DECOMPOSITION OF SOYBEAN OLIGOSACCHARIDES BY INTESTINAL BACTERIA*

I General Introduction and Preliminary Screening of Some Strains of *Escherichia coli* for the Ability of Decomposing Raffinose

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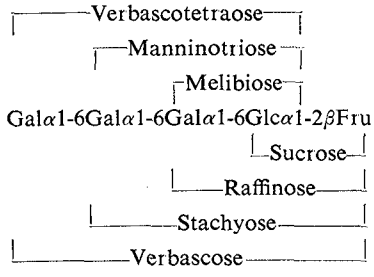
General Introduction

The object of this investigation is to know exactly the α-galactosidase activity in intestinal bacteria, chiefly *Escherichia coli,* and to see the fate of various sugars, especially the oligosaccharides contained in soybeans and those which may be formed from them in the digestive tract of men and animals. The enzyme α-galactosidase is not well studied and its purification should be tried. From the nutritional viewpoint it is aimed to see whether stachyose and raffinose are utilized by men and animals, when soybean foods or feeds are ingested.

Brief summary of previous knowledge is given below concerning (1) soybean oligosaccharides, (2) *E. coli,* and (3) α-galactosidase.

(1) Soybean oligosaccharides. According to the studies made at Kagawa University, whole soybeans contain traces of monosaccharides (glucose, fructose, and arabinose), about 5% sucrose, 1% raffinose, 4% stachyose, and a trace of verbascose.[1] The oligosaccharides were separated by column chromatography,[2,3] centrifugal chromatography,[4] and gel filtration[3] and were identified by the m.p. and specific rotation of free sugars and peracetylated derivatives.[3] Raffinose, stachyose, and verbascose are mono-, di-, and trigalactosidosucrose as shown below.



Gal: D-galactopyranosyl
Glc: D-glucopyranosyl
Fru: D-fructofuranosyl

The three main oligosaccharides (sucrose, raffinose, and stachyose) in soybeans receive only slight changes by heating.[5] These oligosaccharides are, therefore, still present in soybean foods and feeds after the conventional processing methods, unless they are removed or fermented.

In humans, intestinal juice contains sucrase, maltase, and lactase as the oligosaccharide-splitting enzymes, while other digestive fluids apparently do not contain oligosaccharide-splitting enzymes.[12] Recently

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it has been shown that intestinal juice contains two specific maltases as well as isomaltase and trehalase; these are all α-glucosidases.[6] Also according to FRENCH,[7] sucrases "from the animal intestinal tract generally act as D-glucosidases rather than as D-fructosidases.[8] Hence, it is to be expected that those oligosaccharides containing a sucrose unit in which the D-glucose is substituted by D-galactosyl would resist the action of animal sucrase. ..... BIERRY[9] found that mammalian sucrase is without action on raffinose and stachyose. CLARKE et al.[10] also obtained negative results, with both melibiose and raffinose."

However, we should consider the acidity of gastric juice. According to Biochemists' Handbook[11] the pH of human gastric juice varies widely, and normal values are 1.2-2.0. The gastric parietal cells secrete a fluid of constant composition, which is practically a pure solution of 0.17 *N* HCl, the pH being about 0.87.[12] Thus we have examined[13] the rate of hydrolysis (reducing sugar/total sugar) of sucrose, raffinose, and stachyose at various acidities. Apart from sucrose, which is digested freely, raffinose showed the rate of hydrolysis 45.2% at pH 0.86, 32.9% at pH 1.10, and 4.1% at pH 2.10, and stachyose showed 32.2% at pH 0.86, at 37°. Thus at pH 1-2 about 35 to 4% of raffinose and about 25 to 3% of stachyose may be split to liberate fructose.

In any case we may say that while sucrose is completely digested, raffinose and stachyose are only partially hydrolyzed to liberate fructose. The residue of fructose liberation is melibiose from raffinose, and manninotriose from stachyose. These contain 1 and 2 α-galactoside linkages, respectively. We are interested in α-galactosidase activity of *E. coli* in this connection.

(2) *Escherichia coli.* Bergey's Manual of Determinative Bacteriology[14] tells us as follows. The genus *Escherichia* was named by CASTELLANI and CHALMERS (1919) for Prof. Theodor ESCHERICH, who first isolated the type species of this genus, which is *E. coli* (CASTELLANI and CHALMERS, 1919), formerly known as *Bacterium coli commune* ESCHERICH 1885. As to the utilization of sugars, acid and gas are formed from glucose, fructose, galactose, lactose, maltose, arabinose, xylose, rhamnose, and mannitol. Sucrose[15,16] and raffinose may or may not be fermented. *E. coli* is usually destroyed in 30 min at 60°. It shows good growth on ordinary laboratory media. Optimum growth temperature is between 30° and 37°. Though we did not use *E. coli* var. *communis,* this includes the strains which do not ferment sucrose or salicin. Among others we used *E. coli* var. *communior,* including strains which ferment sugars but not salicin.

(3) α-Galactosidase. We have a review of WALLENFELS and MALHORTA[17] on galactosidases (1961). However, only 9 pages out of 60 pages concern α-galactosidase. This enzyme was called melibiase, since melibiose was the commonest substrate α-galactoside. The scientific name is α-D-galactoside galactohydrolase (E. C. 3.2.1.22).

α-Galactosidase is found in some lower animals and higher plants,[17] but for us the occurrence among microorganisms is of more interest. According to WALLENFELS,[17] the activity of this enzyme was first detected in brewers' yeasts (A. BAU (1895), E. FISCHER and P. LINDNER(1895)). However, bakers' yeasts are devoid of it.[18] This review lists the following microorganisms with α-galactosidase activity—*Aspergillus niger,*[19] *A. oryzae,*[20] and *Aerobacter aerogenes.*[21]

As to *E. coli* HOECKNER (1949)[22] seems to be the first to describe α-galactosidase activity. She reported that some strains of *E. coli* split raffinose but not sucrose, and presumed that they contained α-galactosidase. Then CROCKER et al. (1953-61)[23-25] studied on α-galactosidase of *E. coli* B. Very recently SCHMITT and ROTMAN(1966)[26] reported for the first time on α-galactosidase activity in cell-free extracts of *E. coli.*

Other species of microorganisms to be added to the list include a rumen strain of *Streptococcus bovis*,[27] *Diplococcus pneumoniae*,[28,29] and Actinomycetes: *Streptomyces olivaceus*, *S. roseospinus* nov. sp., and *S. fradiae*.[30-33]

It is natural that "all of the naturally occurring α-D-galactopyranosides are hydrolyzed by α-galactosidases. The velocity of hydrolysis seems to be reduced by increase in the D-galactosidic chain-length. A free reducing group in the sugar molecules reduces the rate of hydrolysis; for example, melibiose is hydrolyzed less rapidly than raffinose."[17] This statement is based on the studies of COURTOIS et al. by use of this enzyme of coffee bean,[34] germinating seeds of *Trigonellum foenum graecum*,[35] and seeds of *Plantago psyllium* and *P. ovata*.[36] Transgalactosylation was also noted.[27,29,35,36]

COURTOIS et al.[37] searched for α-galactosidase of bacterial origin, but they could find no α-galactosidase activity in many species of bacteria from the intestinal tract of cattle and sheep, though they obtained evidence for presence of this enzyme in a strain of bacteria in the feces of a guinea pig.

Preliminary Screening of Some Strains of Escherichia coli for the Ability of Decomposing Raffinose

1. Experimental Procedures

Seventeen strains of *E. coli* were used for screening. They were provided by Institute of Applied Microbiology, The University of Tokyo. They are numbered from No. 1 to No. 17 in this report. See Table 1.

Table 1 Strains of *Escherichia coli* examined

No. in this report	No. of IAM	Other descriptions
1	1016	*E. coli*; coli-2
2	1062	*E. coli*; coli-5
3	1101	*E. coli*; coli-6
4	1132	*E. coli*; coli-7
5	1159	*E. coli*; coli-8
6	1182	*E. coli*; 2-7
7	1204	*E. coli*; ML-3
8	1222	*E. coli*; Bordet
9	1239	*E. coli*; Najjar strain
10	1253	*E. coli*; ATCC 3655
11	1264	*E. coli*; K-12
12	1268	*E. coli*; B
13	1272	*E. coli* var. *communior*; ATCC 7009
14	1518	*E. coli* var. *communior*; ATCC 745
15	1519	*E. coli* var. *communior*; ATCC 206
16	1137	*E. coli*
17	—	*E. coli*; ML 308; 8-2

1.1. Method of screening

The 17 strains of *E. coli* were cultivated on nutrient bouillon (Table 2) and the strains showing high consumption of raffinose were selected.

Table 2 Nutrient bouillon

Meat extract	1%
Peptone	1
NaCl	0.5
Raffinose	0.5
in well water	

The mixture is adjusted to pH 7.0 with NaOH solution.

Take 5 ml culture medium in 18 sterilized test tubes (1 for blank test). Sterilize 3 times with the Koch sterilizer. Inoculate with a loop of each strain. Culture at 37° for 72 hours by settling. Remove the bacteria by centrifuging at 4000 rpm for 15 min. Determine residual raffinose in the supernatant by quantitative paper chromatography. Spot 20μl supernatant on a paper strip. Develop with butanol-acetic acid-water (4:1:2) 3 times. Spray with 3% *p*-anisidine hydrochloride in water-saturated butanol. Heat at about 90° for 10-15 min to colorize the sugar spot. Soak the paper strip after coloration evenly with molten paraffin. Determine at 440 mμ with a densitometer. Express the decomposition ratio by calculating from the formula, 100−(amount of raffinose in blank test−amount of raffinose in each test) × 100/(amount of raffinose in blank test).

1.2. Consumption of some sugars by the strain No. 13

Consumption of raffinose, sucrose, glucose, galactose, and fructose was compared by using the medium of Table 3.

Table 3 Culture medium for **1.2.**

KH_2PO_4	0.2%
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
Peptone	2
Sugar	0.6
in distilled water	

The pH adjusted to 7.0 with NaOH solution.

As the seed was used the suspension in which the bacteria were cultivated for 15 hours after inoculation on sugar-free medium. The seed was used in the amount of 2%, and the rest of method is the same as in **1.1.**

1.3. α-Galactosidase activity in the culture solution of the two strains No. 13 and No. 16.

The culture medium of Table 3 containing 0.6% raffinose (as the inducer) was used.

(1) Seed cultivation. —Sterilize the medium without raffinose. Inoculate with a loop of each strain. Cultivate at 37° for 15 hours at settling.

(2) Main cultivation. —Sterilize the medium (Table 3) containing 0.6% raffinose. Add 2% seed. Cultivate at 37° for 24, 48, and 72 hours (by settling). Centrifuge at 10,000 rpm for 15 min at 0 to −5°. The supernatant was used as enzyme solution.

(3) Measurement of α-galactosidase activity. —Incubate a mixture of 2 ml enzyme solution, 2 ml

Sörensen phosphate buffer (4.0 ml $M/15$ KH_2PO_4 plus 6.0 ml $M/15$ Na_2HPO_4, pH 6.98 at 18°), and 1 ml 2.5% melibiose at 37° for 24 hours. Stop the enzymatic reaction by adding 15 ml absolute ethanol. Centrifuge at 3000 rpm for 15 min. Concentrate with a rotary evaporator below 40°. Fill up with water to 2 ml. Spot 10μl on a sheet of filter paper Toyo No. 51. Develop with butanol-pyridine-water(6:4:3) twice. Spray with 3% *p*-anisidine hydrochloride in water-saturated butanol. Heat at about 90° for 15 min. Soak in paraffin. Determine at 440 mμ by a densitometer.

2. Results and Discussion

2.1. Comparison of 17 strains for the ability of decomposing raffinose

The result of the screening made by the method described in **1.1.** is shown in Table 4.

Table 4 Raffinose decomposition by 17 strains

No. of strain	Blank	1	2	3	4	5
Area of spot	7.8	6.3	5.1	4.9	4.3	6.1
Decompn. ratio	0	19.2	34.6	37.2	44.8	21.8
pH	7.0	7.8	7.8	7.4	7.6	8.6
Other spots	−	−	−	−	+	±
No. of strain	6	7	8	9	10	11
Area of spot	5.2	5.3	6.7	3.6	8.2	7.2
Decompn. ratio	33.3	32.1	14.1	53.1	0	7.7
pH	7.8	7.8	7.8	8.4	8.2	8.6
Other spots	−	±	±	+	++?	+
No. of strain	12	13	14	15	16	17
Area of spot	5.8	0	1.0	1.6	0.2	7.0
Decompn. ratio	25.6	100	87.2	79.7	99.7	10.2
pH	8.2	5.6	5.4	5.6	7.0	8.2
Other spots	++	±	±	−	−	±

Thus the strains Nos. 13 and 16 showed the highest degree of consuming raffinose. It may be noted that raffinose of the blank test showed the values 11, 9.8, and 7.8 (area of spot) by the densitometer, when tested soon after preparing culture medium, after sterilization, and after cultivation, respectively. The pH value decreased with most strains, showing the production of acids. The presence or absence of other spots than raffinose is shown.

2.2. Consumption of some sugars by the strain No. 13

The result of the experiment by the method **1.2.** is given in Table 5.

Table 5 Consumption by the strain No. 13

	Decompn. ratio	pH	Degree of browning
Raffinose	100	5.8	−
Sucrose	91.8	4.6	−
Glucose	90.7	4.2	+
Galactose	100	5.2	++
Fructose	83.8	4.4	+
No sugar	−	8.4	−

Thus all the sugars tested were consumed considerably. Especially raffinose and galactose showed 100% decomposition, and sucrose, glucose, and fructose followed in the decreasing order. The browning concerns that after sterilization by the Koch apparatus. It may have some relation with degradation of sugar before seeding.

2.3. α-Galactosidase activity in the culture solution of the two strains Nos. 13 and 16

The result of the experiment **1.3.** is shown in Table 6.

Table 6 α-Galactosidase activity of culture solution
(% decomposition of melibiose)

Strain No.	13	16
24 hrs.	9	0
48	22	9
72	34	0

Thus the strain No. 16 showed no activity, while the strain No. 13 showed slight activity which increased by longer cultivation. In cultivation of 72 hours paper chromatography showed only the spots of galactose and glucose. Increase by longer cultivation may be due to autolysis of bacteria, thus giving activity originally inside the cell.

3. Summary

Out of 17 strains of *E. coli* (Table 1), some strains, especially Nos. 13 and 16 consumed raffinose nearly completely (Table 4), when examined by settling cultivation for 72 hours. The strain No. 13 consumed completely raffinose and galactose, 92% sucrose, 91% glucose, and 84% fructose (Table 5). α-Galactosidase activity (as measured by decomposing melibiose) outside the cell was very low even with No. 13 (Table 6).

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大腸菌による大豆少糖類の分解

I 序説ならびに *Escherichia coli* のラフィノース分解能の予備実験

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要 旨

この研究の目的は大腸菌，とくに *E. coli* の中のα-ガラクトシダーゼ (E.C. 3.2.1.22，α-D- ガラクトシドガラクトヒドロラーゼ) の作用を調べ，大豆少糖類の中のスタキオース (α- ガラクトシル -α- ガラクトシルサッカロース) やラフィノース (α- ガラクトシルサッカロース) の大腸菌による分解の可能性を明らかにすることにある。

そこでまず (1) 大豆少糖類とその消化酵素による分解の可能性，(2) *E coli* の糖類に対する作用，(3) α- ガラクトシダーゼについての従来の研究を検討した。

次に17株の *E. coli* (3株の *E. coli* var. *communior* を含む) につきラフィノースの消費を比較した。72時間の静置培養で No.13 と No.16 とはほとんど完全にラフィノースを消費した。No.13 はラフィノースのほかガラクトースをも完全に消費し，サッカロース92%，グルコース91%，フルクトース84%を消費した。しかし培養液中 (細胞外) の α- ガラクトシダーゼ活性はメリビオースを基質としてはかると No. 13 でも非常に低かった。

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