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Identification and Distribution of Soluble Saccharides in Pickling Cucumber Plants and their Fate in Fermentation[1]

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Abstract. Leaves of *Cucumis sativus* L. contained predominantly, non-reducing sugars which included verbascose, stachyose, raffinose and sucrose. Glucose and fructose were also present. The major sugars of fruit were glucose, fructose, and sucrose. Stachyose was present in small fruit (5 to 7 g fresh weight), but no soluble galactose-containing saccharides were found in larger fruit. Other plant parts also contained the raffinose saccharides. The identities of these sugars were established by partial enzymatic hydrolysis and paper chromatographic examination of the hydrolytic products. Four species of lactic acid bacteria from cucumber fermentations were able to ferment stachyose, raffinose, sucrose, melibiose, galactose, glucose, and fructose.

Bloater formation during cucumber fermentation results in serious quality defects in pickle products and causes major economic losses to the industry. Bloater formation has been attributed to CO_2 produced, in part, from metabolism of sugars by fermenting microorganisms (6). Fermentable sugars should be utilized by lactic acid bacteria, with the exclusion of microorganisms that produce large quantities of CO_2. In natural fermentations, as practiced commercially, lactic acid bacteria frequently are inhibited by low pH prior to complete conversion of the sugars. Yeasts then ferment the residual sugars and yield large quantities of CO_2 (6). A controlled fermentation process for brined cucumbers was recently developed, using a buffering agent, sodium acetate, to insure complete sugar utilization by lactic acid bacteria (8).

A cucumber fruit with a low concn of fermentable sugar would be desirable so that lactic acid bacteria could utilize all the sugar without the addition of buffering agents. One possible approach is genetic selection for low sugar. Genetic variation in sugar content exists; however, environmental influence is great (10). Glucose, fructose, and sucrose occur in cucumber fruits (10) and are readily fermented by lactic acid bacteria. However, genetic manipulation might alter both kinds and quantities of sugars. A soluble carbohydrate complement which is not readily fermented by lactic acid bacteria might arise as a result of genetic selection. Ideally, the sugars in fruit should be readily fermentable by lactic acid bacteria to reduce or eliminate chances for a secondary fermentation by undesirable organisms.

A change in the type of soluble carbohydrate in cucumber fruit is feasible because galactose-containing saccharides are known to be the sugars of transport in vegetative portions of cucurbits (16, 17). This study was conducted to examine the

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soluble sugars of leaves, transport structures, and fruit of cucumber plants. We also tested the ability of various species of lactic acid bacteria, normally present in cucumber fermentations, to ferment all of these sugars.

Materials and Methods

'Chipper', a monoecious cultivar, was used in all studies. The concn of total and reducing sugars in various plant parts were determined first. Samples were taken in mid-summer from a field-grown population of plants in a randomized complete block experiment with 5 replications. Samples included the 6th leaf from a growing tip (leaf), the petiole of the 6th leaf (petiole), internodes between the 6th and 7th leaves (mainstem), peduncles from No. 2 size fruit (2.7 to 3.8 cm diam), and fruit cross sections from the central region of size no. 2 fruit. From 5 to 20 plants were sampled per replication depending upon the plant part. All samples were frozen immediatley upon excision and held at −20°C for not longer than 7 days. For each sample, 3 g of frozen chopped material was boiled in about 20 ml of 80% ethanol, homogenized at medium speed in a VirTis homogenizer for 3 min, and centrifuged at 28,000 × g for 10 min. Ethanol insoluble pellets were extracted twice more with 10 ml portions of 80% ethanol, and the supernatants were combined and reduced to dryness at 37°C *in vacuo*. The residue was washed with diethyl ether (15) and redissolved in 10 ml of water. Total sugar was determined with anthrone reagent, and reducing sugar by the method of Nelson (9). Glucose was used as the standard in both tests.

Samples used to identify individual sugars present in various parts of the plant were similar to those used above, but were from greenhouse-grown plants. Fruits were much smaller than those used in expt. 1, weighing 5 to 7 g, and were typical of "midget-size" pickles in the commercial trade. Larger fruits (10 to 500 g) also were sampled; 10 g of each sample was extracted as above. After washing with ether, the dissolved residues were subjected to ion exchange chromatography (15) to obtain the neutral fraction containing soluble sugars. This fraction was evaporated and dissolved in 4 ml of 50% ethanol. Aliquots (20 to 400 µl) were spotted on Whatman No. 1 filter paper strips (1 × 20 cm) and developed (descending) in a solvent of 5 butanol: 1 benzene: 3 pyridine: 3 water (v/v) for 40 hr (14). Spots were visualized by dipping the dried paper strips in aniline-diphenylamine reagent (2, 14) and heating them in a forced air oven at 100°C for 2 min. Tentative identification was based on coloration and on co-chromatography with known sugars (2, 4).

To identify sugars tentatively thought to be verbascose, stachyose, and raffinose, ethanolic extracts prepared from 40 g of leaves or 40 g of small fruits were processed as described above. The concd extracts were spotted in 20 µl spots along the origin of 8 × 20 cm Whatman No. 1 filter paper, and the chromatograms were developed in the butanol:benzene: pyridine:water. A small vertical strip of each sheet was treated with aniline-diphenylamine reagent to locate the sugars. Horizontal strips about 2 cm width, corresponding to the positions of an unknown and raffinose (leaf extract) or stachyose (fruit extract) were removed. Each strip was eluted with water for several hours, and the combined eluates for each sugar were concentrated *in vacuo* to about 0.5 to 1.0 ml. Concentrated eluate, 100 µl, was incubated in a 10 × 75 mm covered test tube with 100 µl (0.25 units) of coffee bean α-galactosidase (P-L Biochem, Inc., Milwaukee, WI), 100 µl (115 units) of yeast invertase (Grade X, Sigma Chemical Co., St. Louis, MO) or α-galactosidase and invertase together for 8 hr at 35°C. After boiling for 1 min, the samples were chromatographed as described above.

To determine the ability of lactic acid bacteria isolated from cucumber brines (6) to ferment raffinose and stachyose, recommended procedures were used (1). The basal broth medium of Efthymiou and Hansen (5) has been suggested for studying fermentation characteristics of lactic acid bacteria (1). This medium was prepared in a 1.25-fold concn, and 4 ml aliquots were dispensed into 16 mm diam screw-capped tubes and heat-sterilized. Aqueous solutions of the test sugars were filter-sterilized (0.22 pore size membrane filter, Millipore Corp., Bedford, MA), and 1 ml was added aseptically to the basal medium to give final concn of 0.5% for stachyose and 2% for all other sugars. The initial broth pH was 6.5.

Test cultures were incubated in 10 ml of APT broth (BBL, Cockeysville, MD) for 16 hr at 30°C. The cells were collected by centrifugation, washed twice with 10 ml of sterile 0.85% saline and then resuspended in 10 ml of saline. Two drops of this suspension were added to 5 ml of the fermentation broth. Fermentation of the sugars was determined by measuring the broth pH after incubation for 2 to 3 weeks at 30°. A lowering of pH indicated acid production from the sugar by the bacteria. non-inoculated controls were used to ascertain sterility of broth containing filter-sterilized sugars.

Results

From 30 to 73% of the total sugar in vegetative tissues, and all of that in size 3 fruit was reducing sugar (Table 1). Size 3 fruit contained a much greater concn of sugar than did the vegetative parts. In contrast, small size 1 fruit reportedly contain a much lower sugar concn than size 3 fruit, and as much as 40% of the total sugar is non-reducing (11).

Galactose was not found in extracts from plant parts (Fig. 1). Leaves, petioles, and stems contained a polar unknown, stachyose, raffinose, sucrose, glucose, and fructose. The largest sugar spot separated from peduncle extracts was sucrose. The unknown was absent in peduncles, and the spots of sugars other than sucrose were weak. "Midget-sized" fruit (5 to 7 g) extracts yielded a small spot for stachyose and a small spot of a second unknown compound of slightly greater mobility which moved between stachyose and raffinose. Larger fruits (10 to 500 g) contained neither stachyose nor the second unidentified compound (chromatograms not shown). In all fruit extracts, glucose and fructose spots were prominent and sucrose spots were weak. The methods used to differentiate between reducing and non-reducing sugars are not sufficiently accurate when ratios of reducing to non-reducing sugars are exceedingly large (Table 1). When sugar concn is high, a large dilution must be made. Any analytical error is increased by the magnitude of the dilution factor.

Hydrolysis by α-galactosidase of the purified sugar which co-chromatographed with raffinose produced galactose and sucrose, while treatment with invertase yielded melibiose and fructose (Fig. 2A). Use of both enzymes simultaneously produced galactose, glucose, and fructose. Treatment of presumed stachyose from cucumber fruit with α-galactosidase yielded raffinose, sucrose, and galactose (Fig. 2B). Treatment with invertase gave fructose and a blue spot in the position of stachyose that was presumed to be manninotriose. The two enzymes together produced manninotriose, melibiose, galactose, glucose, and

Table 1. Sugar concn in different parts of 'Chipper' cucumber plants[z]

Plant part	Total sugar (mg/g ft wt)	Reducing sugar (mg/g ft wt)	Reducing sugar as % of total sugar
Leaf blade	3.20 ± 0.66	1.22 ± 0.44	38
Leaf Petiole	2.14 ± 0.76	1.26 ± 0.48	62
Internode	4.92 ± 1.54	3.62 ± 1.20	73
Peduncle	2.04 ± 0.50	0.66 ± 0.26	30
Size 3 fruit	15.22 ± 2.94	17.60 ± 3.04	100

[z]Values at the right of each column are 2 × SE.

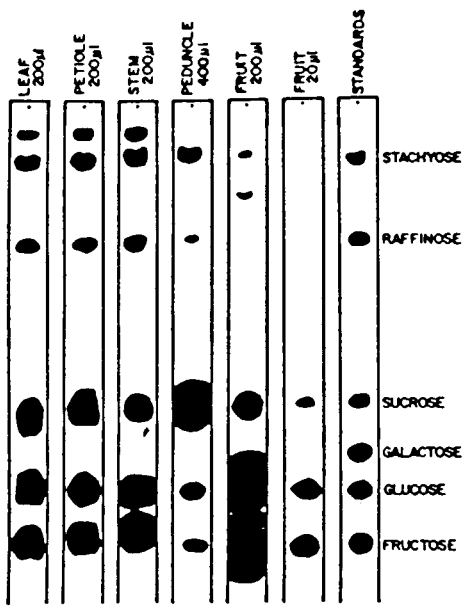

Fig. 1. Chromatograms of 80% ethanol-soluble sugars in extracts from various parts of cucumber plants. Fruit were "midget-size" (5 to 7 g). See Materials and Methods for details.

fructose. Stachyose and galactose appeared after treatment of the purified polar unknown with α-galactosidase (Fig. 2C). Invertase yielded fructose and a blue spot in the position of verbascose that was presumed to be verbascotetraose. Both enzymes together produced the blue spot, manninotriose, galactose, glucose, and fructose. Similar hydrolyses were conducted with commercially available raffinose and stachyose. The products from commercial sugars were identical with those from the saccharides of cucumber plants. Verbascose was not available from a commercial source. However, the hydrolysis products of the polar sugar are those expected from verbascose.

Of the 10 cultures of lactic acid bacteria tested, 7 fermented raffinose and 6 fermented stachyose (Table 2). At least 1 culture from each of the 4 species fermented each of the 2 sugars. Except for *Leuconostoc mesenteroides* FBB-42, all cultures that failed to ferment raffinose also failed to ferment stachyose. Glucose, fructose, and galactose were fermented by all cultures, which is typical of these species of bacteria (3). Sucrose was fermented by all cultures except one of *Lactobacillus brevis*. Melibiose was fermented by 4 of 5 cultures tested.

Discussion

In genetic selection for a low sugar cucumber fruit, raffinose saccharides could presumably become a major component of the soluble carbohydrates if screening were based solely on concn of glucose and fructose. This would appear to be of little consequence for the brining process, because the lactic acid bacteria used in this study, and presumably others involved in natural fermentations, could utilize these sugars to form lactic acid. The fact that raffinose, melibiose, galactose, and sucrose were all fermented by the bacteria in this study indicates that stachyose would probably be completely fermented by a mixture of the bacteria, as opposed to the alternate possibility that only some small fragment of the saccharide might be fermented. Fruit containing a high concn of stachyose might be undesirable for other uses because of dietary problems associated with the raffinose saccharides (4).

Hydrolysis of transported sucrose by invertase at sites of sugar accumulation in corn kernels (13) and in sugar cane stem (12) has been demonstrated. Inversion of sucrose to the monosaccharides was postulated to be an integral step in sugar accumulation in cane (12). Sucrose is the transport species in corn and in cane. Our data show that the predominant sugars in cucumber leaves are non-reducing sugars comprised of galactose-containing oligosaccharides and some sucrose. Conversely, the predominant sugars that accumulate in cucumber fruit are the monosaccharides, glucose and fructose. Stachyose is the predominant, if not the exclusive, transport species in cucumber plants (17). We found stachyose only in very small fruit (7 g or less). If complete hydrolysis of transported stachyose occurs prior to or upon entry into cucumber fruit larger than 7 g,

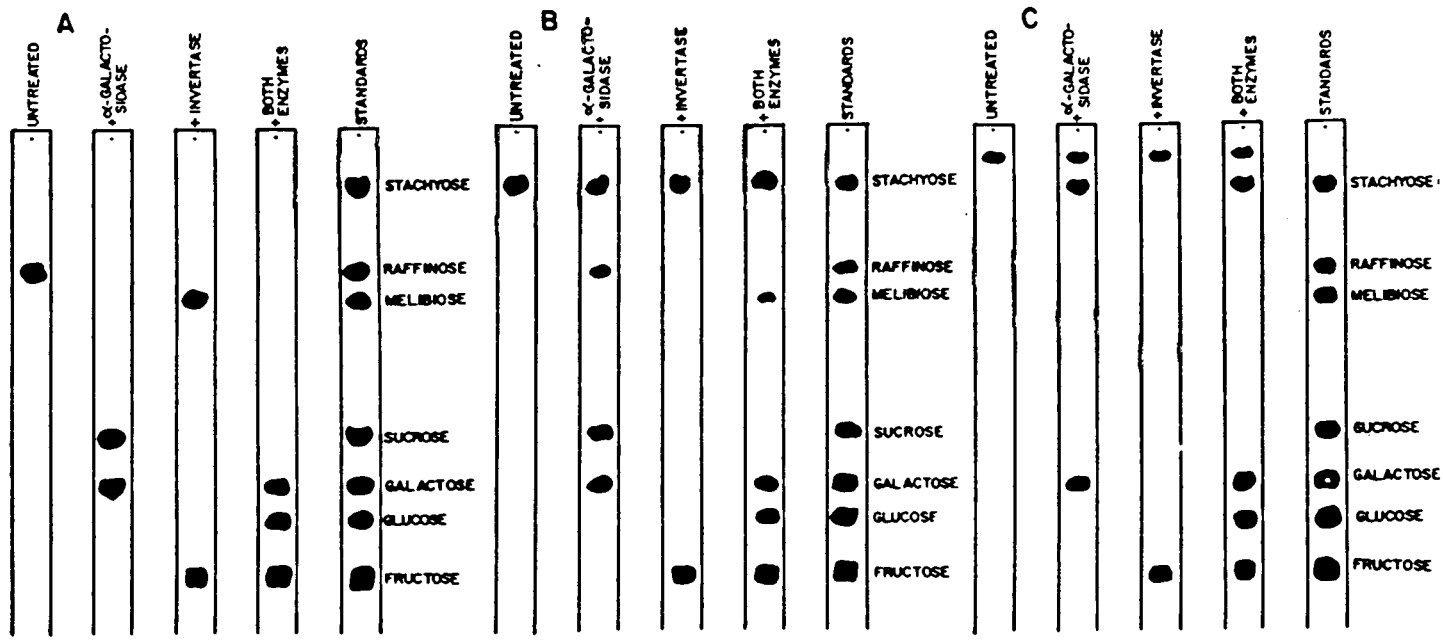

Fig. 2. Chromatograms of saccharides purified from cucumber plants and the products of their hydrolysis by α-galactosidase, invertase and both enzymes together. A – presumed raffinose from cucumber leaves and its hydrolysis products; B – presumed stachyose from small cucumber fruit (5 to 7 g) and its hydrolysis products; C – unknown from cucumber leaves and its hydrolysis products.

Table 2. Sugars fermented by various cultures of lactic acid bacteria isolated from cucumber brines[z].

Species of bacteria	Culture	Sugar						
		Stachyose	Raffinose	Melibiose	Sucrose	Galactose	Fructose	Glucose
Pediococcus cerevisiae[y]	FBB-61	-	-	-	+	+	+	+
	FBB-39	+	+	+	+	+	+	+
	L-7230	+	+	+	+	+	+	+
	L-728	+	+	+	+	+	+	+
Lactobacillus plantarum	WSO	-	-	+	+	+	+	+
	L-442	+	+	ND	+	+	+	+
Lactobacillus brevis	FBB-50	+	+	ND	+	+	+	+
	FBB-70	-	-	ND	-	+	+	+
Leuconostoc mesenteroides	FBB-42	-	+	ND	+	+	+	+
	FBB-73	+	+	ND	+	+	+	+

[z]+ = fermented, final pH of 5.4 or lower; - = not fermented, final pH of 6.3 or higher; ND = not determined.

[y]The *P. cerevisiae* cultures used may be more closely identified with *P. pentosaceus* according to Buchanan and Gibbons (3).

α-galactosidase as well as invertase must be involved. Since free galactose was not present in cucumbers, a mechanism for galactose usage, perhaps phosphorylation and epimerization to glucose, apparently is active in cucumber plants. The regulatory significance of such enzyme systems in sugar accumulation in cucumber fruit merits investigation.

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