Isolation and Identification of Galactinol from Castor Oilseed Meal[1]

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Galactinol, 1-*O*-α-D-galactopyranosyl-D-*myo*-inositol, is an essential intermediate in the biosynthesis of raffinose saccharides in plant tissues. The unavailability of a commercial source for this metabolite has hindered research on raffinose saccharide metabolism to date. The objective of this study was to develop a facile method for obtaining highly purified galactinol from a readily available source. Defatted castor oilseed meal was found to exhibit a simple soluble carbohydrate profile that included galactinol. Purification of galactinol from castor meal was achieved by enzymatic treatment and a series of liquid chromatography steps, including ion-exchange and carbon adsorption. The isolation procedure was reliable with a yield of 278 mg of galactinol from 150 g of defatted meal. The purity of galactinol was estimated at 96.4% by high-performance liquid chromatography. The identity of the purified galactinol was confirmed by enzymatic analysis, identical relative retention times on high-performance liquid chromatography and gas chromatography, and comparison of its mass spectrum to that of authentic galactinol.

KEY WORDS: Castor oilseed, chromatography, defatted meal, galactinol, invertase.

Galactinol, 1-*O*-α-D-galactopyranosyl-D-*myo*-inositol (1), serves as the galactosyl donor in the formation of the abundant oligosaccharides of the raffinose family in higher plants (2,3). In contrast to the raffinose saccharides, galactinol is present at relatively low levels in various photosynthetic leaf tissues (4–6), soybean seeds (7–9), sugar beet molasses (10), and cold-stored potato tubers (11). While galactinol has been isolated from sugar beet molasses by Brown and Serro (10), and McCready *et al.* (12), and from leaf extracts of *Lamium maculatum* by Senser and Kandler (6) and *Cucumis sativus* by Pharr *et al.* (13), it is unavailable commercially. Furthermore, a routine method for obtaining sufficient quantities of galactinol is limited to a few laboratories where suitable source material, techniques and facilities are available. Consequently, studies of the raffinose biosynthetic pathway, necessary for understanding plant carbon metabolism and improving food quality, have been hindered due to the unavailability of this metabolite.

In a previous study (14), we have characterized soluble oligosaccharides in 33 different dry seeds by high-performance liquid chromatography (HPLC). An unknown peak with a retention time similar to authentic galactinol was present in several different seed extracts. The objectives of the present study were to confirm the identity of galactinol in one of these seed extracts, namely castor bean (*Ricinus communis*), and to develop an effective method for its purification from castor meal.

EXPERIMENTAL PROCEDURES

Seed meal and chemicals. Defatted castor (*Ricinus communis*) meal, an industrial processing by-product after oil extraction, was kindly supplied by Dr. K.C. Rhee, Texas A&M University, College Station, Texas. The meal comprised several unknown varieties of castor seeds. Ultrapure authentic sugar standards were acquired from Pfanstiehl Laboratories (Waukegan, IL), except for L-iditol which was from Aldrich (Milwaukee, WI). Galactinol isolated from sugar beet syrup was a gift of Dr. L. Lehl, University of Regensburg, Germany. Activated charcoal was obtained from Ruger Chemical (Irvington, NJ) and Celite 535 from Johns-Manville (Lompoc, CA). Ion-exchange resins (200–400 mesh), AG 50W-X8 (H^+) and AG 1-X8 (Cl^- and OH^-), were obtained from Bio-Rad (Richmond, CA). The Cl^- form of AG-1 was converted to OH^- form by washing the resin with about 20 vol of 1 N NaOH and then with water until free of Cl^- ion. Preswollen microgranular celluloses, DE 52 and CM 52, were from Whatman (Clifton, NJ). Trifluoroacetic acid was obtained from Pierce (Rockford, IL) and absolute ethanol from U.S. Industrial Chemicals (Tuscola, IL). Sugar-Pak I, a Ca^{2+}-loaded cation-exchange HPLC column was purchased from Waters Associates (Milford, MA). Distilled water used in this study was further purified by a Nanopure II ion-exchange system (Sybron/Barnstead, Boston, MA).

Isolation procedures. All isolation steps were performed at room temperature (23°C), unless specified otherwise. Disposable gloves and dust respirators (No. 8710; 3M Co., St. Paul, MN) were used to handle defatted meal in a fume hood, and the residues left after ethanol extraction and the reacted ion-exchangers were treated as biohazardous materials. Sample volumes were routinely reduced *in vacuo* at 40°C with a Buchi Rotavapor (Brinkmann Instruments, Westbury, NY). Galactinol was first isolated and identified from 25-g samples of defatted castor meal and the isolation procedure was subsequently scaled up to routinely handle 150 g of material. The isolation procedure consisted of the following four steps: *i) Carbohydrate extraction.* Soluble carbohydrates were extracted from 150 g defatted castor meal with 700 mL 80% (vol/vol) aqueous ethanol in an Erlenmeyer flask by shaking at 72–75°C for 30 min. The extract was cooled and then passed through a No. 2 Whatman filter paper. The meal was extracted a second time with 650 mL 80% ethanol under the same conditions. Filtrates were combined and the volume was reduced to 250–300 mL and then stored at −20°C until further treatment. *ii) Enzymatic hydrolysis and sample clean-up.* The frozen extract was thawed completely and centrifuged at 15,000 × *g* for 15 min at 10°C to remove insolubles. A 5-mL invertase (EC 3.2.1.26; Sigma, St. Louis, MO; No. I-4504) solution (5 mg/mL of 0.25 M sodium acetate, pH 4.5) was added dropwisely to the supernatant (250–300 mL) in a 600-mL glass beaker. The mixture was incubated for 2 h with gentle shaking in an Orbit-Environ shaker (Lab-Line, Melrose, IL) maintained at 45°C. The enzyme-treated extract was applied onto a Bio-Rad Econo-column (5 cm i.d.), which contained 50 mL CM 52 overlaid by 300 mL DE 52 and was equilibrated with water. The column was eluted with water at a flow rate of 200 mL/h. The first 250 mL of eluent was discarded. A total of 1,200 mL of eluent was

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collected and stored at 5°C overnight. *iii) Ion-exchange column chromatography.* The eluent volume was reduced to about 100 mL before being applied to a Bio-Rad Econo-column (2.5 cm i.d.), which contained 150 mL AG-1 (OH^-) overlaid by 15 mL AG-50 (H^+) and was equilibrated with water. AG-50 ion-exchange resin was washed separately from AG-1 with water prior to column packing. The column was eluted with water at a flow rate of 2 mL/min and 850 mL was collected. The volume of the eluent was reduced to about 80 mL and stored at 5°C overnight. *iv) Charcoal-Celite column chromatography.* The above sample was applied onto an 80-mL charcoal-Celite (1:1) column (3.2 cm i.d.), which was washed with one bed volume of 80% ethanol the day before use and then thoroughly washed with water overnight. After sample application, the column was eluted with 800 mL each of water, 5% (vol/vol) ethanol, and 10% (vol/vol) ethanol at a flow rate of 1.5 mL/min. Each elution was collected and reduced to complete dryness *in vacuo.* The dry samples were resuspended in water, filtered through a 0.2-μm membrane filter, and analyzed by HPLC.

HPLC analysis. A portion of the crude extract of castor meal (usually 1 mL) was first passed through a column of 1 mL CM 52 overlaid by 3 mL DE 52 to remove ionic substances, while other samples obtained during isolation were analyzed directly with an appropriate dilution by the procedures described previously (14,15). Sugars were identified by comparison of retention times with authentic standards and quantitated based on the amount of the internal standard (mannitol) added to the sample with appropriate response factors for each sugar. In this study, the sugars eluted from the HPLC column were monitored by an Evaporative Light Scattering Detector, Model MK II (Varex, Burtonville, MD).

GC-MS analysis. Aliquots containing 1 mg of galactinol purified from the charcoal-Celite column were placed in Kimax (Kimble, Vineland, NJ) test tubes, equipped with screw caps (13 × 100 mm), and lyophilized. The dry sample was mixed with 1 mL of 2 N trifluoroacetic acid. The tube was then capped and heated at 121°C for 1 hr. The hydrolysates were evaporated to dryness at 45°C with a stream of N_2 in a Meyer N-Evap (Organomation Associates, Northborough, MA). An aliquot of 0.5 mg of L-iditol was then added to the sample as an internal standard and the mixture was lyophilized again. Authentic galactinol was treated simultaneously as a reference. Other authentic standards (0.5 mg each) containing L-iditol were directly lyophilized without the trifluoroacetic acid treatment. Subsequent conversion to the acetylated derivatives and analysis by GC-MS were carried out as described previously (15).

Enzymatic hydrolysis of galactinol. Purified galactinol and authentic melibiose and sucrose were treated with invertae and α-galactosidase, and the reaction products were analyzed by HPLC. α-Galactosidase was partially purified from defatted Nutrisoy brand soy flour (Archer Daniels Midland, Decatur, IL) by a procedure modified from that described by Harpaz *et al.* (16). The enzyme preparation had a specific activity of 6.3 units (μmol/min) per mg of protein. The hydrolysis reaction, with 2.5 mL of sugar solution, 0.25 mL of 0.4 M sodium acetate, pH 5.0, and 0.25 mL enzyme solution, was carried out at 37°C. The stock solution for each sugar was 7.5 mg/mL H_2O, while that for invertase was 1 mg/mL H_2O and for soybean α-galactosidase, 0.83 mg/mL in 50 mM sodium acetate, pH 5.5, containing 0.1 mM EDTA and 0.02% NaN_3. The reaction was initiated by adding an enzyme solution, and aliquots (0.5 mL) were taken at zero time, and the remaining mixture was incubated for 2 h. These samples were immediately placed on dry ice, boiled for 3 min, cooled, deionized by passing through mini-columns containing 1 mL CM 52 overlaid by 2 mL DE 52, and the eluents were lyophilized. The dried samples were resuspended in water, filtered through a Uniflo membrane (0.2 μm; Schleicher & Schuell, Keene, NH), and analyzed by HPLC as described above.

Stachyose synthase reaction. The identity of galactinol was confirmed by the reaction of this compound with stachyose synthase (EC 2.4.1.67) to produce stachyose in the presence of raffinose. The preparation of crude enzyme extracts and the analysis of stachyose formation followed the basic procedures described by Pharr *et al.* (13). Briefly, 4 g of fresh, mature, dark green leaves harvested from zucchini plants, which were grown in a greenhouse for 5 wk as described previously (17), were homogenized with a mortar and pestle at 0°C in 10 mL of 50 mM of *N*-2-hydroxyethyl piperazine-*N'*-2 ethanesulfonic acid (HEPES)-NaOH, pH 7.0, containing 5 mM dithiothreitol (DTT). The homogenate was filtered through Miracloth and centrifuged at 26,000 × *g* for 10 min. A portion of the clear supernatant was desalted in a Pharmacia (Piscataway, NJ) PD-10 G25 column equilibrated with 20 mM HEPES-NaOH, pH 7.0, containing 5 mM DTT. Duplicate reaction mixtures containing 0.6 mL desalted enzyme extracts, 0.65 mL of 10 mM galactinol and 20 mM raffinose in the desalting buffer were incubated in a shaking water bath set at 30°C. Control reaction mixtures contained no galactinol. Aliquots of 0.2 mL were taken and mixed with 0.8 mL absolute ethanol in screw-capped Microfuge tubes (PGC Scientific; Gaithersburg, MD) at zero time and at 15-min intervals thereafter. These were immediately placed on dry ice and stored at −15°C. The content of these tubes was boiled in a water bath for 1 min, filtered through 0.45-μm Prep-Disc (Bio-Rad), dried *in vacuo,* and dissolved in 0.4 mL filtered water. HPLC analysis of the resulting solution was carried out as described above.

RESULTS

Isolation. Aqueous ethanol extracts of defatted castor oilseed meal exhibited a simple HPLC profile of soluble carbohydrates as separated by a Sugar-Pak I column eluted with water at 85°C (Fig. 1A). Sucrose (peak 1) was the major eluting sugar (78% of total). A small amount (10%) of galactinol (peak 2), and detectable amounts of glucose (peak 3), fructose (peak 4) and *myo*-inositol (peak 5) were also present. This was equivalent to 17.8 and 2.2 mg/g defatted meal for sucrose and galactinol, respectively. Treatment of crude extracts with purified yeast invertase hydrolyzed sucrose to produce early an equal amount of glucose and fructose, leaving galactinol and two unknown components intact (Fig. 1B). Figure 1B also shows that fructose responded to the light-scattering detector less than glucose, which was confirmed by authentic standards. Sample clean-up with ion-exchange celluloses, DE 52 plus CM 52, removed most ionic substances without affecting the recovery of galactinol. Application of anionic exchange resin, AG-1 in the OH^- form, removed all

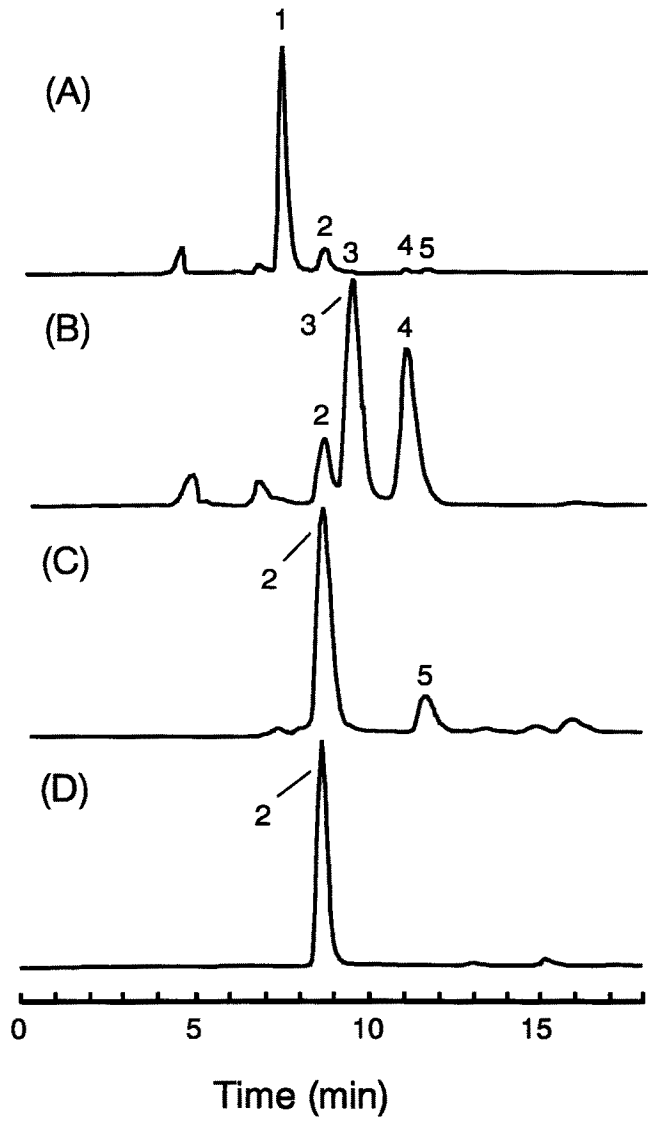

FIG. 1. High-performance liquid chromatograms of carbohydrates in each stage of galactinol purification from defatted castor oilseed meal. (A) Ethanolic extraction, (B) enzymatic treatment and clean-up, (C) anion-exchange resin column elution, and (D) charcoal-Celite column chromatography. Peak 1, sucrose; 2, galactinol; 3, glucose; 4, fructose; and 5, *myo*-inostiol.

monosaccharides and some other unknown components from invertase-treated sugar extracts and facilitated the isolation of galactinol (peak 2) with small amounts of *myo*-inositol (peak 5) and a few unknown minor components, probably sugar alcohols, also present (Fig. 1C). Subsequent chromatography on a charcoal-Celite column and elution with 5% aqueous ethanol resulted in a highly purified galactinol preparation with good yield, after the column had been washed extensively to elute *myo*-inositol and the other unknown components (Fig. 1D). Table 1 summarizes the yield and purity of galactinol isolated from defatted castor meal and compares the procedure with that used to purify galactinol from mature cucumber leaves (13).

Galactinol identity. The purified compound and authentic galactinol had the same retention time, as chromatographed on a Sugar-Pak I column. The relative retention time (RRT) was 0.662 in reference to the retention time of mannitol (12.84 min from six different determinations). Mixing of the isolated compound with an equal amount of authentic galactinol resulted in a single sharp peak after chromatography.

Hydrolysis of the purified compound with trifluoroacetic acid produced the same gas chromatography (GC) profile (Fig. 2A) as authentic galactinol (Fig. 2B), except that the purified fraction also contained a small amount of a contaminant (peak 3, Fig. 2A). In both cases, there were two major hydrolytic products (peak 1, RRT=0.726 and peak 2, RRT=0.938 with respect to the internal standard, L-iditol, from two separate experiments) revealed by GC that corresponded to galactose and *myo*-inositol, respectively (Fig. 2). Peak 1 compound exhibited a mass spectrum that was identical to authentic galactose (Fig. 3A) under the same experimental conditions, whereas peak 2 produced a mass spectrum which was identical to that of *myo*-inositol (Fig. 3B). Enzymatic hydrolysis of the putative galactinol purified from castor meal by soybean α-galactosidase produced two new major peaks on HPLC with retention times identical to those of galactose and *myo*-inositol. On the other hand, the compound was completely resistant to the hydrolysis by yeast invertase, which hydrolyzed sucrose to produce glucose and fructose under the same experimental conditions.

The putative galactinol, purified from defatted castor oilseed meal, was further examined for its effectiveness as a substrate in the formation of stachyose. The formation of stachyose from raffinose by a crude preparation of stachyose synthase from mature zucchini leaves was totally dependent upon the presence of the isolated compound (Fig. 4).

DISCUSSION

The procedure described here offers an effective means of purifying galactinol from defatted castor oilseed meal. The novelty of this procedure is the use of commercial invertase to convert sucrose to monosaccharides to facilitate the isolation of galactinol. Galactinol and sucrose are disaccharides of similar size, and galactinol is present in relatively small amounts in castor meal (Fig. 1A). Without the enzyme treatment, it would undoubtedly require more laborious and defined elution conditions for the subsequent anion-exchange (Fig. 1C) and charcoal-Celite column chromatography steps. In addition, the use of a small charcoal-Celite column for isolating galactinol reported in this study is simpler and more economical than the preparative paper chromatographic method described by Pharr *et al.* (13). Otherwise, the isolation procedure and the result are, in many aspects, similar to the isolation of galactinol from cucumber leaves (Table 1).

This constitutes the first report of the presence of galactinol in castor beans. The identity of galactinol was verified by both acid and enzyme hydrolyses, showing that the isolated compound was composed of galactose and *myo*-inositol, as analyzed by HPLC and gas chromatography-mass spectrometry (GC-MS) (Fig. 2 and 3). In all the aspects tested here, galactinol isolated from castor meal is identical to the authentic galactinol isolated from sugar beet molasses and, therefore, also identical to

TABLE 1

A Comparison of Galactinol Isolation from Castor Meal and Cucumber Leaf

Source	Defatted castor meal	Mature cucumber leaf[a]
Isolation steps	1. Ethanolic extraction 2. Enzymatic hydrolysis 3. Ion-exchange elution 4. Carbon adsorption	1. Ethanolic extraction 2. Ion-exchange elution 3. Preparative paper chromatography
Purity	96.4 ± 1.0%[b]	97%
Yield	278 ± 14 mg/150 g[b]	305 mg/500 g

[a]Results of Pharr *et al.*, *Plant Sci. 50*:21 (1987).

[b]Average of four consecutive isolation results ± standard deviation.

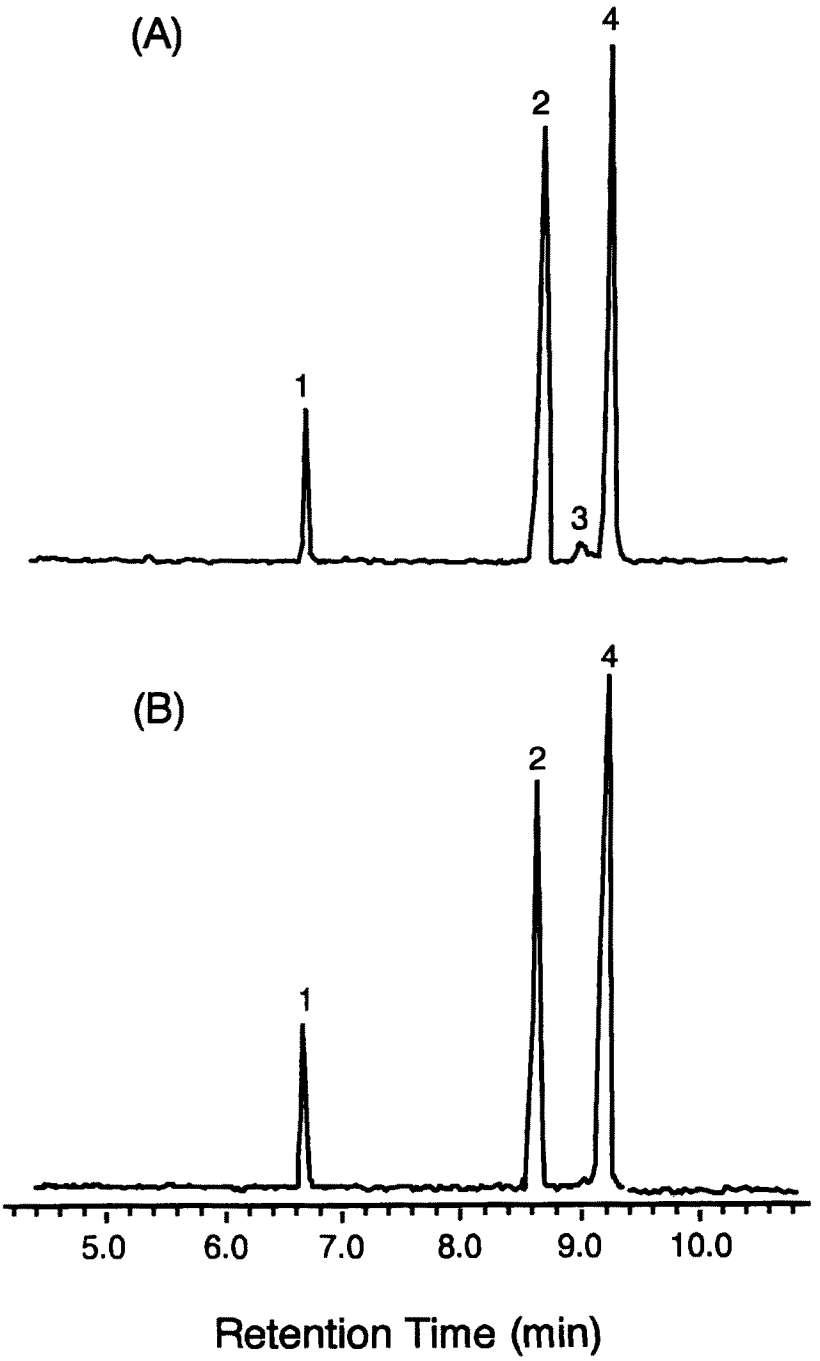

FIG. 2. Gas chromatograms of galactinol upon hydrolysis with trifluoroacetic acid. (A) Isolated compound, and (B) authentic galactinol. Chromatographic peaks are hexaacetate of (1) galactose, (2) *myo*-inositol, (3) unknown contaminant, and (4) L-iditol, internal standard.

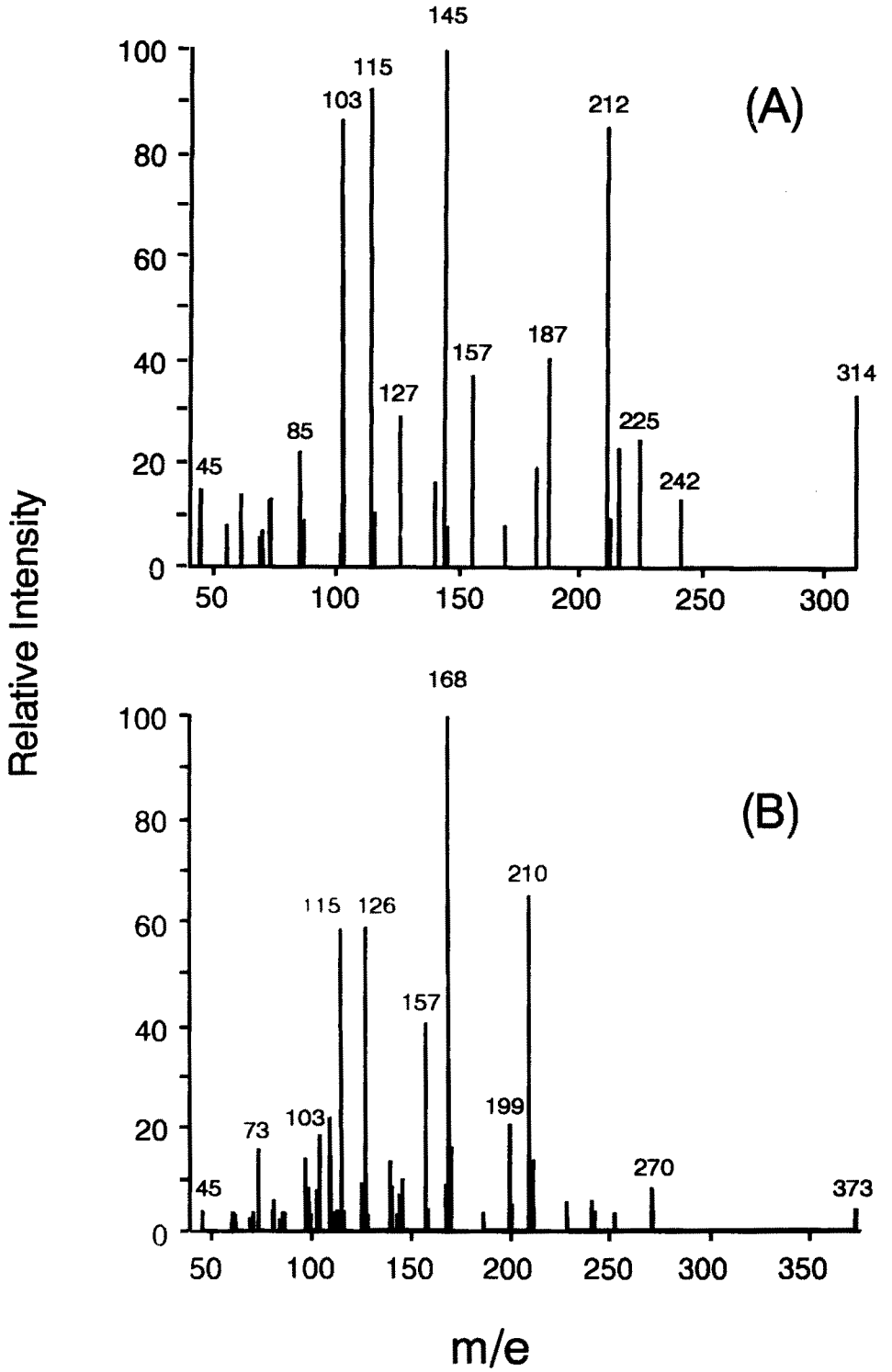

FIG. 3. Mass spectra of galactinol hydrolysates. (A) Peak 1, and (B) peak 2 of acid-hydrolyzed galactinol as separated by the gas chromatography shown in Figure 2.

galactinol isolated from cucumber leaves (13). The dependence of stachyose formation on galactinol isolated from castor meal (Fig. 4) was similar to its dependence on galactinol isolated from cucumber leaves (13).

Defatted castor meal, which is an industrial processing by-product, is a good source material for galactinol. Our previous study (14) suggested that castor meal has one of the simplest soluble carbohydrate profiles known to contain galactinol (Fig. 1A). Cucumber leaves also contain a relatively simple soluble carbohydrate profile in aqueous ethanol extracts, but require cultivation of plants under suitable greenhouse conditions (13) that are difficult to

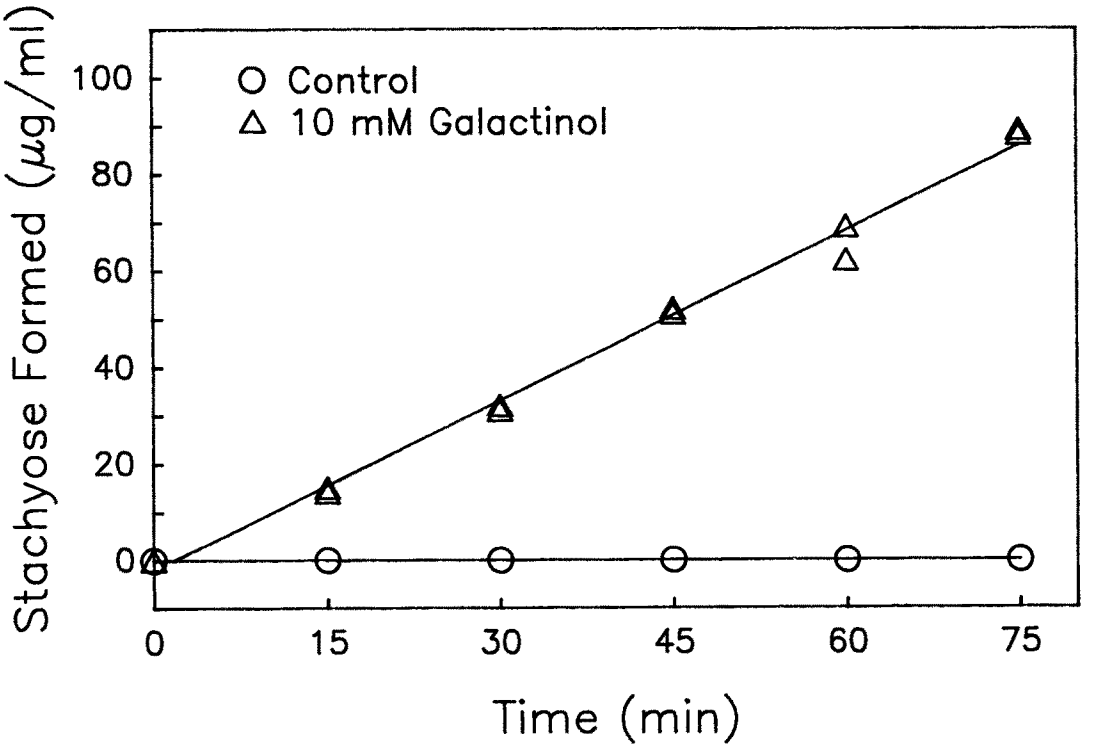

FIG. 4. Stachyose formation in the presence of the isolated galactinol as catalyzed by a crude enzyme preparation from mature zucchini leaves.

maintain in diverse geographical locations. Like defatted castor meal, sugar beet molasses is also an industrial by-product, but processing for sucrose production has resulted in complex and highly variable carbohydrate profiles that require laborious procedures to purify galactinol (10,12). It should be noted that appropriate steps, such as those described in the Experimental Procedures, should be followed to avoid direct contact with the meal, since even processed castor meal still contains a powerful allergen, CB-1A (18). However, efforts are being made to restore castor bean production in the United States (19,20), and a processing procedure for simultaneous detoxification and deallergenation of castor meal has also been developed (21). Thus, in the near future nontoxic and nonallergenic castor meal will be readily available for use in this country.

In conclusion, the discovery of galactinol in castor oilseeds has led to the development of a simple, effective procedure for obtaining galactinol in a highly purified form and in good yield from defatted castor meal.

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