α-Galactosidase Activity of Fungi on Intestinal Gas-Forming Peanut Oligosaccharides

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Neurospora sitophila and *Rhizopus oligosporus*, the two fungi which are used in the preparation of fermented peanut press cake (ontjom), and eight other fungi, most of which are traditionally or industrially used to ferment oilseeds and grains, were examined for their ability to utilize sucrose, raffinose, and stachyose in peanuts. Trimethylsilyl ether derivatives of sugars extracted from unfermented peanut meal, as well as meal fermented for various times ranging to 98 hr, were quantitated by gas–liquid chromatography using gentiobiose as an internal standard. Six fungal strains, including *N. sitophila*, showed definite α-galactosidase activity with a decrease in raffinose and stachyose content of ferments. *R. oligosporus* and three other strains did not utilize these sugars or utilized them only slowly.

It is well known that consumption of legume seeds by humans may result in gastrointestinal distress and flatulence. Nausea, cramps, and diarrhea may also occur in varying degrees (Rackis *et al.*, 1970a), depending upon the legume consumed. Positive identification of all flatulence-causing factors has not been made; however, the presence of raffinose and stachyose in the active gas-producing fraction of beans has been demonstrated (Rackis *et al.*, 1970b; Steggerda *et al.*, 1966). Mammalian digestive juices lack the α-galactosidase (EC 3.2.1.22) required to hydrolyze these low molecular weight oligosaccharides. The sugars pass through the gastrointestinal tract toward the ileum and colon where major production of hydrogen, carbon dioxide, and methane gases by normal intestinal microflora takes place (Richards and Steggerda, 1966).

Several approaches have been taken in attempts to reduce or eliminate the flatus activity of legumes. Ethanolic (Rackis *et al.*, 1970a) and aqueous (Kim *et al.*, 1973) extraction, induced seed germination (Hsu *et al.*, 1973), and adjustment of pH, temperature, and moisture content (Becker *et al.*, 1974) have been reported to result in decreased raffinose and stachyose levels in beans. Antibiotics have been successfully employed to suppress microbial activity in the lower ileum and thereby reduce flatus (Murphy and Calloway, 1972; Richards and Steggerda, 1966).

α-Galactosidase has been reported in yeasts, molds, and plants (Dey and Pridham, 1972; Wallenfels and Malhotra, 1961). Fewer reports exist, however, relating the employment of microorganism α-galactosidase activity for the purpose of reducing raffinose and stachyose in foods prior to their consumption. Suzuki *et al.* (1969) reported that raffinose in beet molasses could be decomposed continuously by α-galactosidase produced by *Mortierella vinacea* in submerged culture. Recently Mital *et al.* (1973) searched for the presence of the enzyme in various lactobacilli that might then be used for the preparation of fermented products from soybeans. α-Galactosidase activity was observed in cell-free extracts of five *Lactobacillus* spp. Calloway *et al.* (1971) found tempeh, a traditional soybean product fermented with *R. oligosporus*, to be essentially nonflatulent. Interestingly, sucrose, raffinose, and stachyose, the three principal sugars in soybeans, are not utilized by the mold or are used only slowly (Hesseltine, 1965).

The Indonesians ferment peanut (*Arachis hypogaea*) press cake to produce ontjom, a product reported to have improved digestibility (Steinkraus *et al.*, 1965). Although the raffinose and stachyose content of peanuts is considerably lower than that of beans and is variety dependent (Aylward and Nichols, 1961; Hymowitz *et al.*, 1972b), it is possible that these oligosaccharides are hydroloyzed during fermentation, thus contributing to increased digestibility. To test this hypothesis, *N. sitophila* and *R. oligosporus*, the two fungi which may be used to produce ontjom, and eight other fungi, most of which are traditionally or commercially used to ferment oilseeds and grains, were examined for their ability to utilize sucrose, raffinose, and stachyose in peanuts.

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EXPERIMENTAL PROCEDURES

Peanut Fermentation. Florunner variety peanuts were blanched at 210° for 6 min and skins were removed by abrasion. Nuts were then finely ground in a Toledo food chopper and oil was partially removed by repeated passes through a Carver press. Meal was then passed through a 30-mesh screen. Citric acid (3.0% peanut meal weight), sodium chloride (1.4%), tapioca (1.0%), and 75 ml of tap water were combined with 25 g of peanut meal in 500-ml erlenmeyer flasks. The mixture was autoclaved 15 min at 121° and cooled; an additional 50 ml of sterile tap water was combined with the peanut substrate.

Ten fungal strains were included in the study. *Aspergillus niger* NRRL 3122, *Aspergillus oryzae* NRRL 1988, *N. sitophila* NRRL 2884, *Rhizopus delemar* NRRL 1472 and NRRL 1705, *R. arrhizus* NRRL 1526, *R. oligosporus* NRRL 2710, *Monascus purpureus* NRRL 2897, *Mucor hiemalis* NRRL 3103, and *Actinomucor elegans* NRRL 3104 were investigated. Cultures were propagated on potato dextrose agar slants for 6 days at 28° with the exception of *M. purpureus* which was cultured for 15 days. Spore suspensions were prepared by washing the culture surfaces with sterile 0.001% Tween 81. Four milliliters of the suspensions were added individually to the peanut substrate and fermentation was carried out at 28° on a gyratory shaker (150 rpm). Samples were aseptically removed after 21, 44, 68, and 98 hr of fermentation, freeze-dried, and stored at −40° until analyzed. Uninoculated peanut substrates which were not autoclaved, or autoclaved and held for 0, 21, 44, 68, and 98 hr at 28°, served as control samples in the investigation.

Sugar Extraction. Freeze-dried samples were pulverized and passed through a 60-mesh screen. Duplicate 0.50-g samples were weighed in 15-ml glass centrifuge tubes. Five milliliters of 80% ethanol in water was added to one tube; 5 ml of 80% ethanol containing 1.0 mg/ml of gentiobiose as an internal standard (Hymowitz *et al.*, 1972a) was added to the second tube. Each sample was extracted at 80° for 15–20 min with occasional stirring and centrifuged at 6000*g* for 10 min; the supernate was then decanted into a vial. The extraction procedure was re-

peated four times using 80% ethanol for all samples. Combined extracts from individual samples were evaporated to near dryness, freeze-dried, and used for quantitation of sucrose, raffinose, and stachyose by gas-liquid chromatography (glc).

Extraction of reducing sugars was made in 5.0 ml of deionized water at 80°. Duplicate 0.10-g samples were extracted 30 min with occasional stirring, centrifuged at 6000*g* for 5 min, and filtered (Whatman No. 2). Filtrates were analyzed for reducing sugars.

Analytical. A preliminary examination of unfermented, unautoclaved Florunner peanut alcohol extracts by glc showed a low level (*ca.* 0.05%) of a naturally occurring saccharide with a retention time identical with that of gentiobiose. In order to correct for this component each sample was analyzed both with and without the addition of gentiobiose as internal standard.

Trimethylsilyl ethers were prepared by treating a quantity (usually 2-4 mg) of freeze-dried ethanol extract containing approximately 50 μg of added gentiobiose with 50 μl each of pyridine and *N*-trimethylsilylimidazole. Samples without added gentiobiose were treated in the same way. The freeze-dried extracts from some samples rapidly absorbed moisture from the air and were difficult to manage; these samples were dissolved in 5 ml of water and 50-μl aliquots were freeze-dried for derivitization.

After treating with pyridine and *N*-trimethylsilylimidazole, the samples were heated at 40° for 30 min and 1-μl aliquots were analyzed on a MicroTek 220 gas chromatograph equipped with dual flame detectors and an Infotronics integrator. Glass columns, 1.85 m × 4.0 mm i.d., were packed with 80-100 mesh Chromosorb W (acid-washed; DMCS treated) coated with 3% (w/w) OV 101. The column temperature was programmed from 210 to 315° at 5°/min. Detector and injection ports were maintained at 315° and the helium flow rate was 100 ml/min.

Identification of sample sugars was based on a comparison of retention times with those obtained with a standard solution containing sucrose, gentiobiose, raffinose, and stachyose and in some cases by co-injection of the sample with sugar standards.

Reducing sugars (as glucose) were quantitated in water extracts of samples using the Nelson microcuprimetric method as outlined by Clark (1964).

Total lipid content of the peanut meal used in the study was determined by diethyl ether extraction using a Goldfisch extractor. Lipid samples were dried in a vacuum oven at 70° prior to weighing.

Calculations. A correction for the naturally occurring sugar with retention time identical with that of gentiobiose was made by determining the response ratio between this component and a peak of similar size eluting immediately thereafter in samples not containing added gentiobiose. This ratio was used to compute the contribution (integrator count) of the naturally occurring sugar to the gentiobiose internal standard peak in the duplicate sample containing added gentiobiose.

Trimethylsilyl ethers were prepared from 50 μl of a standard solution containing 10 mg of sucrose, 1 mg of gentiobiose, 1 mg of raffinose, and 1 mg of stachyose per ml, and chromatographed to determine weight response ratios relative to gentiobiose. The weight response ratios were then used to adjust the response (integrator count) obtained for each sugar under investigation. Finally, weight determinations for each sugar were made by relating the adjusted integrator count obtained for each sugar to the corrected integrator count obtained for the internal standard according to the following equation: $\% S = (W_{i.s.} C_s 100)/(C_{i.s.} W_s)$, where $W_{i.s.}$ = weight in milligrams of internal standard (5 mg) added to sample, C_s = adjusted integrator count obtained for the sugar being quantitated, $C_{i.s.}$ = corrected integrator count obtained for gentiobiose internal standard, and W_s = weight of sample analyzed (500 mg).

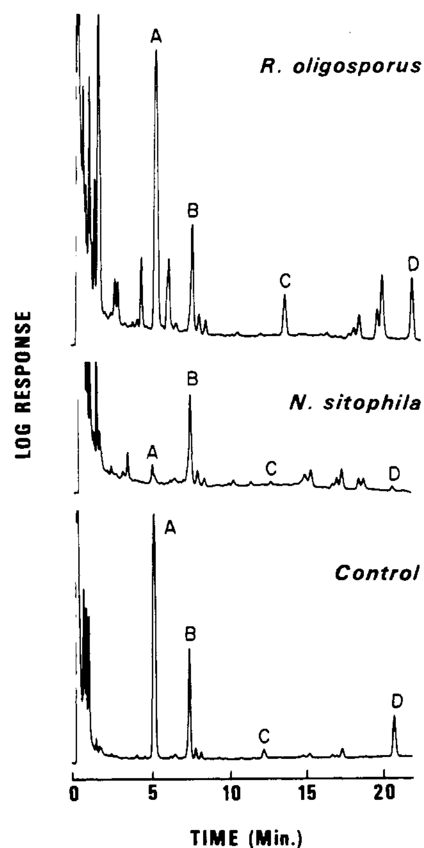

Figure 1. Gas-liquid chromatograms showing sucrose (A), gentiobiose (B, internal standard), raffinose (C), and stachyose (D) peaks from control (unfermented) peanuts and peanuts fermented with *Rhizopus oligosporus* and *Neurospora sitophila* for 98 hr.

RESULTS AND DISCUSSION

Analysis showed that the initial lipid content of partially defatted peanut meal used in this study was 22.3%. This is a reduction from the 50.8% which we earlier reported to be present in the same lot of full-fat Florunner peanuts (Beuchat and Worthington, 1974). Sugar levels reported from control and fermented samples in this study are on a partially defatted basis. Conversion of these data to a full-fat basis would require multiplication by a factor of approximately 0.7.

According to Hymowitz *et al.* (1972b), the sugar content of peanut cultivars is highly variable. Raffinose and stachyose were reported to range from less than 0.1 to 0.3% and less than 0.1 to 0.5%, respectively, depending upon the cultivar under examination. Florunner peanuts were listed to contain 0.1% raffinose and 0.4% stachyose. Since it was desirable to have measurable quantities of both saccharides initially present in the unfermented material, the Florunner cultivar was chosen for this study.

Figure 1 shows a composite of three chromatograms depicting peaks for sucrose, gentiobiose, raffinose, and stachyose in 98-hr fermentation samples of *R. oligosporus* and *N. sitophila* as well as the unfermented, unautoclaved control sample. A number of peaks indicating the presence of mono- and oligosaccharides were observed in these and other fermented samples. In general these components reached maximum levels, as indicated by peak heights, during early or intermediate fermentation periods, and declined at 98-hr fermentation. Oligosaccharide peaks were especially prominent in ferments from the four

Rhizopus spp. Although individual identification and quantitation of these mono- and oligosaccharide components was beyond the scope of this study, saccharide moieties such as manninotriose, galactobiose, melibiose, galactose, glucose, and fructose may have resulted from hydrolytic breakdown of polysaccharides. These sugars would be expected to appear with various retention times on gas-liquid chromatograms.

Reducing sugars increased from 0.14 to 1.16% upon autoclaving the peanut substrate in preparation for fermentation. With the exception of *A. niger* and *N. sitophila* ferments, reducing sugars decrease in all other ferments after 98 hr. Decreased values may be attributed to utilization of easily metabolized low molecular weight reducing sugars in preference to nonreducing sugars as sources of energy.

Concentrations of sucrose, raffinose, and stachyose in control and fermented peanut meal are shown in Table I. Slight reduction in levels of all three oligosaccharides occurred upon autoclaving, probably due to hydrolysis catalyzed by heat and the acidic nature (pH 4.9) of the substrate. Little change in saccharide concentration in autoclaved control samples was noted during the incubation periods of 21, 44, 68, and 98 hr. Data from the 21-, 44-, and 68-hr autoclaved control samples have therefore been omitted from Table I.

Both aspergilli included in the study rapidly utilized sucrose, raffinose, and stachyose in peanut meal. A number of investigators have reported on related saccharolytic activities of *A. niger* (Cadmus *et al.*, 1966; Perzow *et al.*, 1973; Takenishi and Tsujisaka, 1973). The organism produces high levels of oxalic acid under certain conditions and is not used in Oriental fermented foodstuffs. *A. oryzae*, on the other hand, is used widely in the production of miso and shoyo, both fermented soybean-based products. Because the beans are soaked and drained prior to fermentation, water-soluble sugars such as sucrose, raffinose, and stachyose are undoubtedly extracted. Data shown in Table I from fermented peanut substrate indicate that any of these sugars remaining in the soaked soybeans would be hydrolyzed by *A. oryzae* during fermentation.

N. sitophila, the mold most often used to ferment peanut press cake in the production of ontjom, was demonstrated to essentially eliminate sucrose and the intestinal gas-forming sugars, raffinose and stachyose, during the first 21 hr of fermentation. It is possible that utilization of flatulence-causing sugars by *N. sitophila* may contribute to the suggested increased digestibility of ontjom (Steinkraus *et al.*, 1965).

The four *Rhizopus* spp. included in the investigation gave varying results with respect to their ability to utilize sucrose, raffinose, and stachyose. Data from *R. delemar* NRRL 1472 are shown in Table I and are identical with NRRL 1705 (not shown). The organism exhibits strong amylase activity and it is not surprising that other carbohydrases such as α-galactosidase are produced. Reduction in stachyose content occurred slowly in peanuts fermented with *R. arrhizus*. This was accompanied by an apparent increase in raffinose and sucrose. It is possible that the terminal galactose moiety is removed from stachyose more rapidly than galactose or fructose is removed from the resulting raffinose molecule, thus resulting in an elevation of raffinose content. The apparent increase in sucrose is not due to the presence of trehalose, a disaccharide synthesized by some fungi, or melibiose, a possible decomposition product of raffinose and stachyose, since both trehalose and melibiose had glc retention times dissimilar to sucrose. The increase in sucrose may be due in part to galactosidase activity on raffinose and stachyose. Loss of volatiles during fermentation would result in an apparent increase in sucrose and other nonutilizable peanut constituents. *R. oligosporus* may have utilized small amounts of stachyose, but only after 68 hr of fermentation. Raffinose and sucrose were not utilized. These data are consistent with those in a report by Sorenson and Hesseltine (1966) in which the same strain of *R. oligosporus* was shown not to utilize raffinose and stachyose as sole sources of carbon. Shallenberger *et al.* (1967), on the other hand, reported a decrease in stachyose and sucrose content without apparent change in raffinose content in soybeans fermented over a 72-hr period with a *Rhizopus* mold.

M. purpureus grew very slowly on the peanut substrate; however, it does have α-galactosidase activity. A steady decrease in stachyose content occurs after 21 hr with an accompanying increase in raffinose up to 68 hr fermentation and then a precipitous decrease. Reasons for the increasing sucrose content throughout the 98-hr fermentation may be similar to those described for *R. arrhizus*. A sequential hydrolysis of stachyose followed by raffinose is

Table I. Sucrose, Raffinose, and Stachyose Content of Control and Fermented Peanuts

Fungus	Fermentation time, hr	g per 100 g of sample		
		Sucrose	Raffinose	Stachyose
Control	*a*	6.9	0.15	0.65
	b	5.3	0.10	0.60
	c	5.2	0.10	0.47
A. niger	21	0.29	0.04	0.30
	44	0.01	0.02	T[e]
	68	0.08	ND[d]	T
	98	0.08	ND	ND
A. oryzae	21	0.07	0.01	0.24
	44	0.04	T	T
	68	T	ND	ND
	98	0.05	ND	ND
N. sitophila	21	0.03	ND	0.03
	44	0.02	ND	T
	68	T	ND	T
	98	0.06	T	0.02
R. delemar	21	0.02	0.04	0.35
	44	0.51	ND	T
	68	0.15	ND	ND
	98	0.06	ND	ND
R. arrhizus	21	5.9	0.11	0.47
	44	6.6	0.18	0.41
	68	7.7	0.30	0.32
	98	9.3	0.61	0.19
R. oligosporus	21	5.8	0.08	0.45
	44	6.8	0.12	0.53
	68	6.7	0.17	0.62
	98	6.5	0.27	0.39
M. purpureus	21	5.4	0.08	0.48
	44	5.7	0.16	0.37
	68	7.8	0.20	0.09
	98	9.1	0.06	T
M. hiemalis	21	0.53	0.13	0.51
	44	0.22	0.09	0.58
	68	0.34	0.13	0.60
	98	0.20	0.24	0.61
A. elegans	21	5.6	0.10	0.40
	44	5.9	0.10	0.44
	68	6.7	0.13	0.39
	98	6.7	0.28	0.34

[a] Unfermented sample, not autoclaved, freeze-dried without incubation. [b] Unfermented sample, autoclaved, freeze-dried without incubation. [c] Unfermented sample, autoclaved, incubated 98 hr at 28° before freeze-drying. [d] Not detected. [e] Trace, less than 0.01 g/100 g of sample.

likely. Extended fermentation times beyond 98 hr possibly would have revealed a reduction in sucrose content.

M. hiemalis utilized sucrose early in the fermentation but failed to use stachyose and raffinose. *A. elegans* used stachyose after 44 hr of fermentation; the decrease in stachyose was accompanied by an increase in raffinose. Hydrolysis of stachyose after 44 hr occurred at a faster rate than did raffinose utilization and may account for the latent buildup of raffinose. Sucrose levels appeared to increase slightly over the 98-hr fermentation.

Optimal cultural conditions for the production of α-galactosidase by fungi included in this study may not have been achieved. Fatty acids with more than 12 carbon atoms have been shown to stimulate α-galactosidase production by *M. vinacea* (Kobayashi and Suzuki, 1972). In an earlier paper we reported that several of the fungi included in this study had strong lipolytic activity on full-fat Florunner peanuts (Beuchat and Worthington, 1974). A study relating free fatty acid accumulation to induction of fungal α-galactosidase production on a peanut substrate would be of interest. Other nutrient requirements, pH, temperature, and aeration were not investigated with regard to their effect on enzyme production in the peanut substrate. We can state, however, that several fungi are capable of decreasing the sucrose, raffinose, and stachyose content of peanuts. The most notable of these is *N. sitophila*, the ontjom fungus.

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Distribution of Volatile Compounds between the Pulp and Serum of Some Fruit Juices

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The results of an investigation of the distribution of volatile flavor compounds between the pulp and sera of orange, grapefruit, lemon, and apple juices are reported. The trends observed with the natural juices were investigated further using model systems. The data obtained suggest that pulp may have an important effect on the flavor of certain, but not all, fruit juices. The findings are also of some relevance to the selection of methods for the isolation of flavor volatiles from juices.

As part of an investigation of certain fruit flavors, we have studied the distribution of volatile flavor constituents between the pulp and serum fractions which can be obtained from some fruit juices by high-speed centrifugation. This study was prompted by our desire to (1) determine how pulp affects headspace concentrations, and therefore aromas, of fruit juices and purees; (2) investigate statements in the literature which imply that the essential flavor constituents of orange juice are associated with the pulp; (3) determine whether centrifugation offers any advantages as a preliminary step in the isolation of flavor volatiles from fruit juices.

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EXPERIMENTAL SECTION

Preparation of Fruit Juices. Juice was extracted from orange, lemon, and grapefruit by removing the flavedo and albedo from the fruit and squeezing by hand. Vesicles were removed from the juice by filtering through cheesecloth. In the case of apple, the fruit was peeled, cored, sliced, and minced through a household grinder. Juice was obtained by pressing the mash through two layers of cheese cloth.

Separation of Pulp and Serum. Freshly prepared juice (1 l.) was centrifuged at 250,000*g* using a Beckman L2-65B ultracentrifuge. The serum was filtered to separate juice vesicle remnants. The pulp was removed from the centrifuge tubes using distilled water.

Isolation of Volatiles. The serum (~960 ml) was evaporated on a Rinco rotary evaporator at 30° under reduced