Purification and Characterization of a Beta-Glucosidase from *Citrus sinensis* var. Valencia Fruit Tissue

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A preliminary survey demonstrated activity for α-D-glucosidase, α-D-mannosidase, α-L-arabinosidase, β-D-glucosidase, β-D-xylosidase, and β-D-galactosidase in orange fruit flavedo and albedo tissue. α-L-Rhamnosidase was not detected. Subsequently, a β-glucosidase was purified from mature fruit rag tissue (composed of intersegmental septa, squeezed juice sacs, and fruit core tissue) of *Citrus sinensis* var. Valencia. The β-glucosidase exhibited low levels of activity against *p*-nitrophenyl-β-D-fucopyranoside (13.5%) and *p*-nitrophenyl-α-D-glucopyranoside (7.0%), compared to its activity against *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, 100%). The enzyme was purified by a combination of ion exchange (anion and cation) and gel filtration (Superdex and Toyopearl HW-55S) chromatography. It has an apparent molecular mass of 64 kDa by denaturing electrophoresis or 55 kDa by gel filtration chromatography (BioGel P-100). Hydrolysis of *p*NPG demonstrated a pH optimum between 4.5 and 5.5. At pH 5.0 the temperature optimum was 40 °C. At pH 5.0 and 40 °C the K_m for *p*NPG was 0.1146 mM and it had a V_{max} of 5.2792 nkatal·mg^{-1} protein (katal = 0.06 International Units = the amount of enzyme that produces, under standard conditions, one μmol of product per min). Of the substrates tested, the enzyme was most active against the disaccharide cellobiose (1⟹4), but was not active against *p*-nitrophenyl-β-D-cellobioside. High levels of activity also were observed with the disaccharides laminaribiose (1⟹3), gentiobiose (1⟹6), and sophorose (1⟹2). Activity greater than that observed with *p*NPG was obtained with the flavonoids hesperetin-7-glucoside and prunin (naringenin-7-glucoside), salicin, mandelonitrile-β-D-glucoside (a cyanogenic substrate), and sinigrin (a glucosinolate). The enzyme was not active against amygdalin, coniferin, or limonin glucoside.

Keywords: *Citrus; fruit; glucosidase; enzyme; glycosidase*

INTRODUCTION

Glycosidases are capable of hydrolyzing a wide variety of substrates present in processed fruit juice and processing residues. Among these naturally occurring substrates are residual cell-wall materials, which comprise a considerable proportion of processing byproducts (*1*), glycosidic flavor and aroma precursors (*2*), and phytonutrient components (*3*,*4*).

A limited survey of glycosidase activity present in sweet orange fruit juice vesicles (*5*) revealed relatively low levels of α-galactosidase, β-galactosidase, α-glucosidase, and β-glucosidase activity. No activity for α-arabinosidase, β-xylosidase, α-mannosidase, or β-mannosidase was observed. In a similar survey of grapefruit peel and juice vesicles (*6*), minimal amounts of activity for α-arabinosidase and α-glucosidase were detected (quantitative data were not reported). Low activity levels were observed for α-galactosidase, β-galactosidase, and α-mannosidase. Burns and Baldwin (*6*) also reported that activity for β-glucosidase, α- and β-fucosidase, β-xylosidase, and β-mannosidase was not detected in their acetone powder extracts.

Citrus glycosides of potential economic importance include cell wall polysaccharides which may be fermented to ethanol (*7*), limonoid glucoside (*8*), hesperidin (*9*), and naringenin (*10*). Citrus flavor components (e.g., linalool and α-terpineol, *11*) may also occur in a glycosylated form as reported for other fruits (*2*). Because the majority of these glycosides have glucose as the glycan moiety we elected to pursue the isolation and characterization of a β-glucosidase from *Citrus sinensis* var. Valencia fruit tissue following a preliminary survey of seven different fruit peel glycosidases. Our specific objectives were (1) to screen citrus fruit tissue for various glycosidase activities, (2) to characterize a β-glucosidase present in citrus fruit for the reasons noted above, and (3) to attempt to identify the types of glucosides on which the β-glucosidase was active.

MATERIALS AND METHODS

Tissue Extracts. Fruit tissue extracts were prepared from fresh Valencia oranges as previously described (*12*). Briefly, the fruit were halved and hand juiced with a kitchen-type juicer. Rag tissue (intersegmental septa, squeezed juice sacs, and fruit core tissue) was hand-separated from peel (flavedo and albedo). Rag (4 kg) and peel tissue were frozen at −20 °C until needed. Protein in the rag tissue was extracted with three volumes of 0.1 M Tris (pH 8.0 at 27 °C), 1 M NaCl, and 0.02% NaN_3 (w/v). After the sample was centrifuged to pelletize the remaining solids, the supernatant was brought to 75% ammonium sulfate saturation to precipitate solubilized proteins. After pelleting the precipitated proteins and resolubilizing them in 10 mM Tris (pH 7.5 at 31 °C), 20 mM NaCl, and 0.02% NaN_3 (TBS), the protein solution was dialyzed (6000 Da molecular weight cutoff) overnight at 4 °C against a total of four 4-L buffer changes.

Chromatographic Separations. Anion-exchange chromatography (DEAE Sephacel, Sigma) was used to survey peel

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tissue extracts for various glycosidase activities. DEAE Sephacel chromatography, CM-Sepharose (Amersham Pharmacia Biotech), Superdex 200 (Amersham Pharmacia Biotech), Toyopearl HW-55S (TosoHaas), and BioGel P-100 chromatography were used to purify and characterize β-glucosidase. Chromatography was performed at room temperature and fractions were stored at 4 °C as they were collected.

(a) Anion Exchange Chromatography. Peel or rag protein extracts were loaded onto the DEAE Sephacel column (2.6 × 9.5 cm). For surveying the peel extract for glycosidase activity, 3 mL of the extract was loaded and the column was washed with 30 mL of TBS. A gradient of 20 mM to 1 M NaCl in TBS was run over 90 mL of buffer. The buffer was held at 1 M NaCl for 30 mL and then reduced to 20 mM NaCl over 5 mL. Fractions (3 mL) were collected throughout the run at a flow rate of 1 mL·min^{-1}. DEAE chromatography of rag extracts involved loading 20 mL of the extract onto the column (2.6 × 10 cm) at 1.5 mL·min^{-1}, washing with 285 mL of TBS, and eluting bound proteins with a 270-mL gradient from 20 mM to 250 mM NaCl in TBS. The column buffer was then brought to 1 M NaCl over 7.5 mL, and the remaining bound proteins were eluted over 262.5 mL of 1 M NaCl in TBS. Fractions (6-mL) were collected throughout the run. Bound fractions containing β-glucosidase activity were pooled, and proteins were precipitated with 75% ammonium sulfate saturation. After the sample was centrifuged at 12100*g* for 30 min at 4 °C, the pellets were resolubilized in 10 mM $NaPO_4$ (pH 6.0) and 0.02% NaN_3. The resolubilized protein was dialyzed against 1.5 L of 10 mM $NaPO_4$ (pH 6.0 buffer), and 0.02% NaN_3 for 24 h with two more changes of buffer.

(b) Carboxymethyl Sepharose Chromatography. The resolubilized β-glucosidase material from DEAE chromatography was applied to a CM-Sepharose column (2.6 × 10 cm) at 10 mL per run. The column was washed at 1.0 mL·min^{-1} with 40 mL of 10 mM $NaPO_4$ (pH 6.0), 0.2 M NaCl, and 0.02% NaN_3. Proteins remaining bound to the matrix were eluted with a linear gradient of 0.2 to 0.5 M NaCl in 10 mM $NaPO_4$ (pH 6.0) and 0.02% NaN_3 over 110 mL. The salt concentration was then brought to 1 M NaCl and washed with 40 mL of 10 mM $NaPO_4$ (pH 6.0), 1 M NaCl, and 0.02% NaN_3 before regenerating the column with 10 mM $NaPO_4$ (pH 6.0) and 0.02% NaN_3. Fractions of 1 mL each were collected throughout the run.

Following gel filtration chromatography and a buffer change, the β-glucosidase-containing fractions were rechromatographed over a smaller CM-Sepharose column (1.0 × 20.5 cm) at 0.5 mL·min^{-1}. The column was washed with 20 mL of 10 mM $NaPO_4$ (pH 6.0), 0.3 M NaCl, and 0.02% NaN_3. Bound proteins were eluted with a 60 mL linear gradient of 0.3 to 0.5 M NaCl in 10 mM $NaPO_4$ (pH 6.0) and 0.02% NaN_3. Fractions (1-mL) were collected throughout the run.

(c) Superdex 200 Gel Filtration Chromatography. Superdex 200 gel filtration chromatography (1.6 × 59 cm) of the β-glucosidase-containing material from the initial CM-Sepharose chromatography was accomplished after a buffer change to 0.2 M NaCl in TBS. The enzyme was loaded in a 2-mL volume and run at 1.0 mL·min^{-1}. One-mL fractions were collected.

(d) Toyopearl Gel Filtration Chromatography. Toyopearl HW-55S gel filtration chromatography (1.6 × 70 cm) of β-glucosidase obtained after Superdex chromatography was run with 1 M NaCl in TBS. The flow rate was 1.0 mL·min^{-1} and 1-mL fractions were collected.

(e) BioGel P-100 Gel Filtration Chromatography. BioGel P-100 gel filtration chromatography (1.6 × 50 cm) of the β-glucosidase activity peak from Toyopearl gel filtration chromatography was run with 1 M NaCl in TBS. The flow rate was 0.15 mL·min^{-1} and 0.5-mL fractions were collected.

Activity Assays. *(a) Spectrophotometric.* Peel extracts were screened for α-D-glucosidase, α-D-mannosidase, α-L-arabinosidase, α-L-rhamnose, β-D-glucosidase, β-D-xylosidase, and β-D-galactosidase activities using respective *p*-nitrophenyl glycosides as a substrate (Sigma). These assays were conducted in 50 mM sodium acetate buffer (pH 4.5) containing 3.5 mM of the appropriate glycoside (*6*) and 25 μL of the column fraction being assayed in a final volume of 500 μL. The assay was run for 30 min at 37 °C. The reaction was stopped by the addition of 1 mL of 0.2 M $NaCO_3$ and the absorbance was read at 405 nm on a Shimadzu UV160U spectrophotomer. The amount of *p*-nitrophenol formed was determined by constructing a standard curve with *p*-nitrophenol.

Column fractions were screened, and kinetics, pH optimum, and temperature optimum for β-D-glucosidase were determined with *p*-nitrophenyl-β-D-glucoside. The pH optimum was determined at 37 °C in 50 mM citrate−phosphate buffer at the appropriate pH and was run for 15 min. The temperature optimum was determined with 50 mM citrate−phosphate buffer at pH 5.0. Kinetics were determined with 50 mM citrate−phosphate buffer (pH 5.0, 40 °C) and substrate concentrations ranging from 0.025 mM to 5 mM. Activity against other glycosides was determined with 3.5 mM *p*-nitrophenyl derivatives in 50 mM citrate−phosphate buffer (pH 5.0, 40 °C), and run for 10 min. Values reported for pH and temperature optimum, kinetics, and glycoside specificity are means of three replicates. The effect of various salts, EDTA, glucose, and gluconolactone on activity were tested by inclusion in the reaction cocktail along with *p*-nitrophenyl-β-D-glucoside.

(b) Determination of β-D-Glucosidase Activity by Release of Glucose. Hydrolysis of β-oligosaccharides or β-glucosides produces one or two molecules of glucose per each molecule of substrate cleaved. Glucose was separated and determined by ion exchange chromatography using dilute sodium hydroxide as the eluant (*13*, *14*) with a pulsed amperometric detector (Dionex Corp., Sunnyvale, CA) as previously described (*1*). The detector response was calibrated with glucose (Sigma), and 2-deoxy-D-galactose was used as an internal standard. This methodology was used to determine relative activity against cellobiose, sophorose, laminaribiose, gentiobiose, prunasin (mandelonitrile β-D-glucoside), salicin, sinigrin, arbutin, amygdalin, indoxyl β-D-glucoside, and *p*-nitrophenyl-β-D-glucoside (used as standard for comparison).

(c) Determination of Citrus Phenols. The reaction mixture contained 1 mg·mL^{-1} of the corresponding substrate and one unit of enzyme in 50 mM citrate−phosphate buffer (pH 5.0) and reacted at 40 °C. The reaction was run for 2 h, and it was stopped by immersing the mixture in a boiling water bath for five min. Hydrolysis of phenolic glucosides was carried out with 0.9 unit of enzyme activity in 50 mM citrate−phosphate buffer (pH 5.0) and reacted at 40 °C for 1.5 h. Substrate concentration was 0.1 mg·mL^{-1}. The reaction was stopped by immersion in a boiling water bath for five min. Measurements of the production of aglycons from their corresponding glucosides were made with high-pressure liquid chromatography (HPLC) using a Perkin-Elmer 250 binary LC pump with a Hewlett-Packard System 1050 photodiode array detector and 1050 Chromatography Workstation. The phenolic glucosides were analyzed with an Alltech Altima 5 μm analytical column (4.6 mm × 10 cm), using a two-solvent gradient composed of 10 mM phosphoric acid/methanol (90/10, v/v), and increased to 10 mM phosphoric acid/methanol (22/78, v/v) with a linear gradient over 35 min. A final composition of 90% methanol was achieved by a subsequent linear gradient over 20 min with flow rates of 0.75 mL·min^{-1}. This method was used for hesperetin dihydrochalcone glucoside, hesperetin-7-glucoside, prunin, phlorin, coniferin, limonin glucosid, and *p*-nitrophenyl-β-D-glucoside (used as standard for comparison).

Electrophoresis. *SDS−PAGE.* Denaturing electrophoresis (25 μL·fraction^{-1} were loaded per lane) was conducted according to Laemmli (*15*) in a Mini-Protean II dual-slab cell electrophoresis unit (Bio-Rad) on 12.5% acrylamide gels. The gels were stained with Coomassie Brilliant Blue R. Denatured molecular weight was estimated using low-range molecular weight standards (Bio-Rad).

Protein Assays. Protein concentrations were estimated by the bicinchoninic acid method according to the manufacturers directions (micro BCA protein assay reagent kit, Pierce)

RESULTS AND DISCUSSION

Following DEAE chromatography of peel extracts, activity was observed for six of the seven *p*-nitrophenyl

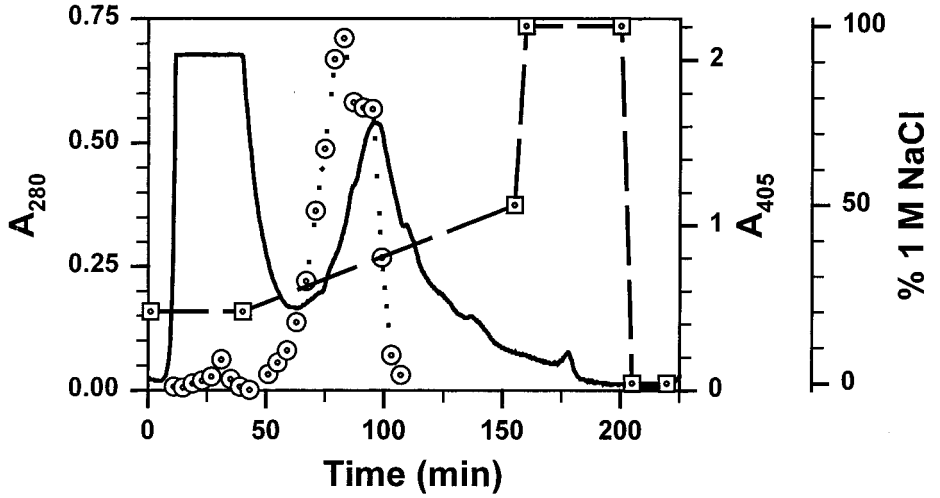

Figure 1. CM-Sepharose chromatography of pooled, active fractions from DEAE chromatography. — = A_{280}, ⊙ = A_{405}, ⊡ = NaCl.

Table 1. Purification Table for DEAE Bound β-D-glucosidase from 4 kg of Fruit Rag Tissue.

sample	μg pro·μL^{-1}	μMol PNP·min^{-1}·μL^{-1}	μMol PNP·min^{-1}·μg^{-1} pro	purification factor
DEAE	9.297	0.0217	0.0023	1
CM-Seph	0.647	0.0056	0.1600	69.6
Superdex	0.047	0.0314	0.6681	290.5
Toyopearl	0.043	0.0483	1.1259	489.5

glycosidases tested (α-rhamnosidase, β-xylosidase, β-galactosidase, α-manosidase, and α-arabinosidase, data not shown, β-glucosidase, α-glucosidase). Rhamnosidase activity was the only one not observed. Five of the glycosidases exhibited two peaks of activity, one which did not bind to the matrix and one which did and could be eluted with a NaCl gradient. Mannosidase activity, while present in the flow-through, demonstrated a higher peak in the bound material. Arabinosidase and xylosidase activity were higher in the unbound material than in the bound material.

The combination of ion exchange and gel filtration chromatography resulted in a purification factor of 489.5 following Toyopearl gel filtration chromatography for this β-D-glucosidase (Table 1). The specific activity following this step (1125.9 Units·mg^{-1} protein) is within the range of previously reported values following purification (*3*, *16*). The purification factor prior to DEAE chromatography could not be determined because of the presence of phenolics in the crude extract that masked absorption at 405 nm.

Two peaks of β-D-glucosidase activity were observed after DEAE chromatography (data not shown). The first peak did not bind to the matrix and eluted early in the flow-through. The second activity peak did bind to the matrix and was eluted between 50 and 180 mM NaCl. Fractions contained within this second peak were pooled for further chromatography whereas the first activity peak was discarded.

Elution of β-D-glucosidase activity from the CM-Sephadex column occurred between 380 and 450 mM NaCl (Figure 1). Fractions within this activity peak were pooled, concentrated, and then applied to a Superdex 200 gel filtration column (Figure 2). Elution of β-D-glucosidase from this column occurred at a time which correlated with a molecular weight of 11 kDa. Preliminary electrophoresis of these fractions demonstrated a densely staining polypeptide band at approximately 64 kDa, in close agreement for β-glucosidase from other published reports (*17*−*20*). This led us to believe that the β-D-glucosidase was interacting with the Superdex 200 matrix which is composed of dextran (glucose) cross-linked to porous agarose beads. Consequently, the fractions containing β-D-glucosidase activity were loaded onto a Toyopearl HW-55S gel filtration column (Figure 3) which is composed of spherical beads produced by the copolymerization of ethylene glycol and

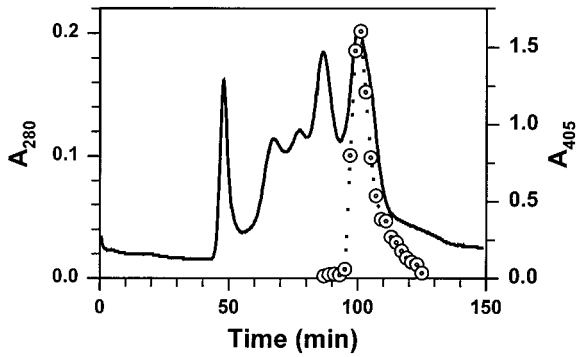

Figure 2. Superdex gel filtration chromatography of pooled, active fractions from CM-Sepharose chromatography. — = A_{280}, ⊙ = A_{405}, ⊡ = NaCl..

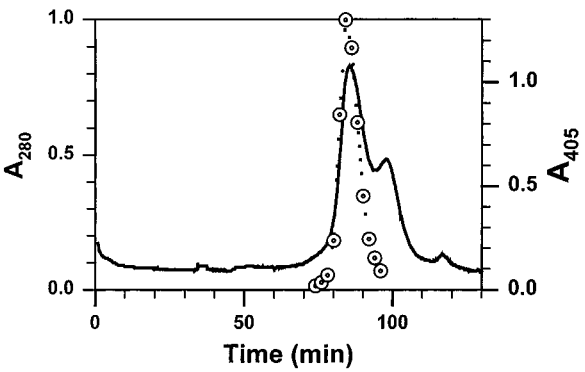

Figure 3. Toyopearl gel filtration chromatography of pooled, active fractions from Superdex chromatography. — = A_{280}, ⊙ = A_{405}, ⊡ = NaCl.

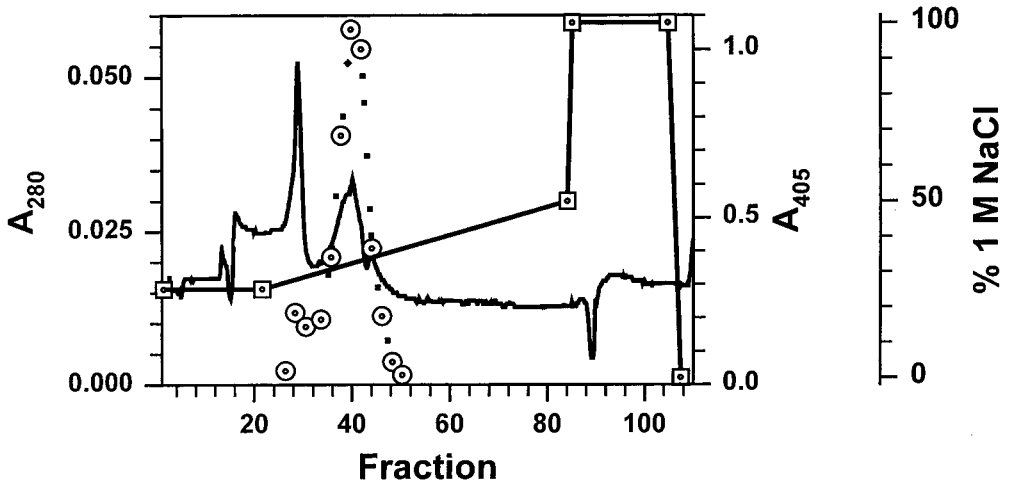

Figure 4. CM-Sepharose rechromatography of pooled, active fractions from Toyopearl chromatography. — = A_{280}, ⊙ = A_{405}, ⊡ = NaCl.

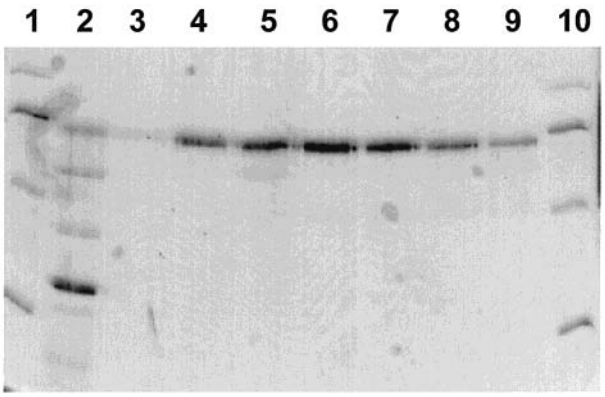

Figure 5. Denaturing electrophoresis of β-glucosidase active fractions following CM-Sepharose rechromatography (Figure 4). Lane 1 and 10 = MW Markers (BioRad, Low Range; 97.4, 66.2, 45.0, and 31 kDa from top to bottom); lane 2 = fraction 31; lane 3 = fraction 40; lane 4 = fraction 42; lane 5 = fraction 44; lane 6 = fraction 46; lane 7 = fraction 48; lane 8 = fraction 50; and lane 9 = fraction 52. A 25-μL portion of each fraction was loaded onto the gel which was stained with Coomassie Brilliant Blue.

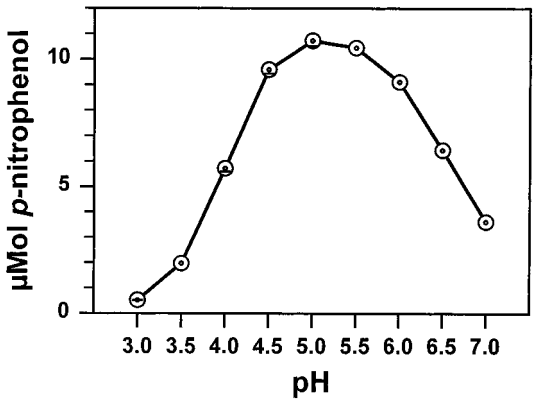

Figure 6. β-Glucosidase activity at various pH values (50 mM citrate−phosphate buffer, 37 °C, 15 min, 20 μL of purified enzyme).

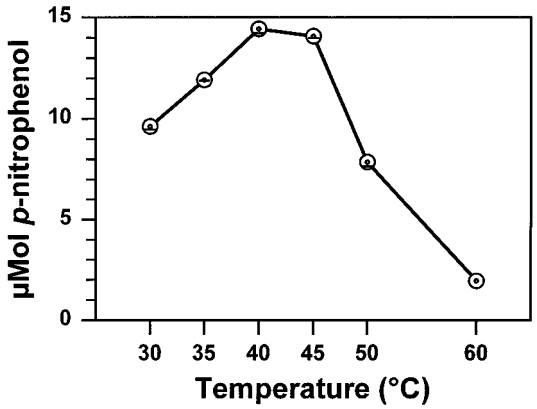

Figure 7. β-Glucosidase activity at various temperatures (50 mM citrate−phosphate buffer, pH 5.0, 20 μL of purified enzyme).

methacrylate type polymers. Elution of the activity peak on the Toyopearl HW-55S column corresponded to a molecular weight estimate of 24.5 kDa, larger than that of the Superdex but still much smaller than that suggested by denaturing electrophoresis. An aliquot of this sample was then run on the BioGel P-100 column and had an elution volume equivalent to a molecular weight of 55 kDa (data not shown).

Following Toyopearl gel filtration chromatography, that portion of the β-D-glucosidase peak that was not run on the BioGel P-100 column was rechromatographed on the CM-Sepharose column (Figure 4). Denaturing electrophoresis of an equal volume from fractions within the β-D-glucosidase peak indicated the enzyme had been purified and suggested a molecular weight of 64 kDa (Figure 5). Optimum pH for this enzyme was between 4.5 and 5.5 (Figure 6), which is in close agreement to values reported for a cell-wall-bound β-glucosidase from rice (*17*) and carrot suspension culture cells (*16*), and leaves of several tropical plants (*21*). At pH 5.0 the optimum temperature was 40−45 °C (Figure 7). At pH 5.0 and 40 °C the K_m for *p*-nitrophenyl-β-D-glucoside was 0.1146 mM, which is very close to that reported for cell walls of carrot suspension cells (*12*, *14*, *16*) but lower than found for β-glucosidases from leaves of three tropical plants (0.30−3.89 mM, *21*). It had a V_{max} of 5.2792 nkatal·mg^{-1} protein (katal = 0.06 International Units = the amount of enzyme that produces, under standard conditions, one μmol of product per min).

A survey with other *p*-nitrophenyl glycosides indicates this enzyme is very specific for β-D-glucose (Table 2). Of the other glycosides tested the most active besides β-D-glucose (100%) were β-D-fucoside (13.5%), α-D-glucoside (7.0%), and α-L-arabinoside (3.7%). Metal ions

Table 2. Relative Activity of β-D-glucosidase on Various *p*-Nitrophenol Glycosides (3.5 mM substrate concentration, 50 mM citrate−phosphate, pH 5.0, 40 °C, one unit enzyme).

substrate	relative activity (%)
p-nitrophenol-β-D-glucopyranoside	100
p-nitrophenol-β-D-cellobioside	0.1
p-nitrophenol-β-D-fucopyranoside	13.5
p-nitrophenol-β-D-xylopyranoside	1.9
p-nitrophenol-β-D-galactopyranoside	0.1
p-nitrophenol-β-D-galactouronide	0.0
p-nitrophenol-α-D-glucopyranoside	7.0
p-nitrophenol-α-D-mannopyranoside	1.1
p-nitrophenol-α-D-rhamnopyranoside	1.9
p-nitrophenol-α-D-arabinofuranoside	3.7

Table 3. Effect of the Addition of Various Substances on Relative Activity of β-D-glucosidase (assay conditions same as described for Table 2)

substance	relative activity (%)
p-nitrophenol-β-D-glucopyranoside	100.0
1 mM $FeCl_3$	100.0
1 mM $CaCl_2$	100.0
1 mM $ZnSO_4$	112.0
1 mM $MgCl_2$	98.0
1 mM EDTA	104.0
10 mM EDTA	105.0
1 mM Indoxyl β-D-glucoside	111.0
10 mM gluconolactone	9.3
1 mM gluconolactone	29.4

Table 4. Relative Activity of β-D-glucosidase on Various β-D-glucosides.

substrate	relative activity
p-nitrophenol-β-D-glucopyranoside[a, b]	100
cellobiose (1⇒4)[a]	609
laminaribiose (1⇒3)[a]	519
gentiobiose (1⇒6)[a]	483
sophorose (1⇒2)[a]	430
prunasin (mandelonitrile-β-D-glucoside)[a]	309
sinigrin[a]	325
arbutin[a]	104
salicin[a]	390
hesperitin dihydrochalcone glucoside[b]	91
hesperitin-7-glucoside[b]	199
naringenin-7-glucoside[b]	138
amygdalin[b]	0
coniferin[b]	0
limonin glucoside[b]	0

[a] Assayed in 50 mM citrate−phosphate buffer, pH 5.0, 40 °C with one Unit of enzyme for 2 h. [b] Assayed in 50 mM citrate−phosphate buffer, pH 5.0, 40 °C for 1.5 h with 0.9 units of enzyme.

had minor, if any, effect on its activity as did EDTA (Table 3). Gluconolactone was an effective inhibitor (Table 3) as was glucose (Figure 8). Interestingly, whereas *p*-nitrophenyl-β-D-cellobioside was not an effective substrate (Table 2), the highest observed activity was against cellobiose (Table 4). The position of the bond between two glucoses did have an effect on activity, although all three (1⇒2; 1⇒3; and 1⇒6) β-disaccharides were hydrolyzed (Table 4). The enzyme was also able to hydrolyze a cyanogenic mono-glucoside (prunasin, but not the diglucoside parent compound amygdalin), a thioglucoside (sinigrin), phenolic glucosides (arbutin and salicin), and three of five flavonoid glucosides tested (hesperetin dihydrochalcone glucoside, hesperetin-7-glucoside, and naringenin-7-glucoside, Table 4). There was no activity against coniferin or limonin glucoside.

The wide variety of substrates from which this enzyme was capable of hydrolyzing glucose makes it problematic to assign its in vivo role. The highest activity observed was with the glucose disaccharides, suggesting it may have a role in cell-wall metabolism and hydrolysis of fruit processing residues. Its activity against phenolic and flavonoid glucosides indicates a potential for utilization in phytonutrient modification.

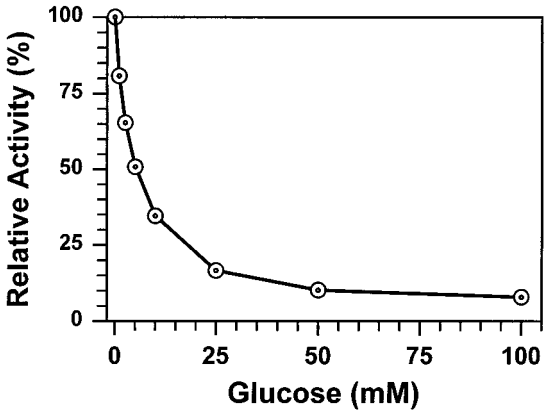

Figure 8. Inhibition of β-Glucosidase by glucose (50 mM citrate−phosphate buffer, pH 5.0, 40 °C, 20 μL of purified enzyme).

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