Isolation of Ethyl α-D-Galactopyranoside and Pinitol from Hexane-Ethanol Extracted Soybean Flakes

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Ethyl α-D-galactopyranoside and pinitol were isolated during an investigation of the flavor components extracted from dehulled, full-fat, soybean flakes with an azeotropic mixture of hexane:ethanol 81:19. These two compounds were identified by carbon-hydrogen analysis, chromatographic behavior, spectroscopy, optical rotation, and melting point. Full-fat and defatted flakes contain about 0.6 and 0.3% pinitol, respectively. Nearly 0.2% ethyl galactoside was formed in full-fat flakes during extraction with the hexane-ethanol azeotrope; only 0.03% was formed during extraction of defatted flakes. Ethyl α-D-galactopyranoside is bitter, and pinitol is somewhat sweet. Taste panel tests indicated that the bitter threshold of the galactoside is 0.5% in a water solution, which level indicates that it would not contribute to the bitterness of soy products. L-Tryptophan, also present in soy flakes in its free state at less than threshold levels, is more bitter than the galactoside. The addition of ethyl α-D-galactopyranoside had an additive rather than potentiating effect on the bitterness threshold of either a tryptophan or a soy protein isolate solution.

Flavor is a factor that limits the use of soy protein products in foods. Honig *et al.* (1969) showed that extraction of defatted soybean flakes with an azeotropic mixture of hexane-ethanol removes most of the flavor along with a complex mixture of residual lipids. Extracting 99.8% of the oil from full-fat soybean flakes with pentane-hexane removed little, if any, of the beany, bitter, green flavors. During the preparation of defatted soy flakes from whole soybeans, some lipid oxidation occurs. The apparent low level of oxidation that does occur, as measured by thiobarbituric acid assay, contributes little to the original soybean flavor (Sessa *et al.*, 1969).

This paper reports the isolation and characterization of ethyl α-D-galactopyranoside and pinitol (5-*O*-methyl-D-inositol), from azeotropic extracts of full-fat and defatted flakes, and their relationship to the flavor of soybeans. Evidence is also presented to show that ethyl galactoside is formed during extraction of soybean flakes with ethanolic solvents, and that when other alcohols are used as extraction solvents the corresponding galactosides are formed.

MATERIALS AND METHODS

Extraction of Soybean Flakes with Hexane-Alcohol Azeotrope. Certified, seed-grade, Amsoy soybeans (1968 crop) were used to prepare both dehulled full-fat and defatted flakes, according to the procedure described by Sessa *et al.* (1969). The procedure for extracting full-fat flakes with hexane-ethanol azeotrope and for isolating ethyl galactoside is outlined in Figure 1. The Soxhlet apparatus was operated at a rate of 10 cycles per hr. Each fraction was concentrated on a rotary evaporator at 40° C and then freeze-dried. Only fraction 1b was used to isolate and identify ethyl galactoside and pinitol. The procedure was similar for extraction with hexane:methanol (75:24 v/v) and hexane:2-propanol (79:21 v/v). The hexane-methanol azeotrope separates into two layers at room temperature. This azeotrope removes most of the oil corresponding to fraction 1a in 1 hr.

Thin-Layer and Silicic Acid Column Chromatography. Samples of 50 to 200 mg were chromatographed on Silica Gel F-254 plates (Brinkmann Instruments, Inc.) by one-dimensional ascending chromatography. The two solvent systems were: chloroform:methanol:water (75:25:4) for general separation and butanol:ethanol:water (6:3:2) for separation of carbohydrate fractions. Spots were visualized by spraying with 0.5% potassium dichromate in 50% sulfuric acid, followed by heating for 10 min at 160° C. Carbohydrates were also visualized by spraying with aniline-diphenylamine reagent (Smith, 1958).

A slurry of 30 g silicic acid and 15 g Celite 545 in chloroform: methanol (50:50) was poured into a 3 × 40 cm glass column. The poured column was washed with 100 ml chloroform and 100 ml acetone before the meal extracts were fractionated. Samples of 0.5 to 1.5 g of 1b (Figure 1) in 3 to 10 ml chloroform:methanol (50:50) were chromatographed. Eluents are listed in Table I. Effluents were collected in 15-ml fractions. The presence of ethyl galactoside and pinitol in the effluents was determined by thin-layer chromatography (tlc), and the phenol-sulfuric acid procedure of Dubois *et al.* (1956).

Gas–Liquid Chromatography. Trimethylsilyl derivatives of pinitol and various glycosides were prepared by adding a tenfold amount of bistrimethylsilylacetamide (BSA) and trimethylchlorosilane reagent (TMCS) (Supelco, Inc., Supelco Park, Bellefonte, Pa.). Two days at room temperature were necessary for complete silylation. The derivatives were chromatographed with a Packard 824 instrument on 6 ft × 1/4 in. o.d. glass columns packed with 15% Carbowax 20M on 80–100 mesh Chromosorb W/AW or 3% OV-101 on 100-110 mesh Anakrom SD at a helium flow rate of 20 to 25 ml per min. A flame ionization detector was used. The injection port temperature was 210° C, the oven temperature was 150° C, and detector 210° C.

Proton Magnetic Resonance. All spectra were obtained on a Varian H-100 nuclear magnetic resonance spectrometer. Concentrations of all samples in D_2O or dimethyl sulfoxide (DMSO) were 7%. Shifts in the O-H hydrogen peaks were made by adding a small amount of D_2O to the DMSO solutions.

Quantitation of Pinitol and Ethyl Galactoside. Full-fat flakes were extracted with hexane-ethanol and then fractionated with $CHCl_3$:CH_3OH:H_2O to remove the oil and other lipids, as shown in Figure 1. The residual flakes were then re-extracted with 90% acetone for at least 11 hr to remove most of the remaining pinitol and ethyl galactoside. With defatted

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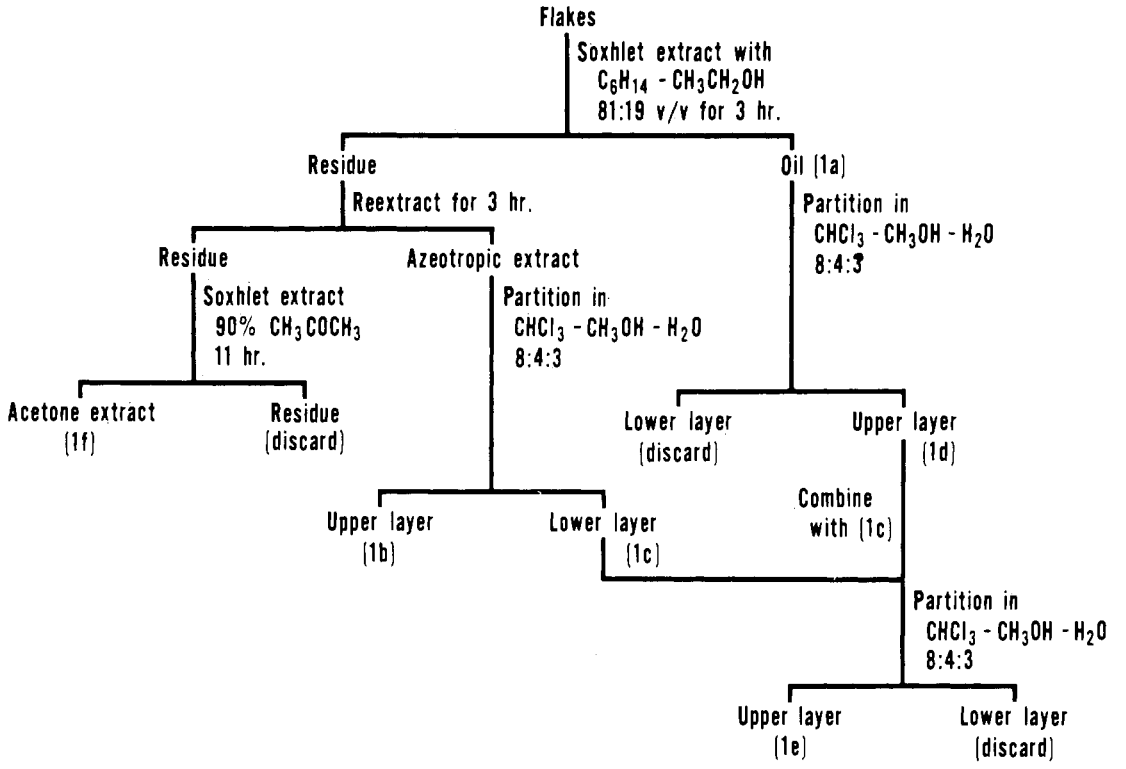

Figure 1. Extraction of ethyl α-D-galactopyranoside and pinitol from dehulled full-fat flakes

flakes the oil fraction (1a, Figure 1) had already been removed during the defatting process. Fractions 1b, 1e, and 1f were then silylated with the BSA-TMCS reagent and analyzed by gas–liquid chromatography (glc). Known quantities of silylated ethyl α-D-galactopyranoside and pinitol were also chromatographed on the same day to standardize retention times and response factors. The amount of galactoside or pinitol was then determined from the area under each glc peak.

Synthesis of Methyl, Ethyl, and Isopropyl α-D-Galactosides. In a modification of the methods of Frahm and Mills (1965) and of Fischer and Beensch (1894), 10 g of D-galactose in 100 ml of the appropriate alcohol containing 4% dry HCl was heated for 72 hr at 30° C on a magnetic stirrer and then refluxed for 4 hr. The reaction mixture was neutralized with $BaCO_3$, filtered through activated carbon black, and concentrated to a syrup on a rotary evaporator at 40° C. The syrup was taken up in a minimum volume of 90% acetone, allowed to stand at room temperature for 24 hr, and then placed in a refrigerator (4° C) until crystallization occurred. The solids were separated, filtered, and then recrystallized from 90% acetone.

Taste Panel Evaluations. The taste threshold of ethyl galactoside was determined by a 17-member panel. All tasters received five 5-ml samples. One of the randomized samples contained only charcoal filtered tap water, and the other four samples varied in amounts of ethyl galactoside, caffeine, or L-tryptophan. For a determination of the relative degree of bitterness of ethyl galactoside, its threshold was compared to those of caffeine and L-tryptophan. The threshold of each compound was based on the concentration at which at least 50% of the members said the sample was bitter. Ethyl D-galactoside (mp 135–138° C) was used in the tests. Caffeine was USP grade and L-tryptophan was Mann assayed grade (Mann Research, Inc.).

Table I. Isolation of Ethyl α-D-Galactopyranoside and Pinitol on Silicic Acid Column[a]

Fraction	Eluent	Weight, Ml	Weight, Mg	Main Components
1	Acetone	75	67	Triglycerides, sterols, isoflavones
2	Acetone	30	92	Ethyl α-D-galactopyranoside
3	Acetone	105	76	Pinitol and galactoside
4	Acetone	150	125	Pinitol
5	Acetone	240	32	Pinitol and oligosaccharides
6	$CHCl_3$:CH_3OH: NH_4OH 9:9:2	300	107	Phospholipids and unknowns

[a] 740 Mg of starting material, fraction 1b (see Figure 1), in 3 ml of $CHCl_3$:CH_3OH 50:50. Column: 30 g silicic acid; 15 g Celite 545.

RESULTS AND DISCUSSION

Isolation of Ethyl α-D-Galactopyranoside and Pinitol from Hexane-Ethanol Extracts. The hexane-ethanol azeotrope extracts large amounts of oil and lipids, together with the galactoside and pinitol. The procedure used for their extraction and separation from the lipids is shown in Figure 1. The highest concentration of galactoside and pinitol is in fraction 1b which amounted to 0.35% of the flakes. More than half the total still remained in fractions 1c, 1e, and 1f.

To isolate crystalline ethyl α-D-galactopyranoside and pinitol, fraction 1b was chromatographed on a silicic acid column. The elution schedule and the major components present in the effluent fractions are given in Table I. Fractions 1 and 6 are a complex mixture of components, primarily lipids. Many of them have been previously identified (Honig *et al.*, 1969). Fractions 4 and 5 containing pinitol and carbohydrates had a somewhat sweet taste. All other fractions had a bitter taste, but fractions 1 and 6 were the most bitter.

Yields of up to 0.05% ethyl α-D-galactopyranoside crystallized from fraction 2 on standing and during removal of sol-

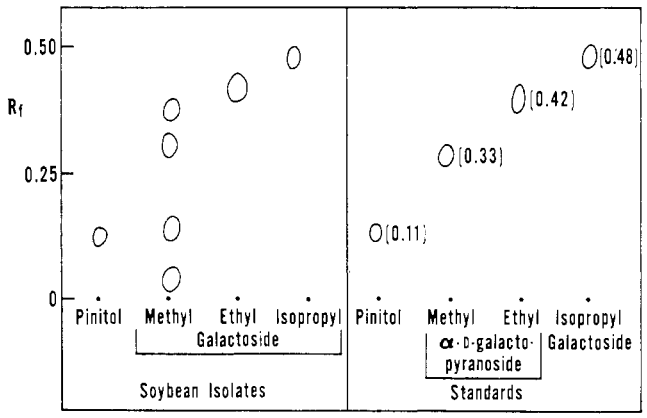

Figure 2. Thin-layer chromatogram of pinitol and methyl, ethyl and isopropyl galactosides isolated from soybean flakes. Elution solvent, $CHCl_3$-CH_3OH-H_2O. The galactoside standards were prepared by synthesis; those for the pinitol were furnished by the Southern Regional Research Laboratory. Figures in parentheses are R_f values

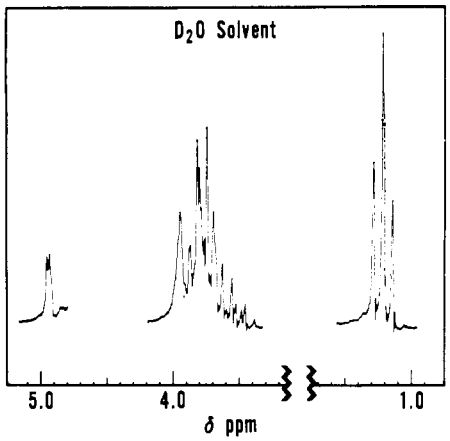

Figure 3. Proton magnetic resonance (pmr) of ethyl α-D-galactopyranoside in D_2O

vents, after which the galactoside was purified by sublimation. A yield of 0.08% crystalline pinitol was obtained from fraction 4.

To determine whether ethyl α-D-galactopyranoside might be an artifact or a natural component, full-fat meal was also extracted with hexane-2-propanol azeotrope and a hexane-methanol azeotrope. Each of these extracts was then fractionated on silicic acid. No ethyl α-D-galactopyranoside was isolated from the hexane-2-propanol extracts. Instead, a compound resulted with the same R_f on tlc and an mp in the 145–152° C range as a synthesized 2-propanol galactoside. Since both tlc and glc indicate the presence of methyl galactoside in the methanol-hexane extracts, apparently in each alcoholic extract a galactoside is formed of the corresponding alcohol.

Thin-Layer Chromatography of Pinitol and Methyl, Ethyl and Isopropyl Galactosides. No methyl galactoside was obtained in crystalline form from soybean flakes. However, a fraction was isolated that on tlc had a spot with the same R_f as methyl α-D-galactopyranoside and also the same reaction toward aniline-diphenylamine spray reagent. In Figure 2 is a diagrammed chromatogram of this fraction, along with pinitol and ethyl α-D-galactopyranosides isolated by hexane-ethanol extraction as well as the material isolated by hexane-2-propanol extraction. The standards were pinitol, supplied by the Southern Regional Research Laboratory; methyl α-D-galactopyranoside; ethyl α-D-galactopyranoside, and a 2-propyl galactoside, prepared in our laboratory. The respective R_f's of pinitol, methyl α-D-galactopyranoside, and the isopropyl derivative were 0.11, 0.33, 0.42, and 0.48, with $CHCl_3$:CH_3OH:H_2O 75:25:4 as eluent.

Gas-Liquid Chromatography. The four peaks that appeared when crude synthetic ethyl galactoside was chromatographed on a Carbowax 20M column indicated separation of the four major isomers. Further purification by recrystallization yielded a single chromatographic peak. Chromatography of a mixture of the ethyl α-D-galactopyranoside isolated from soybean meal and the purified synthetic material gave only one peak. Relative retention times with respect to BSA on the Carbowax 20M column were: pinitol, 1.12; ethyl α-D-galactopyranoside, 1.49; and methyl α-D-galactopyranoside, 1.55.

Proton Magnetic Resonance Spectrum of Ethyl α-D-Galactopyranoside. The proton magnetic resonance (pmr) spectrum of ethyl galactoside isolated from soybeans was the same as that of the synthesized material in D_2O shown in Figure 3. The chemical shift and J value of the anomeric proton peaks centered at 4.95 ppm correspond to those of the α-anomer of a glycoside (Casu *et al.*, 1966). In DMSO (Figure 4), all peaks are shifted to higher field, and the hydroxyl proton peaks are separated from those of protons attached to C–O. These spectra confirm an ethoxyl glycoside in the α configuration. Integration of the proton peaks was consistent with the expected 16 protons.

Chemical-Physical Data for Ethyl α-D-Galactopyranoside and Pinitol. Melting points, specific rotations, and carbon-hydrogen analyses are summarized in Table II. They are consistent with values published for ethyl α-D-galactopyranoside by Fischer (1914) and for pinitol by Anderson *et al.* (1952). The samples isolated from soybean flakes were somewhat impure according to optical rotation measurements.

Quantitation of Ethyl α-D-Galactopyranoside and Pinitol. The values obtained by glc analysis of full-fat and defatted soy flakes are shown in Table III. The data indicate that much more ethyl galactoside is produced in full-fat than in defatted flakes. Because pinitol content in defatted flakes is somewhat lower than in full-fat flakes, some must be re-

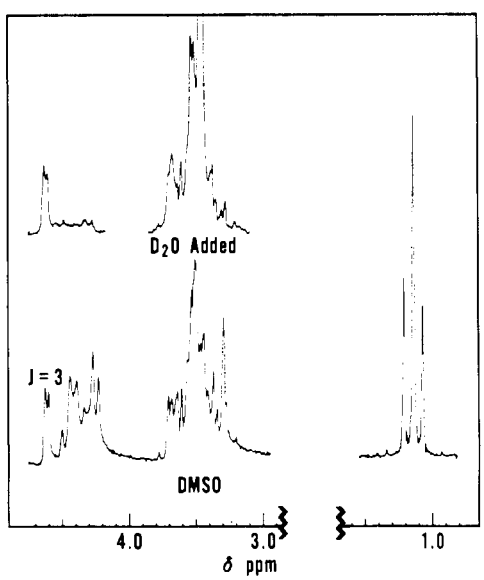

Figure 4. Pmr of ethyl α-D-galactopyranoside in dimethyl sulfoxide

Table II. Physical and Chemical Properties of Ethyl α-D-Galactopyranoside and Pinitol

Property	Galactoside		Pinitol	
	Found	Lit.	Found	Lit.
Mp, °C	142	142	184	187
Specific rotation, deg	+170	+186	+61.3	+65.5
C analysis, %	45.2	46.2[a]	43.0	43.3[b]
H analysis, %	7.98	7.69[a]	7.42	7.21[b]

[a] Calculated for $C_8H_{16}O_8$. [b] Calculated for $C_7H_{14}O_6$.

Table III. Pinitol and Ethyl α-D-Galactopyranoside Content of Soy Flakes

Sample	Compound	g/100 g Flakes
Full-fat	Pinitol	0.6
	Ethyl galactoside	0.2
Defatted	Pinitol	0.3
	Ethyl galactoside	0.03

Table IV. Bitter Response of Ethyl α-D-Galactopyranoside *vs.* Caffeine and L-Tryptophan

Compound	Concentration[a] %	Bitter Response,[b] %
Caffeine	0.04	65
Caffeine	0.08	91
Ethyl galactoside	0.30	38
Ethyl galactoside	0.60	57
Ethyl galactoside	1.00	81
L-Tryptophan	0.05	19
L-Tryptophan	0.10	63
L-Tryptophan	0.30	100
Ethyl galactoside + L-Tryptophan	0.30[c] + 0.05[c]	52

[a] In charcoal-filtered tap water. [b] Percentage of panelists responding yes to the question of whether a sample was bitter or not. [c] Each compound at half the concentration that produces over 50% bitter response.

moved during defatting. Less than half of either component is extracted from full-fat and defatted flakes with hexane-ethanol azeotrope. Extraction for 10 hr with 90% acetone or for 6 hr with methanol removes most of the extractable pinitol and galactoside.

Taste Panel Results. Responses (Table IV) indicate that the bitterness of a 1% solution of ethyl α-D-galactopyranoside is close to that of a 0.067% concentration of caffeine. The bitterness threshold of the galactoside of about 0.5% is higher than the 0.2% amount found in hexane-ethanol extracted flakes. The galactoside is much less bitter then L-tryptophan.

To determine whether the presence of ethyl galactoside has a potentiating effect on the bitter threshold response of tryptophan, a solution of 0.3% ethyl galactoside containing 0.05% tryptophan was compared to the bitterness of either 0.6% ethyl galactose or 0.1% tryptophan. As shown in Table IV, the solution containing both substances gave a bitter response of 52%, compared to 57% for 0.6% ethyl galactoside and a 63% response for 0.1% tryptophan. Based on these results, the presence of both ethyl galactoside and tryptophan in the same solution, each at half the concentration that would normally elicit at least a 50% yes response, have only an additive effect on the bitterness threshold. A potentiating effect would evoke a much greater increase in bitterness response. Also, in one taste panel test, the addition of ethyl galactoside to a solution of soy protein isolate had no effect on its bitterness threshold.

CONCLUSION

Ethyl α-D-galactopyranoside has been reported to have a bittersweet taste (Fischer, 1914). Our taste panel results indicate that this galactoside has a bitter threshold of 0.5% and no sweetness was reported at the 1% level. Since its level in full-fat or defatted soybean flakes was not found to be more than 0.2%, and since raw defatted soy flour has a bitterness threshold of 0.04% (Kalbrener *et al.*, 1970), ethyl α-D-galactopyranoside would seem to have little effect on soybean flavor unless highly concentrated during processing. Although ethyl α-D-galactopyranoside has been identified as a natural product in *Eleutherococcus senticoccus* max (Ovodov *et al.*, 1967), the galactoside from hexane-ethanol extracts of soybeans is an artifact of the extraction process. An α-galactosidase of sweet almonds reportedly is involved in hydrolysis, synthesis, and trans glycosylation of galactosides (Dey, 1969). A similar enzyme may be involved in the formation of ethyl α-D-galactopyranoside during alcoholic extraction of soybean flakes. No other evidence, however, has yet been found to support this explanation.

Free L-tryptophan was quantitated in defatted flakes by extraction with a 7 hr 95% ethanol reflux and then it was separated from other components on Sephadex G-10; its concentration was determined from its ultraviolet absorption at 280 mμ. Only 7 ppm was found. Since, as shown in Table IV, a 0.10% concentration was considered bitter by only 63% of those tasting, L-tryptophan does not appear to be a source of bitterness in soybean products.

Pinitol has been previously reported in hexane-ethanol extracts of soybean flakes (Nielsen, 1960); however, his identification was somewhat incomplete and he did not report its isolation or quantitation. Pinitol does not appear to have enough sweetness to affect the flavor of soybean products.

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