	7.73	Leu	4.72
Ala	10.68	Phe	6.76
Pro	4.36	Lys	4.14

Since α-D- and β-D-xylosidases have been detected in the protein fraction extracted with 3 M LiCl from cell walls of carrot cell cultures (H. Konno, unpublished results), we must further consider a simultaneous action of β-glucosidase and xylosidases towards polymers and oligosaccharides [21] enriched in glucosyl and xylosyl residues. Finally, although the cell wall preparation was exposed to the action of β-glucosidase, no mono- and oligo-saccharides were released, even after an exhaustive reaction time.

EXPERIMENTAL

Plant material and enzyme source. Cells of carrot (*D. carota* L. cv. Kintoki) were cultured in the basal medium of ref. [22] containing 3% (w/v) sucrose as C source and 4.5 μM 2,4-D as growth regulator [3]. After 15–20 days of culture, cells were harvested by filteration through filter paper, washed with H_2O and stored at $-20°$ until required.

Enzyme extraction and purification. Cells (*ca* 800 g fresh wt) were suspended in 100 mM K-Pi (pH 7.0) containing 10 mM 2-mercaptoethanol and disrupted by sonication for 10 min at 0°. All subsequent steps and the purification procedure were conducted at 2–4°; all buffers used contained 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 8000 *g* for 15 min, and the ppt. (cell wall material) was collected, washed with 50 mM NaOAc (pH 5.2), resuspended in 50 mM NaO Ac (pH 5.2) containing 3 M LiCl and stirred overnight. Following centrifugation, the supernatant soln was concd by an Amicon Untrafiltration Cell (PM-10 membrane) and dialysed overnight against 50 mM NaO Ac (pH 5.2) containing 10 mM NaCl.

Protein in the LiCl-soluble fr. was ppted by addition of solid $(NH_4)_2SO_4$ until 3.4 M was reached. The resulting ppt. was collected by centrifugation, resuspended in 20 mM NaOAc (pH 5.2) containing 10 mM NaCl and dialysed against the same buffer. The dialysed soln was applied to a CM-Sepharose CL-6B column (3 × 20 cm) equilibrated with 20 mM NaOAc (pH 5.2) containing 10 mM NaCl. The column was then eluted with a 10–600 mM NaCl gradient in 20 mM NaOAc (pH 5.2); 15-ml frs were collected. The frs with the higher sp. act. were pooled, concd and dialysed against 20 mM NaOAc (pH 5.2) containing 1 mM methyl-α-D-mannopyranoside and 10 mM NaCl. The dialysed soln was applied to a Con A-Sepharose column (2 × 15 cm) equilibrated with the same buffer. The bound activity was eluted with 20 mM NaOAc (pH 5.2) containing 200 mM methyl-α-D-mannopyranoside and 1 M NaCl. Active frs were pooled, concd, dialysed against 50 mM K-Pi (pH 7.0) containing 150 mM NaCl and applied to a Sephacryl S-200HR column (1.5 × 90 cm) equilibrated with the same buffer; 2-ml frs were pooled. The most active frs were pooled, concd and further purified by prep. PAGE.

Enzyme assays. β-D-Glucosidase was measured by determining the release of *p*-nitrophenol from PNP-β-D-glucopyranoside. The reaction mixt. contained 0.1 ml enzyme and 2.5 mM substrate in 1 ml 50 mM NaOAc (pH 5.0). After incubation for 20 min at 37°, the reaction was terminated by addition of 2 ml 100 mM Na_2CO_3 and liberated *p*-nitrophenol was measured by *A* at 400 nm. A unit of activity is defined as that amount which hydrolysed 1 μmol of PNP-β-D-glucopyranoside hr^{-1} at 37°. Other glycosidase and glycanase activities were assayed using the corresponding PNP-glycosides and polysaccharide substrates, respectively [23]. The substrates tested were as follows: arabinan, arabinogalactan, carboxymethylcellulose, galactan, laminarin, lichenan, pectin, polygalacturonate, xylan and PNP-glycosides, such as α-L-arabinofuranoside, β-D-cellobioside, α-D and β-D-glucopyranosides, α-D and β-D-galactopyranosides, β-D-mannopyranoside, α-D- and β-D-xylopyranosides. Protein concn was determined by the method of ref. [24] using BSA as standard.

Preparation of cell walls and hemicellulosic polymer. Cell walls were prep. from *ca* 15-day-old cultures as described in ref. [3]. After cell walls were treated with CDTA and Na_2CO_3 to remove pectic polysaccharides [25], depectinated walls were extracted with 1 M KOH containing 20 mM $NaBH_4$ under N_2 for 2 hr at 20° [20]. The alkaline extract was acidified to pH 5 with HOAc, dialysed against H_2O and centrifuged at 10 000 g for 20 min. The supernatant soln was concd by evapn under red. pres. Polysaccharides were dialysed against 50 mM K-Pi (pH 6.0) and applied to a DEAE-Sepharose CL-6B column (2.0 × 20 cm), previously equilibrated with the same buffer, by sequential elution with buffer and buffers containing 125 and 250 mM NaCl. The polymer eluted with buffer alone was further purified by gel-permeation chromatography on a Bio-Gel A-1.5 m coulmn (1.5 × 90 cm) previously equilibrated with 50 mM NaOAc (pH 5.2) containing 20 mM EDTA [3]. Carbohydrates were eluted using the same buffer; 2-ml frs were collected. The contents of total carbohydrate and galacturonic acid in each sample were estimated by the PhOH–H_2SO_4 [12] and *m*-hydroxydiphenyl [26] methods, respectively. Neutral sugars were analysed by GC as their alditol acetate derivatives following trifluoroacetic acid hydrolysis [27].

Enzyme treatment of KOH-*soluble polymer and cell walls.* The KOH-sol. polymer (*ca* 4 mg sugar content) was incubated for 2 days at 30° with purified enzyme (2.7 units) in 10 ml 50 mM NaOAc (pH 5.0) containing 0.02% (w/v) NaN_3 and a drop of toluene. During prolonged incubation, further enzyme was added to the reaction mixt. after 2 days of reaction. After the reaction was terminated by heating at 100° for 3 min, the vol. was reduced by evapn, and the sample applied to a Bio-Gel P-2 column (1.5 × 90 cm), previously equilibrated with 50 mM NaOAc buffer (pH 5.2) containing 20 mM EDTA. Cell walls (50 mg dry wt) were treated with β-glucosidase as described above. After incubation, wall residues were separated from the digestion products by filtration on glass fibre filters. Products were further filtered in a Molcut L (UFP2-LGC filter) to remove the enzyme.

Electropheresis. Native enzyme was analysed by PAGE (7.5% gel) at pH 4.3 using the buffer system described in ref [28], and by isoelectric focusing in polyacrylamide gels (7.5%) as described in ref. [29]. Prep. PAGE was carried out on the gel slab (7.5% gel, 8 cm long, 2 mm thick) using the buffer system as described above under non-denaturing condition. After electrophoresis, part of the gel was strained for protein with 0.25% Coomassie Brilliant Blue R-250. The other part was used for enzyme prepn by soaking in ice-cold 50 mM NaOAc (pH 5.0) for 30 min; slices corre-

sponding to protein bands were cut with a slicer. Protein in each gel slice was eluted overnight with 50 mM NaOAc (pH 5.2). SDS–PAGE (10% gel) of denatured enzyme was conducted according to the method of ref. [30].

M_r *determination.* The M_r of native enzyme was estimated by gel-permeation chromatography on Sephacryl S-200HR according to ref. [25]. The M_r of denatured enzyme was determined by SDS–PAGE, following the conditions described above using a LMW Calibration Kit (Pharmacia).

Amino acid composition. The enzyme was filtered through a membrane with a M_r 10 000 cut-off, lyophilized and hydrolysed for 20 hr at 110° in 6 M HCl–PhOH vapour. After hydrolysis, amino acid composition was determined by analysis of PTH-derivatives [31].

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