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PURIFICATION AND PARTIAL CHARACTERIZATION OF β-GLUCOSIDASE FROM PAPAYA FRUIT

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Key Word Index—*Carica papaya*; Caricaceae; papaya fruit; β-glucosidase.

Abstract—β-Glucosidase (I) was isolated from *Carica papaya* fruit pulp and purified *ca* 1000-fold to electrophoretic homogeneity. The procedure used ammonium sulphate fractionation followed by chromatography on Phenyl-Sepharose CL-4B and Sephacryl S-200 to separate α-mannosidase (II) and, in part, β-galactosidase (III) from (I). Final separation of (III) from (I) was achieved by preparative isoelectric focusing (PIEF). The glycosidases had pI of 5.2 (I), 4.9 (II) and 6.9 (III). M_rs of 54 000 (I), 260 000 (II) and 67 000 (III) were determined by gel filtration. The M_r of (I) estimated by SDS-PAGE was 27 000 suggesting that (I) consisted of two subunits. The optimum pH and optimum temperature of (I) were 5.0 and 50°, respectively, and the enzyme followed typical Michaelis kinetics with K_m and V_{max} of 1.1×10^{-4} M and 1.8×10^{-6} mol/hr, respectively, for *p*-nitrophenyl-β-D-glucoside (40°).

INTRODUCTION

Although the primary role of glycosidases is considered to be the hydrolysis of secondary plant glycosides, the exact physiological role is in most cases far from understood [1]. With our present state of knowledge, an essential prerequisite for the evaluation of glycohydrolases is thorough purification of the enzymes. Since different activities of a single type may occur in a single plant tissue [2], a clear-cut separation of the enzymes under study from other activities has to be achieved before determination of their properties is possible.

In the course of our studies on flavour precursors in plants, recently first results about the occurrence of terpenoid and other β-D-glucosides in *Carica papaya* fruit were obtained [3, 4]. Continuing this work, we were interested to investigate the enzymes responsible for their formation and hydrolysis [5], i.e. glucosyltransferases and glucosidases. The present paper concerns the β-glucosidase (EC 3.2.1.21) from papaya fruit pulp.

RESULTS AND DISCUSSION

Among the glycosidases present in *Carica papaya* β-galactosidase (EC 3.2.1.23) together with α-mannosidase (EC 3.2.1.24) and β-glucosidase showed the highest activity in extracts of the fruit pulp (Table 1). A clear dependence of activity on the ripening stage could not be observed; nearly identical variations of activity were determined in a series of batches of different ripening stage. Three other glycosidases, β-*N*-acetyl-glucosaminidase (EC 3.2.1.36) (100–200 nkat/kg fruit pulp), β-*N*-acetylgalactosaminidase (EC 3.2.1.53) and β-xylosidase (EC 3.2.1.37) (each 80–160 nkat/kg fruit pulp) had lower activity in the extracts. α-Glucosidase (EC 3.2.1.20), β-fucosidase (EC 3.2.1.38), β-glucuronidase (EC 3.2.1.31) and β-thioglucosidase (EC 3.2.3.1) could not be detected in papaya fruit pulp. The last mentioned has been found previously in papaya seeds [6].

The extraction of the glycosidases from papaya fruit pulp was done with 0.5 M ammonium sulphate solution, and about 95% of the β-glucosidase activity was recovered in the subsequent ammonium sulphate cut (Table 2). When the redissolved and dialysed (1 M ammonium sulphate) preparation was applied to a Phenyl-Sepharose CL-4B column (Fig. 1), from the bound glycosidases about 60% of the total α-mannosidase was eluted by washing with 1 M sodium chloride solution. Elution of β-glucosidase was achieved using 50 mM phosphate buffer (pH 7.6). The β-glucosidase fractions still contained the remaining α-mannosidase and about 30% of the total β-galactosidase activity. The pooled fractions, enriched 47.6-fold in β-glucosidase activity (Table 2), were ultrafiltered and applied to a Sephacryl S-200 column. Figure 2 shows the elution profiles of the three glycosidases during gel filtration. while α-mannosidase was completely separated from β-glucosidase by this step, in spite of an additional 3-fold enrichment of β-glucosidase (Table 2), these fractions still showed β-galactosidase activity.

As ultrathin-layer isoelectric focusing (UIEF) of the ammonium sulphate fractionation from papaya fruit pulp showed pI of 5.2, 4.9 and 6.9 for β-glucosidase, α-mannosidase and β-galactosidase, respectively (Fig. 3), isoelectric focusing should be a powerful tool for the separation of the remaining β-galactosidase activity from β-glucosidase. In fact, subsequent use of preparative isoelectric focusing (PIEF) led to electrophoretic homogeneity of the β-glucosidase (Fig. 3). The purified enzyme was enriched 952-fold with a recovery of 30.7 % (Table 2). At the same time an UIEF homogeneous β-galactosidase was obtained by this step, but as already demonstrated by different authors [7, 8] including our group [9], in some cases, affinity column chromatography delivered faster results, e.g. one-step purification of β-galactosidase. However, in our recent work on papaya fruit glycosidases, this technique failed to separate abundant β-galactosidase activity from β-glucosidase [9].

The M_r of the α-mannosidase was found to be 260 000

Table 1. Activity of three glycosidases in crude extracts of papaya fruit pulp

Ripening stage	Activity (nkat/kg fruit pulp)*		
	α-mannosidase	β-galactosidase	β-glucosidase
Unripe	660–1500	3200–5000	300–470
Ripe	1000–1830	2800–5500	330–500
Overripe	1000–1500	2800–5000	300–500

*Ranges each determined from ten batches of different ripening stage.

Table 2. Purification of β-glucosidase from papaya fruit pulp

Purification step	Vol. (ml)	Total act. (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)	Purification factor*	Recovery* (%)
Crude extract	5300	842	14300	0.059	1	100
1.0–2.8 M ammonium sulphate cut	350	804	920	0.874	14.8	95.4
Phenyl-Sepharose CL-4B	210	492	175	2.811	47.6	58.4
Sephacryl S-200	20	353	38	9.289	157.4	42.0
Preparative isoelectric focusing	25	258	4.6	56.174	952.1	30.7

*Calculated with respect to the crude extract.

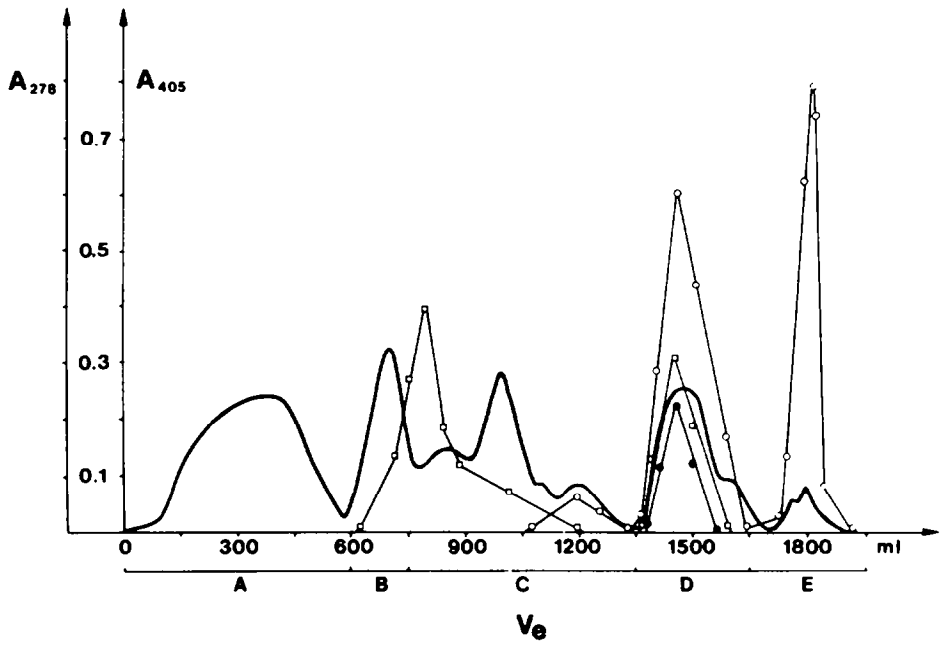

Fig. 1. Hydrophobic chromatography on Phenyl-Sepharose CL-4B of papaya fruit pulp glycosidases. A, 1 M $(NH_4)_2SO_4$; B, 1 M NaCl; C, linear gradient from 1 M to 100 mM NaCl; D, 50 mM phosphate buffer (pH 7.6); E, 50 mM phosphate buffer (pH 7.6) containing 50% ethylene glycol. (—) $A_{278\,nm}$; (□) α-mannosidase; (○) β-galactosidase; (●) β-glucosidase.

by gel filtration; with the same technique the M_rs of β-galactosidase and β-glucosidase were determined to be 67000 and 54000, respectively. Similar M_rs have been established previously for these glycosidases from different plant origin [2]. The M_r of (I) estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was 27000 suggesting that (I) consisted of two subunits.

The purified β-glucosidase showed half maximal activity at pH 3.9 and 5.7 and its pH optimum (5.0) was

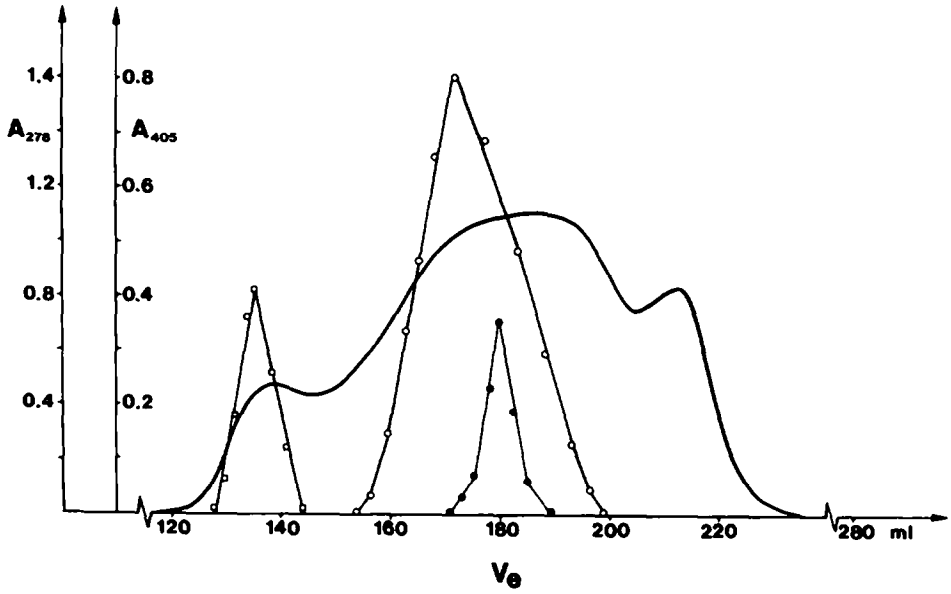

Fig. 2. Elution profiles of papaya fruit pulp glycosidases during gel filtration on Sephacryl S-200. (—) $A_{278\ nm}$; (□) α-mannosidase; (○) β-galactosidase; (●) β-glucosidase.

similar to that of some other plant β-glucosidases [2]. The values of apparent K_m and V_{max} were 1.1×10^{-4} M and 1.8×10^{-6} mol/hr for *p*-nitrophenyl-β-D-glucoside (40°). The few times that they have been determined, similar data have been found for different plant β-glucosidases [2].

In contrast to several plant glycosidases, which have been found to exhibit multiple forms or activities [1, 2], the three above mentioned glycosidases each showed a single activity and a single pI value. Thus, the β-glucosidase from papaya fruit pulp clearly differs, e.g. from the common almond enzyme (emulsin), which has been described to display multiple forms and β-glucosidase/β-galactosidase activity [10–12]. While β-glucosidase from *Carica papaya* has not been studied as yet, α-mannosidase and β-galactosidase have been partially purified from papaya seeds [13]. Since the published M_rs and pI values do not correspond with our findings obtained from the fruit pulp enzymes, it seems to be likely that different enzymes are active both in seeds and pulp.

EXPERIMENTAL

Fruits. Fresh papaya fruits (Solo variety) were obtained by air-freight from Brazil. Unripe fruits (green colour; pale, firm pulp) were separated from ripe ones (green–yellow colour; pink, soft pulp). In part, the fruits were allowed to overripen until developing an 'off-flavour' (cf. Table 1).

Enzyme assays. The glycosidases [β-glucosidase (I), α-mannosidase (II), β-galactosidase (III)] were determined by measuring release of *p*-nitrophenol (PNP) from PNP-conjugated substrates. Assays containing 20–100 μl enzyme and 1 ml 4 mM substrate in 100 mM NaOAc buffer (I: pH 5.0; II/III and other glycosidases: pH 4.5) were incubated at 40°. After an appropriate time (10–30 min), the reaction was stopped by the addition of 2 ml 200 mM borate buffer (pH 9.8). The reactions were linear for at least 60 min and were directly proportional to the amount of enzyme present. The $A_{405\ nm}$ was measured and the amount of PNP was determined from $\varepsilon = 18.5$ M/mN/cm.

Separation and purification of β-glucosidase. Ripe papaya fruits (2 kg) were peeled and cut into two pieces; the seeds were carefully separated. The initial extraction was carried out by placing the fruits and 4 l. 500 mM $(NH_4)_2SO_4$ in a blender and homogenizing for 1 min. The extract was centrifuged at 20000 *g* for 30 min and the supernatant decanted. Solid $(NH_4)_2SO_4$ was added to the supernatant and the material precipitated until 2.8 M was collected by centrifugation. The $(NH_4)_2SO_4$ pellet was suspended in 50 mM NaPi buffer (pH 7.6) and dialysed against 1 M $(NH_4)_2SO_4$. The material that precipitated was removed by centrifugation and discarded.

The dialysed soln was loaded onto a Phenyl-Sepharose CL-4B column, 2.5 × 30 cm, equilibrated with 1 M $(NH_4)_2SO_4$. The gel was treated with 1 M $(NH_4)_2SO_4$ until unbound protein was completely washed out. Subsequent washing was carried out using one column vol. of 1 M NaCl, a linear gradient of four column vols from 1 M to 100 mM NaCl, and two column vols of 50 mM NaPi buffer (pH 7.6) as well as finally two column vols of the same buffer containing 50% ethylene glycol.

The β-glucosidase active fractions from the Phenyl-Sepharose CL-4B column were pooled and ultrafiltered (3 bar, N_2) to 6 ml using an Amicon cell (Kalle TU-AN-4045.380 membrane). The ultrafiltrate was applied to a Sephacryl S-200 column, 1.6 × 140 cm, equilibrated with 50 mM NaPi buffer (pH 7.0). Gel filtration was performed using the same buffer. The peaks of the glycosidases were separated and collected as shown in Fig. 2.

M_r *determinations.* The M_rs of the glycosidases I–III were determined by gel filtration [14] on Sephacryl S-200 as described above. The column was calibrated and a standard curve of V/V_0 vs log M_r for the calibration proteins was used to determine the M_rs of I–III. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified (I) was carried out according to ref. [15].

Preparative isoelectric focusing (PIEF). Biogel P 60 (4 g),

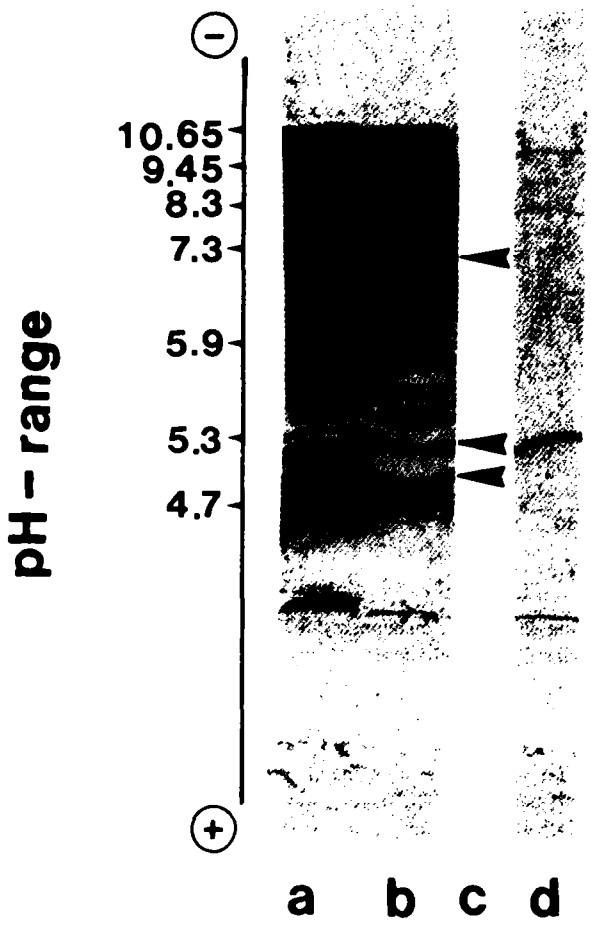

Fig. 3. Ultrathin-layer isoelectric focusing of papaya fruit pulp glycosidases in 50 μm polyacrylamide gels [17]. (a) Protein staining of marker proteins; (b) protein staining of papaya fruit pulp [$(NH_4)_2SO_4$ cut]; (c) arrows: positions of papaya fruit pulp glycosidases after enzyme visualization (from top to bottom, β-galactosidase; β-glucosidase; α-mannosidase); (d) protein staining of PIEF purified β-glucosidase. Separation distance, 10 cm; sample, 10 μl of the ultrafiltered enzyme preparation. Carrier ampholytes, 1:1 mixture of pH 2–11 and 4–9 Servalyt AG. Conditions: prefocusing, 400 V, 30 min; focusing, 400 V, 20 min; 1000 V, 10 min; 1500 V, 5 min.

swollen in H_2O for a few days and thoroughly washed with H_2O was added under stirring to the gel filtered β-glucosidase active fractions (20 ml + 40 ml H_2O). Carrier ampholytes (2 ml; Servalyt T, pH 4–9) was added and the gel applied to a 20 × 20 cm glass trough. PIEF was carried out according to the procedure of ref. [16] using a Desaga Mediphor chamber. Voltage was applied through platinum electrodes which were in contact with the gel layer through pads of MN 866 paper (Macherey & Nagel) soaked with the corresponding electrode solns (0.5 M H_2SO_4 at the anode and 2 M ethylene diamine at the cathode). The focusing program was 200 V for 15 hr; 400 V/1 hr; 600 V/1 hr; 800 V/1 hr and 1000 V/1 hr using a Desaga power supply 1200/200. Cooling water at 4° was circulated through the apparatus. After focusing, the pH gradient was measured in the layer at regular 1 cm intervals along the focusing track (pH electrode EA 156; Deutsche Metrohm). The gel was divided into 1 cm strips corresponding to the pH measurement points. Each gel strip was removed from the trough and transferred to a small elution column. Elution was performed using 50 mM NaPi buffer (pH 7.0) to yield solns suitable for subsequent enzyme assay.

Ultrathin-layer isoelectric focusing (UIEF). UIEF in 50 μm PAG gels was carried out as previously described [17]. For the visualization of enzymes after UIEF the substrates for ultrathin-layer agarose gels on polyester films [17] were prepared as follows. Agarose soln (2%) in 100 mM NaOAc buffer (I: pH 5.0; II/III: pH 4.5) was prepared under heating. After cooling to about 40°, 10 mg 4-methylumbelliferyl-conjugated glycoside was added. After UIEF the PAG gel was immersed in satd $(NH_4)_2SO_4$ soln (5 min), washed with H_2O, covered with the agarose gel and incubated at 40° for 10–20 min. Blue fluorescent bands in UV-light (366 nm) exhibiting glycosidase activities were detected after NH_3 treatment.

Protein measurements. Protein content was determined according to the method of ref. [18] with bovine serum albumin as standard.

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