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Myrosinase Activity in Bacteria as Demonstrated by the Conversion of Progoitrin to Goitrin.* (30181)

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Progoitrin is a thioglycoside present in high concentration in the seeds of most Brassicaceous plants and in the edible parts of some, particularly rutabaga and turnip. It is hydrolyzed by enzymes (myrosinases) in these plants to yield goitrin, a potent antithyroid compound(1,2,3) (Fig. 1).

Although it had been assumed for many years that hydrolysis of thioglycosides similar to progoitrin could be accomplished only through the action of myrosinase of plant origin, recent studies have indicated that this view is incorrect. Reese *et al*(4) reported that the fungus *Aspergillus sydowi* produced a β-thioglycosidase (termed "sinigrinase") active on the mustard oil thioglycosides sinigrin, sinalbin, and progoitrin; however, the enzyme could not be obtained from other fungal genera or from the bacteria, *Bacillus subtilis* or *Pseudomonas aeruginosa,* which also were found to "consume" sinigrin on prolonged (14-day) incubation with this compound. Thioglycosidase activity hydrolyzing several purinyl thioglycosides has been reported by Goodman *et al*(5) to be widespread in mammalian species, and to occur in the microorganisms *Tetrahymena pyriformis* and *Escherichia coli.*

Because of the possible importance of goitrin derived from commonly ingested vegetables in the production of human non-toxic goiter, studies were undertaken of the metabolic fate of pure crystalline progoitrin in man after oral ingestion. It was found that in such cases goitrin could be demonstrated in blood and urine(6). These results suggested that the conversion of progoitrin to goitrin might have been carried out by thioglycosidase activity of bacteria in the gastrointestinal tract. This hypothesis was reinforced by the finding that incubation of rat or human feces with progoitrin resulted in goitrin formation(3). This myrosinase activity in feces was destroyed by boiling, filtration, or sonoration, and was markedly reduced by prior oral administration of Amphotericin B and Neomycin(3), a regimen which has been shown to effect a striking decrease in viable fecal bacterial flora(7).

The data reported here concern experiments on the isolation and identification of fecal bacteria with myrosinase activity, comparison of such organisms with stock strains of various bacterial species, and conditions necessary for formation and activity of bacterial myrosinase.

Materials and methods. Progoitrin was prepared from rutabaga seed by the method of Greer(2). Bacterial cell suspensions were obtained by growing the organisms, unless otherwise stated, in nutrient broth containing 1% glucose for 18 hours at 37°C without shaking. The cells were harvested by centrifugation, washed once with water, and resuspended in water at a concentration between 1.0 and 2.0 mg bacterial nitrogen per ml. The cell suspensions were incubated with progoitrin and buffer at concentrations noted in the legends, in a total volume of 5.0 ml contained in 50 ml Erlenmeyer flasks which were shaken in a water bath at 37°C. Length

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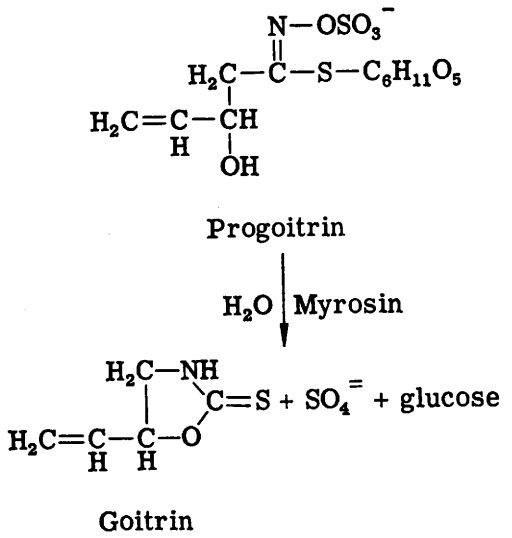

FIG. 1. Enzymatic conversion of progoitrin to goitrin. Myrosin = myrosinase.

of incubation time is noted in each Table or Figure. The assay method for the goitrin formed from progoitrin has been previously reported(6). Control flasks incubated without bacteria showed no goitrin formation except for those containing K ascorbate noted in Table III.

Results. The first approach to the isolation of organisms with myrosinase activity from mixed fecal flora was an enrichment procedure, such as is employed in the isolation from soil of organisms with specific degradative pathways. A synthetic medium containing KH_2PO_4, Na_2HPO_4, NaCl, $MgSO_4$, NH_4Cl and 0.08% progoitrin was inoculated with 10^{-3} to 10^{-7} final dilutions of a fresh fecal sample possessing myrosinase activity, and incubated at 37°C. It was hoped that the liberation of glucose on the conversion of progoitrin to goitrin would provide a carbon source for selective outgrowth of myrosinase-producing organisms. However, no growth was obtained even on several days' incubation, although growth obtained in the same medium supplemented with 0.05% glucose indicated that the negative results were not due to toxicity of progoitrin.

Similar fecal dilutions were then inoculated into tubes containing 5 ml trypticase-soy, heart infusion, nutrient, or synthetic-glucose broth, each containing 0.025% progoitrin; the tubes were incubated at 37°C for 3 days. The cultures in the last 2 tubes showing growth (usually 10^{-6} and 10^{-7} dilutions) were inoculated in duplicate at 10^{-4} to 10^{-6} final dilution into the same type of medium, and these sub-cultures incubated at 37°C for 3 days. Assays of the goitrin content after the latter incubation showed that goitrin was produced only by the nutrient broth cultures, but not by those in trypticase-soy, heart infusion, or synthetic-glucose broth. The goitrin yields ranged from 91% to 94%, indicating that organisms with significant myrosinase activity were present in these nutrient broth cultures.

The active cultures, which contained mixtures of Gram-positive cocci and Gram-negative rods, were streaked out on nutrient, brain heart infusion, MacConkey, and azide agar plates, each medium containing 0.025% progoitrin. Twenty-seven isolates, of which 9 were Gram-positive cocci and 18 Gram-negative rods, were then tested for myrosinase activity on incubation of nutrient broth cultures containing 0.075% progoitrin. None of the cocci were active. Twelve of the rods produced detectable goitrin on 24 hours' incubation. One of these positive cultures (designated as strain 55-6) was selected for further study of the optimum cultural conditions for myrosinase formation. This organism was determined to be a strain of *Escherichia coli,* with delayed lactose fermentation in 48 hours.

Resting cell suspensions of *E. coli* strain 55-6 harvested from a variety of media incubated under different conditions were tested for their ability to hydrolyze progoitrin, with the formation of goitrin. As shown in Table I, the addition of 1% glucose to nutrient broth stationary cultures resulted in a marked increase in myrosinase content of the cells, but did not improve the enzyme content of aerated cultures. The myrosinase activity appeared to be independent of the presence of progoitrin in the medium, at least in the concentrations employed. Reese *et al* noted that, while the sinigrinase of *A. sydowi* was produced in the absence of sinigrin in the growth medium, addition of sini-

TABLE I. Effect of Cultural Conditions on Myrosinase Activity of *Escherichia coli* Strain 55-6.

Conditions for growth			% Progoitrin converted to goitrin	
Broth medium	Progoitrin, μg/ml	Manner of incubation	Exp 1 24 hr incubation	Exp 2 48 hr incubation
Nutrient	0	Stationary	10	21
	50	"	8	—*
	100	"	—	23
	250	"	10	—
Nutrient + 1% glucose	0	Stationary	38	—
	250	"	35	64
	0	Aerated	8	—
	250	"	14	—
Trypticase soy	250	Stationary	—	4
Thioglycollate	250	Stationary	—	23

* Not done.

Flask contents: 0.04 M K phosphate pH 7.0, 3.75 mg progoitrin, and cell suspensions containing 3.6 mg bacterial nitrogen.

TABLE II. Comparison of Myrosinase Activity of Various Bacterial Species.

	% Progoitrin converted to goitrin					
	Experiment No.					
Organism	1	2	3	4	5	6
Escherichia coli, strain 55-6	14, 17*	14, 24	24, 26			
" " " B	20, 25					
" " " K12	12, 15					
" " " Texas	3, 3	4, 5	15, 16			
Paracolobactrum aerogenoides	41, 43	73, 81	54, 57	42, 67	24, 24	33, 38
" *coliforme*	17, 18					
" sp.				47, 48		
Aerobacter aerogenes					26, 28	0, 0
Proteus vulgaris				42, 48		0, 0
Alcaligenes faecalis				13, 15		
Pseudomonas aeruginosa					0, 11	0, 14
Bacillus subtilis				59, 72		0, 38
" *cereus*					0, 21	0, 26
Staphylococcus epidermidis				19, 19		
Streptococcus faecalis					0, 0	0, 0

* Values obtained on duplicate flasks.

Flask contents: 0.04 M K phosphate pH 7.0, 3.75 mg progoitrin, and cell suspensions containing 1 mg bacterial nitrogen. Incubation for 48 hr.

grin or mustard flour to the medium resulted in several-fold increase in enzyme yield(4). No such effect of progoitrin on bacterial myrosinase was detected in our experiments.

Since myrosinase activity was exhibited by several fecal isolates which were designated as *Paracolobactrum* species because they fermented lactose only after prolonged incubation, it was of interest to compare the activity of stock strains of the coli-aerogenes-paracolon group. A variety of other bacterial species was also tested for myrosinase content. The comparative activity of resting cell suspensions harvested from stationary cultures in nutrient broth with 1% glucose is shown in Table II.

It is apparent from these data that considerable variation was observed between replicate runs in different experiments, and even between duplicate runs on the same day, particularly with the later experiments. Despite intensive efforts, no explanation for these variations, *e.g.*, glassware washing procedure, bacterial contaminants, or the assay methods, has been found.

Even with the unusual degree of variability, the data in Table II do indicate that enzyme activity is widely distributed among

TABLE III. Effect of Sonication and Ascorbate on Myrosinase Activity of *P. aerogenoides*.

Preparation	% Progoitrin converted to goitrin	
	−ascorbate	+ascorbate
Intact cells	58, 64	2, 12
Sonicate	63, 69	7, 12

Flask contents: 1 ml 0.2 M K phosphate buffer pH 7.4, 1 ml progoitrin solution (5 mg/ml), 2 ml bacterial cell suspension (1.8 mg bacterial N/ml) or sonicate thereof, and 1 ml either H_2O or K ascorbate (25 mg/ml, pH 7). Incubation for 40 hr. Data corrected for control flasks without bacterial preparations: 0% progoitrin converted without ascorbate, 5% with ascorbate.

TABLE IV. Effect of Buffers on Myrosinase Activity of *P. aerogenoides*.

Buffer	pH	Concentration	% Progoitrin converted
K phosphate	7.4	.12 M	50, 67
"	7.4	.006 M	15, 15
"	5.6	.12 M	4, 8
"	5.6	.006 M	0, 4
Tris	7.4	.03 M	0, 0
Na acetate: acetic acid	5.6	.03 M	0, 0

Flask contents: 3.75 mg progoitrin, and cell suspension containing 2.0 mg bacterial nitrogen in 5.0 ml volume of appropriate buffer. Incubation for 48 hr.

bacterial genera and is somewhat higher in the *Paracolobactrum, Proteus vulgaris,* and *Bacillus subtilis* strains tested. As noted above, strains of the latter organism were reported by Reese *et al*(4) to "consume" sinigrin, a related mustard oil thioglycoside, but whether or not this organism hydrolyzed sinigrin is not clear from their data.

Since the strain of *Paracolobactrum aerogenoides* showed consistently high activity, it was employed in further studies on bacterial myrosinase activity. The activity was found to be retained in acetone-dried preparations of *P. aerogenoides*. Water extraction of the dried preparation by shaking for 2 hours eluted only a small fraction of the activity. Toluene treatment of cell suspensions for 15 minutes resulted in loss of about ⅔ of the myrosinase. However, sonication of cell suspensions for 5 minutes in the 10-kc Raytheon sonicator provided a crude extract with myrosinase content equivalent to that of intact cells (Table III). The equivalent activity of intact cells and sonicate of *P. aerogenoides* on progoitrin is in contrast to the results with the purinyl thioglycosidase activity of *E. coli* strain B reported by Goodman *et al* (5), who found enzyme activity only in the sonicates.

Ascorbic acid has been shown to serve as an essential co-factor for the myrosinase extracted from mustard seed(8). It was therefore of interest to determine whether it was also effective on bacterial myrosinase. As shown in Table III, the addition of L-ascorbate to *P. aerogenoides* cells or sonicates markedly decreased the production of goitrin; in other experiments, ascorbate had a less pronounced inhibitory effect on enzyme activity. While the ascorbate may be oxidized during the prolonged incubation period, and thus be unavailable for function with the enzyme, it is noteworthy that Ettlinger found fungal sinigrinase not to be affected by L-ascorbate(8).

The amount of progoitrin conversion to goitrin by *P. aerogenoides* was found to be dependent not only on the pH, but also on the type and concentration of buffer employed (Table IV). It thus appears that potassium phosphate serves not only in its buffering capacity, but may also participate in the reaction sequence as well.

The degree of conversion was also found to be dependent on the progoitrin concentration, as might be expected. However, the shape of the curve obtained (Fig. 2) indicates that the conversion rate is particularly slow at low concentrations of progoitrin. It is interesting that straight lines can be ob-

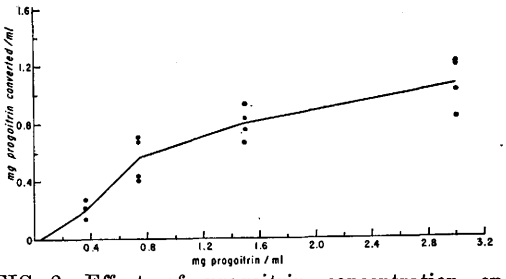

FIG. 2. Effect of progoitrin concentration on myrosinase activity of *P. aerogenoides*.

Flask contents: *P. aerogenoides* cell suspension containing 1.0 mg bacterial nitrogen, and appropriate progoitrin concentrations in 5.0 ml 0.12 M K phosphate pH 7.4. Incubation for 48 hrs.

tained by plotting the reciprocal of the progoitrin converted on a linear scale (1/V) against the reciprocal of the square of the progoitrin concentration on a linear scale ($1/S^2$) or of the progoitrin concentration on a logarthmic scale (log 1/S), whereas a curve is obtained if 1/V is plotted against 1/S on linear scales. The straight lines in themselves are not significant for the derivation of kinetic data, but they do suggest some degree of complexity in the reaction assayed. It is not known whether this complexity arises from the use of intact cells, the long incubation period, the possibility of two enzymatic reactions in sequence, the possibility of progoitrin serving as both activator and substrate of a single enzyme, or indeed from all or from none of these.

Summary. Myrosinase activity could be demonstrated in a variety of bacteria, particularly *Paracolobactrum,* which commonly inhabit the intestinal tract of man. Intact cells and sonicates were equally active. Ascorbate inhibited rather than potentiated this bacterial myrosinase activity.

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Connective Tissue XIII. Effect of Estradiol Benzoate upon Collagen Synthesis by Sponge Biopsy Connective Tissue.* (30182)

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Recently, we reported(1) a stimulating effect by several estrogenic substances on collagen synthesis in rat uterine tissue slices. Estradiol was the most potent. The effect was more pronounced in the hormone deficient animal than in the normal animal and was elicited only when the estrogens were given *in vivo*. Only a few studies have been made on the effects of estrogens on collagen synthesis in tissues other than uterus. Most of these reports have been concerned with changes in ground substance(2-5). An inhibitory effect of estrogen on the synthesis of DNA and protein in L-strain fibroblast tissue cultures has been recently reported by Kuchler *et al*(6). The parenteral administration of large doses of estrogen in mice has been reported to stimulate the deposition of new bone(7). This was believed to be a reaction of the primitive marrow to the injury caused by large doses of estrogen. On the other hand, a decrease in collagen in the bones of rats after estrogen administration was reported by Sobel *et al*(8). Recently, based on the application of histological techniques, Kelly has concluded that estrogen stimulates the growth of collagen in sponge connective tissue of normal rats(9).

Since any influence of estrogens can be easily demonstrated with a technique for *in vitro* synthesis of collagen(10,11), we have employed sponge connective tissue slices to determine the effect of *in vivo* administration of estradiol benzoate upon *in vitro* collagen synthesis in normal and ovariectomized rats. Our study included an estimation of 1) total amount and concentration of col-

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