Phytochemistry, Vol. 29, No. 8, pp. 2425–2429, 1990.
Printed in Great Britain.

0031–9422/90 $3.00 + 0.00

α- AND β-GALACTOSIDASE ACTIVITIES IN JUICE VESICLES OF STORED VALENCIA ORANGES*

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(*Received in revised form* 12 *December* 1989)

Key Word Index—*Citrus sinensis*; Rutaceae; glycosidase activity; postharvest; cell wall.

Abstract—Several glycosidase activities were assessed in extracts of Valencia juice vesicles of citrus fruit. Of the enzymes assayed, α- and β-galactosidase were most active, but α- and β-glucosidase were also found. The galactosidases were partially purified and their activities assessed during eight weeks of storage at 10°. Total α-galactosidase activity remained constant but β-galactosidase declined after the eight-week storage period. Both galactosidases were resolved into two enzymatic forms by ion-exchange and gel filtration chromatography. Each enzymatic form of the galactosidases had different substrate specificities, pH optima, and responses to inhibitors. α-Galactosidase I, α-galactosidase II, β-galactosidase I, and β-galactosidase II had K_m values of 0.47, 0.23, 0.27 and 0.77 mM, and apparent M_rs of 36 300, 39 800, 56 200 and 57 200, respectively. Only α-galactosidase II was able to release reducing groups from isolated cell wall material of juice vesicles.

INTRODUCTION

Limited information is available on the activities of enzymes which modify oligo- and polysaccharides present in juice vesicles of fresh citrus fruit. Of the enzymes that modify the extracellular components, pectinesterase (EC 3.1.1.11) is more abundant [1] and has received the most attention. During development and maturation of Valencia oranges, pectinesterase activity present in juice vesicles remained relatively unchanged until an increase occurred at the latter portion of the maturation period [2]. Substantial polygalacturonase (EC 3.2.1.15) activity in fresh citrus peel and juice vesicles is lacking [1, 3], and therefore, major alterations of the polygalacturonic acid backbone before postharvest storage appear unlikely. Intracellular enzymes that hydrolyse storage oligosaccharides (with the exception of disaccharides) have not been studied because the substrates are apparently not present in juice vesicles [4].

Glycosidases have been studied from a wide variety of plant sources and can occur both extra- and intracellularly. In the present work, several glycosidase activities were determined in juice vesicles of Valencia orange during eight weeks of storage. Of the enzymes tested, α-galactosidase (EC 3.2.1.22) and β-galactosidase (EC 3.2.1.23) activities were most prevalent, and the enzymes were partially purified and characterized.

RESULTS

The glycosidase activities of juice vesicles of freshly harvested Valencia oranges are shown in Table 1. β-Galactosidase was most active, followed by α-galactosidase, α-glucosidase (EC 3.2.1.20), and β-glucosidase (EC 3.2.1.21). No other glycosidase activities were found when the corresponding *p*-nitrophenyl glycosides (α-arabinoside, β-xyloside, α-mannoside, or β-mannoside) were used as substrate. The specific activity and the activity on a fresh weight basis of β-galactosidase decreased significantly after eight weeks storage at 10° (Fig. 1). α-Galactosidase activity remained constant throughout the storage period. Polygalacturonase activity was absent in the crude extracts at all storage periods evaluated.

Two peaks of α-galactosidase and one peak of β-galactosidase activity were resolved on DEAE-Sephadex (Fig. 2I). One peak of α-galactosidase (α-gal I) and the β-galactosidase activity were eluted together in the void volume, whereas the remaining α-galactosidase activity (α-gal II) was partially retained and eluted in later fractions. Further chromatography of the galactosidase activity in the DEAE-Sephadex void volume on CM-Sephadex (Fig. 2II) resulted in the separation of two peaks of β-galactosidase activity. Fractions 11–16 contained one peak of β-galactosidase activity (β-gal I) and fractions 28–32 a second peak of β-galactosidase (β-gal II) and the α-gal I activity. Chromatography of α-gal II on CM-Sephadex (Fig. 2III) did not result in

Table 1. Glycosidase activities in crude extracts of freshly harvested Valencia orange juice vesicles

Glycoside	Enzyme activity	
	nkat 200 g^{-1} fr. wt	nkat mg^{-1} protein
β-galactosidase	27.1 ± 5.9	1.2 ± 0.3
α-galactosidase	14.9 ± 3.4	0.7 ± 0.1
α-glucosidase	4.6 ± 1.5	0.1 ± 0.1
β-glucosidase	3.2 ± 0.3	0.1 ± 0.1

The values represent the means ± s.e. of at least four separate experiments.

*Florida Agricultural Experiment Station Journal Series No. R-00244.

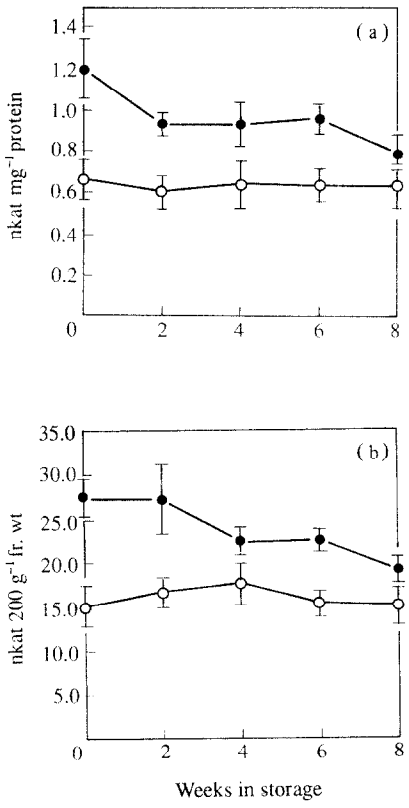

Fig. 1. Levels of α-galactosidase (○) and β-galactosidase (●) in juice vesicles of stored Valencia oranges. (A) Activity mg^{-1} protein and (B) activity per 200 g juice vesicles.

further resolution of additional enzyme forms but did serve to remove contaminating material. Satisfactory separation of α-gal I and β-gal II was achieved by Sephadex G-100 chromatography (Fig. 2IV). Column chromatography of β-gal I and β-gal II resulted in 18- and 14-fold purification, whereas 60- and 98-fold purification was achieved with α-gal I and α-gal II, respectively. Preliminary results suggest that β-galactosidase loses activity with time, even at −15°, as has previously been reported [5]. A summary of the purification scheme is shown in Table 2.

The calculated M_rs of the α-galactosidases were 36 000 and 39 800 for α-gal I and α-gal II, respectively. These values are similar to reported M_r of α-galactosidase in tomato [6] and soybean seeds [7]. Molecular weights were also calculated for β-gal I (56 200) and β-gal II (57 200), and are similar to some enzymatic forms found in soybean seeds [8] and tomato [5].

The *p*-nitrophenyl galactosides were hydrolysed more efficiently by the galactosidases than any other substrate tested (Table 3). Stachyose was utilized more efficiently as substrate by α-gal I than by α-gal II, whereas raffinose was more efficiently hydrolysed by a α-gal II than α-gal I. Of the gums used as substrate, only minimal hydrolysis was observed by α-gal II with locust bean gum. Myo-inositol was a more effective inhibitor of the hydrolysis of *p*-nitrophenyl α-galactoside with α-gal II ($K_i = 143$ mM) than α-gal I ($K_i = 319$ mM). Galactose and galactolactone inhibited the hydrolysis of substrate, although more effectively with β-gal II ($K_i = 3$ and 0.6 mM, respectively) than β-gal I ($K_i = 5$ and 0.7 mM, respectively). Lactose was not a substrate for β-gal I, but was an effective substrate for β-gal II.

The pH optima were assessed for each form of galactosidase studied, and the enzymatic activities were similar in acetate and citrate buffers. The maximum activity of α-gal I was determined to be pH 5.0. A broad effective pH range was detected for α-gal II, with the optimum pH being 4.5. The pH optimum for β-gal I was 2.0 and for β-gal II was 4.0. Reaction rates were measured for each enzymatic form with *p*-nitrophenyl galactoside as substrate. The K_m values were calculated to be 0.47, 0.23, 0.27 and 0.77 mM for α-gal I, α-gal II, β-gal I, and β-gal II, respectively.

Extraction of the total galactosidase activity sequentially in water and 1.0 M NaCl indicated that 75 and 89% of the α- and β-galactosidase activity, respectively, was found in the salt-soluble fraction. Both water and salt-soluble fractions contained similar proportions of α- and β-galactosidase isozymes. Incubation of partially purified α-gal II (1.0 nkat m^{-1}) with isolated cell wall material prepared from freshly harvested juice vesicle tissue resulted in release of 0.72 μg reducing groups mg^{-1} cell wall material hr^{-1}. No release of reducing groups occurred when α-gal II (1.0 nkat m^{-1}) was incubated with water-soluble pectin isolated from the same source. Partially purified α-gal I and β-galactosidase isozymes failed to release reducing groups from either water-soluble pectin or cell wall material isolated from fresh mature Valencia juice vesicles.

DISCUSSION

Galactosidases have been found in a number of plant sources. In rapidly growing tissues such as those of germinating seeds, galactosidase activities are associated with hydrolysis of storage vacuolar oligosaccharides [9], cell wall polysaccharides [10], and galactoproteins in protein bodies [11, 12]. Cell wall modification as a role for galactosidases has been implicated during growth of germinating seeds [13], carrot cell suspension cultures [14], and in fruit tissues such as apple [15] and tomato [5], where hydrolysis of specific cell wall galactans has been demonstrated. Extracts of citrus fruit juice vesicles contain two galactosidases, each with two enzymatic forms. Only α-gal II, however, was able to release reducing groups from isolated cell walls of juice vesicles. This suggests that cell wall modification and/or turnover may occur in the juice vesicles of citrus. That α-gal II can hydrolyse larger substrates is also indicated by the release of reducing groups from locust bean gum, a galactomannan polymer.

Over 75% of the α- and β-galactosidase activities were recovered in the salt-soluble fraction which suggests that the majority of the activity is bound to the cell wall. Caution is warranted in this interpretation, because compartmentalized soluble enzymes can easily adhere to the insoluble fraction [16]. Because α-gal I and the β-galactosidase forms were incapable of acting on cell wall material, a role for the enzymes in cell wall modification appears doubtful. More likely would be their role in galactoprotein modification, but this was not explored in this study. Storage oligosaccharide hydrolysis is also unlikely, because these substrates are not present in juice vesicles of citrus [4]. The fact that inositol is present in

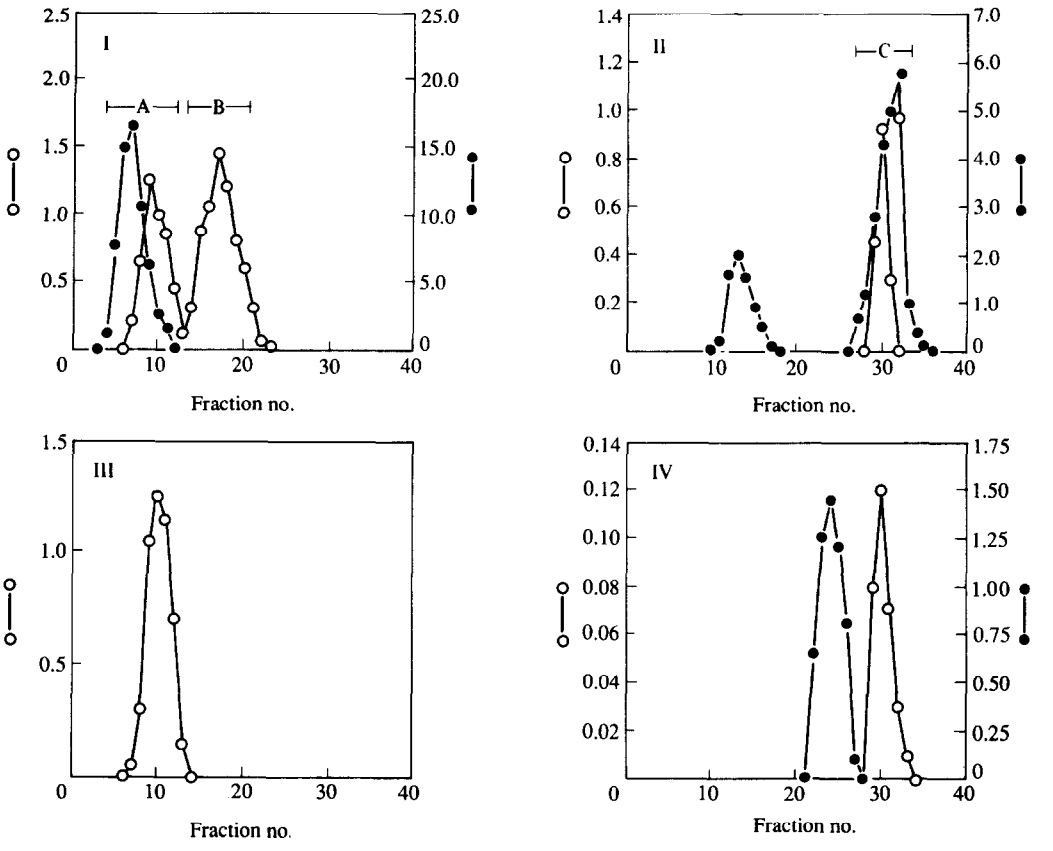

Fig. 2. Chromatographic separation of α-galactosidase (○) and β-galactosidase (●) and their enzymatic forms. Activities of the galactosidases are expressed in nkat fraction^{-1}. Panel I, crude extract on DEAE-Sephadex; Panel II, peak A on CM-Sephadex; Panel III, peak B on CM-Sephadex; Panel IV, peak C on Sephadex G-100.

Table 2. Summary of the purification of α-galactosidase and β-galactosidase from fresh 'Valencia' orange juice vesicles

	Enzyme form					
	I			II		
	Total protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1})	Total protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1})
α-Galactosidase						
Lyophilized pellet	100.0	20.9*	0.2	—	—	—
DEAE-Sephadex	16.4	6.3	0.4	4.3	11.8	2.7
CM-Sephadex	5.7	3.4	0.6	0.4	7.8	19.5
Sephadex G-100	0.2	2.4	12.0	—	—	—
β-Galactosidase						
Lyophilized pellet	100.0	72.1†	0.7	—	—	—
DEAE-Sephadex	16.4	30.9‡	1.9	—	—	—
CM-Sephadex	0.6	7.5	12.5	5.7	11.7	2.1
Sephadex G-100	—	—	—	0.9	9.1	10.1

*Total α-galactosidase activity in the lyophilized pellet.
†Total β-galactosidase activity in the lyophilized pellet.
‡Total β-galactosidase activity recovered by DEAE-Sephadex chromatography.

Table 3. The effect of various substrates on galactosidase activity

Substrate	% relative activity of galactosidases			
	α-gal I	α-gal II	β-gal I	β-gal II
p-Nitrophenyl-α-galactoside	100	100	0	0
Stachyose	62	14	—	—
Raffinose	39	74	—	—
Locust bean gum	0	4	—	—
Guar gum	0	0	—	—
p-Nitrophenyl-β-galactoside	0	0	100	100
Lactose	—	—	0	88

juice vesicles [4] suggests that this compound may play a role in modifying the activity of both forms of α-gal. However, reported concentrations of inositol in this tissue are less than 6 mM and is far less than the measured K_i of both α-gal I and α-gal II.

In conclusion, the results show that two enzymatic forms of α- and β-galactosidase are present in the juice vesicles of citrus. The enzymes can be resolved by ion-exchange and gel filtration chromatography. Each form differs in pH optima, substrate specificity, and response to inhibitors. Each form of α- and β-galactosidase has similar M_r, but can be separated by net charge using ion-exchange chromatography. Further research is needed to clarify the role of these enzymes in the juice vesicles of citrus.

EXPERIMENTAL

Plant material. Mature Valencia oranges were obtained from groves at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. Fruit were washed, fungicide applied, waxed according to established commerical procedure and placed at 10°. After a 16 hr equilibration period, fruit for the initial time period were removed for evaluation which was repeated every 2 weeks for 8 weeks. At least 5 fruit were used at each time period, and the experiment replicated × 4.

Preparation of extract. Juice vesicles were removed from mature Valencia oranges and homogenized in cold H_2O (100 g tissue: 150 ml H_2O). All subsequent steps were carried out at 4°. The homogenate was stirred for 30 min, then brought to 1 M salt by addition of solid NaCl. After the pH was adjusted to 6.0 with 1 M KOH, the homogenate was stirred for an additional hour. The homogenate was clarified by centrifugation at 10000 *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 65% satn over a 2 hr period. The ppt. proteins were collected by centrifugation at 10000 *g* for 20 min, and dialysed overnight against 4 l 0.1 M NaCl. Insoluble material which was formed during dialysis was removed by centrifugation. The supernatant was lyophilized and stored at −15° until needed. Lyophilized powder was resuspended in 0.05 M Na acetate buffer, pH 5.0, (10 mg powder ml^{-1}) and centrifuged to remove insoluble material. The resulting supernatant represented the crude extract.

Cell wall and alcohol-insoluble solids preparation. Juice vesicles (100 g) were homogenized and filtered through miracloth. The retained material was washed twice with 2 l distilled H_2O and twice with 1 l Me_2CO. The retained material was then transferred to 200 ml $CHCl_3$–MeOH (2:1) and stirred for 10 min. Cell wall material was recovered by filtration and stored until needed in a vacuum dessicator at room temp. Alcohol-insoluble solids were prepared from juice vesicles by sequential extraction in H_2O, chelate (2 mM EDTA), and NaOH (0.05%) as previously described [2]. Uronic acids present in the cell wall material or AIS were determined by the method of ref. [17].

Estimation of protein. Protein concentration was determined by the method of ref. [18]. Bovine serum albumin was used as standard. The amount of protein present in chromatographic fractions was measured by absorbance at 280 nm.

Enzyme assays. α-D-Galactosidase and β-D-galactosidase activities were measured by following the release of *p*-nitrophenol from *p*-nitrophenyl α-D-galactopyranoside and *p*-nitrophenyl β-D-galactopyranoside substrates (Sigma). Other glycosidase activities (α-arabinosidase, α- and β-mannosidase, α- and β-glucosidase and β-xylosidase) were assessed by the use of appropriate *p*-nitrophenyl glycoside substrates. The reaction mixture contained 1.0 ml of 0.05 M NaOAc (pH 5.0), 0.1 ml of enzyme solution, and 0.4 ml of 3.5 mM substrate (final concentration). Boiled enzyme solution was used for a blank. After incubation at 40° for 15 min, the reaction was terminated by the addition of 2 ml of 0.2 M Na_2CO_3, and the liberated *p*-nitrophenol was measured by absorbance at 405 nm [5, 6]. Enzymatic activity was expressed in nkat (nmol *p*-nitrophenol liberated sec^{-1}), and specific activity in nkat mg^{-1} protein. Polygalacturonase activity was assayed as previously described [19], with incubation times of up to 3 days.

Partial purification of galactosidases. Lyophilized powder which contained 50–60 mg protein was resuspended in 5.0 ml of 0.05 M NaOAc buffer in 0.1 M NaCl, pH 6.0. Insoluble material was removed by centrifugation, and the resulting supernatant was applied to a column of DEAE-Sephadex A-50 (2.5 × 40 cm) equilibrated in the same buffer. The proteins were eluted from the column with the equilibration buffer at a flow rate of 25 ml hr^{-1}. Active fractions containing α- or β-galactosidase activity were pooled and lyophilized. The lyophilized powders (20 mg protein) were resuspended in 5 ml 1 mM NaOAc buffer and applied to a column of CM-Sephadex A-50 (2.5 × 40 cm) equilibrated in 0.05 M NaOAc and 0.25 M NaCl, pH 5.2. Elution of proteins was with the equilibration buffer at a flow rate of 25 ml hr^{-1}. Fractions of 6.8 ml were collected. Appropriate fractions were collected and lyophilized.

Molecular weight determination. Lyophilized column fractions (6–10 mg protein) were dialysed against 0.1 M NaCl overnight and then applied to a column of Sephadex G-100 (2.5 × 95 cm). Proteins were eluted with 0.1 M NaCl at a flow rate of 30 ml hr^{-1}. Fraction size was 8.3 ml. Standards and their elution volumes were blue dextran 137 ml, bovine serum albumin 203 ml, egg serum albumin 237 ml, and cytochrome *c* 344 ml. Fractions which contained enzymatic activity were pooled and lyophilized.

Substrate specificity and inhibitors. Lyophilized powder from CM-Sephadex or Sephadex G-100 column fractions were resuspended in a small volume of 0.05 M NaOAc buffer. The ability of α-galactosidase to hydrolyse 0.05 M stachyose, raffinose, 0.1% locust bean gum, guar gum, water-soluble pectic fractions (125 μg galacturonic acid ml^{-1}) and cell wall material (15 mg ml^{-1}) was determined. Oligosaccharides were incubated for 1 hr and polysaccharides for 24 hr with enzyme (0.86 nkat ml^{-1}) at 40°. Substrate specificities of the β-galactosidases were assessed by incubation of the enzyme (0.9 nkat ml^{-1}) with 3.5 mM lactose, water-soluble pectin and cell wall material isolated from juice vesicles and prepared as above. The reaction was terminated by boiling and the insoluble material removed by centrifugation. Release of reducing groups in the resulting supernatant was measured by the method of ref. [20]. Galactose was used as standard. Boiled enzyme mixture incubated with substrate was used as control. The effectiveness of inhibitors (myo-inositol, galactose, and galacto-lactone) was determined by preincubation of the enzyme with inhibitors of various concentrations for 15 min at 40°, and then hydrolysis of *p*-nitrophenyl substrates was measured as described above. The pH optima of each of the enzymatic forms was determined at pH ranges between 1.0 and 7.0 with 50 mM Na acetate or Na citrate buffers.

Extractability of the galactosidases. Juice vesicles were homogenized in H_2O (100 g tissue: 150 ml H_2O) and stirred for 30 min at 4°. Soluble and insoluble (bound) components were collected by centrifugation. Insoluble components were further extracted with 1.0 M NaCl at pH 6.0, stirred for 30 min as above, and collected by centrifugation. $(NH_4)_2SO_4$ was added to the soluble and insoluble fractions to satn (soluble 69%, insoluble 75%). The pptd proteins were collected by centrifugation, dialysed against 0.01 M NaCl, and lyophilized.

Acknowledgement—The excellent technical assistance of Ms Kim Bates is gratefully appreciated.

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