ISOLATION AND PROPERTIES OF THE ENZYME OF *LEUCONOSTOC MESENTEROIDES*. PRODUCING RIBOFLAVINYLGLUCOSIDE

Yukio Suzuki

In the previous paper (3), it was found that a large amount of riboflavinylglucoside was produced from sucrose and riboflavin in the growing culture of *Leuconostoc mesenteroides*. The present experiments were instituted in order to investigate some properties of the enzyme producing riboflavinylglucoside.

METHODS

A strain of *Leuconostoc mesenteroides* L. 20 was used throughout the experiments. Assay of riboflavin compounds was performed by the method mentioned in the previous paper (3). Protein was determined by the procedure of Lowry et al. (1).

RESULTS

1. *Formation of Riboflavinylglucoside by Cell-free Culture Fluid*

The bacterium was grown in a medium containing 4 % sucrose, 0.5 % yeast extract, 0.75 % KH_2PO_4, 1.5 % K_2HPO_4, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.002 % NaCl and 0.002 % $MnSO_4$. Two hundred and fifty milliliters of the medium were placed in a 300 ml Erlenmeyer flask and then sterilized. A loopful of the bacterium from a stock culture was inoculated into 10 ml of a sterilized seed medium and incubated at 25°C for one to two days. This culture (10 ml) was used as the inoculum for the experiments. The compositions of the seed medium were as follows: 3 % sucrose, 1 % yeast extract, 0.1 % bonito extract, 0.5 % KH_2PO_4, 1 % K_2HPO_4, 0.5 % NaCl and 4 % tomato juice. After inoculation, the medium was incubated at 25°C for 16 hours under stationary conditions. During this period the bacterial growth occurred abundantly and pH was observed to be dropped from the initial value of about 7.2 to the final value of about 5.0, while dextran remained at such lower concentration as the bacterial cells could easily be removed from the culture without any disturbance. The cell-free culture fluid was obtained by centrifugation for 30 minutes at 10,000 $\times$g using a Kubota's supercentrifuge. The supernatant was recentrifuged to ensure complete removal of the cells and made up to the original voulme of the culture (1,000 ml) with distilled water. After washing twice with 0.005 M acetate buffer

(pH 5.3) by centrifugation, the bacterial cells were suspended in 20 ml of the same buffer and subjected to Kubota's 10 kc oscillator for 20 minutes. To 2 ml each of the cell-free fluid or of the sonicated cell suspension, 2 mmoles of sucrose and 3.2 μmoles of riboflavin dissolved in 4 ml of the buffer (0.2 M acetate buffer, pH 5.3, or McIlvaine's buffer, pH 7.2) were added, the total volume was made up in both cases to 6 ml, and the whole was incubated at 25°C for 9 hours. From the results of the experiments given in Table 1, it was found that most of the enzyme activity existed in the cell-free culture

TABLE 1

Formation of riboflavinylglucoside by cell-free culture fluid and by sonicated cell suspension

	*Riboflavinylglucoside formed by	
	1000 ml of cell-fee culture fluid	20 ml of sonicated cell suspension
	μmoles	μmoles
Acetate buffer (pH 5.3)	77.2	0.9
McIlvaine's buffer (pH 6.7)	31.9	0.3

*In the previous papers (3; 2) the quantities of riboflavinylglucoside and riboflavinyloligosaccharides produced were represented by the amount of riboflavin itself in the riboflavin compounds.

fluid, showing that *Leuc. mesenteroides* secreted a riboflavinylglucoside-producing enzyme as an extracellular enzyme.

The bacterial growth was readily attained with various kinds of sugar media such as glucose, glucose plus fructose or maltose. However, it is worthy of note that the enzyme activity of riboflavinylglucoside production has never been pointed out in any of the cell-free fluid obtained from such sugar media. The enzyme formation was observed only in the sucrose medium.

2. *Isolation of Crude Enzyme from Cell-free Culture Fluid*

For isolation of the enzyme, solid ammonium sulfate was added to the cell-free culture fluid (5,000 ml) at 0°-5° C to 0.45 saturation, and the precipitate was discarded. Further addition of the salt was carried out to 0.88 saturation and the mixture was adjusted to pH 4 with sulfuric acid. After standing for one hour, the precipitate was collected by centrifugation (10,000 ×g) at 0°-5°C for 30 minutes, dissolved in 200 ml of 0.2 M acetate buffer (pH 5.3) and the insoluble residue was discarded. The solution was dialyzed for 24 hours at 2°C against 0.05M acetate buffer (pH 5.3) containing 100 μmoles of sucrose and 68 μmoles of $CaCl_2 \cdot 2H_2O$ per 100 ml. The dialyzed solution of the enzyme was centrifuged and diluted as to contain 4 mg of protein per ml of 0.05 M acetate buffer (pH 5.3). To this solution,

acetone cooled previously to $-20°C$ was added dropwise until the final concentration attained to 40 per cent and kept stirring at about $-5°C$ throughout the experiments. This turbid solution was then centrifuged at high speed in the cold. The precipitate, which was found to contain most of the active enzyme together with a small amount of colored material derived from yeast extract of the culture medium, was redissolved in 50 ml of 0.05 M acetate buffer (pH 5.3) and clarified by centrifugation. The preparation thus obtained was used in the following experiments to investigate the natures of the enzyme.

3. *Some Fundamental Conditions on the Formation of Riboflavinylglucoside by the Enzyme of Leuc. mesenteroides*

Effect of incubation time. Experiments were carried out with the reaction mixture composed of the enzyme preparation, sucrose, riboflavin and acetate buffer, and the amount of riboflavinylglucoside formed during various incubation times was determined. Control experiments were made

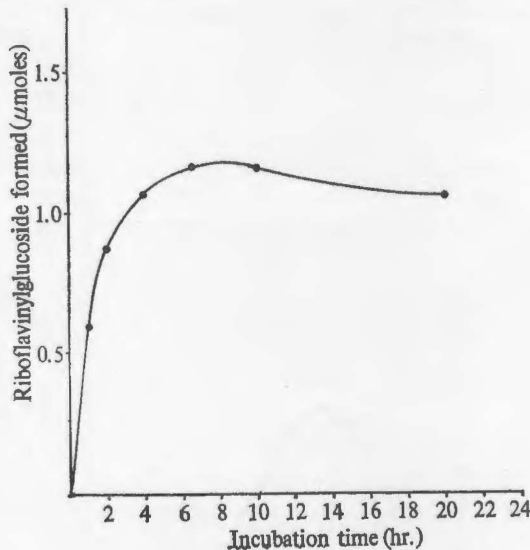


Fig. 1. Effect of incubation time
Reaction mixture (3ml) containing enzyme(0.5 ml), riboflavin (1.60 μmoles), sucrose (200 μmoles) and acetate buffer (400 μmoles, pH 5.3) was incubated at 25°C.

with the reaction mixture of (a) enzyme only, (b) boiled enzyme, sucrose and riboflavin and (c) sucrose and riboflavin but no enzyme. The results (Figure 1) show that the reaction reached nearly to the maximum equilibrium after 6.5 hours at 25°C. On prolonged incubation, however, a secondary reaction, which riboflavinylglucoside acted as an acceptor of the glucosyl group, occurred. On this reaction it will be discussed later.

Effect of sucrose concentration. Various amounts of sucrose were

added to the reaction mixture so as to give the final concentrations of 0.1, 0.5, 2, 10 and 30 %, respectively. All the reaction mixtures were incubated in the dark for 6.5 hours at 25°C. A higher yield of riboflavinylglucoside was obtained when the concentration of sucrose was in the range of 2-10 per cent, as will be seen in Table 2.

Effect of pH. The enzyme was incubated for 6.5 hours at 25°C with the reaction mixture, whose pH had been adjusted to 4.0—7.2. It will be seen in Figure 2 that the maximum yield of riboflavinylglucoside was obtained at pH 5.0-5.6.

TABLE 2

Effect of sucrose concentration

Reaction mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.60 μmoles), acetate buffer (400 μmoles, pH 5.3) and sucrose was incubated at 25°C for 6.5 hours.

Concentration of sucrose	Riboflavinylglucoside formed
per cent	μmoles
0.1	0.41
0.5	0.77
2.0	1.08
10.0	1.17
30.0	0.93

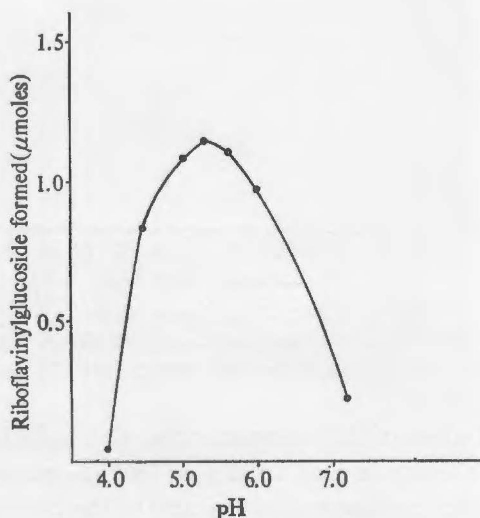
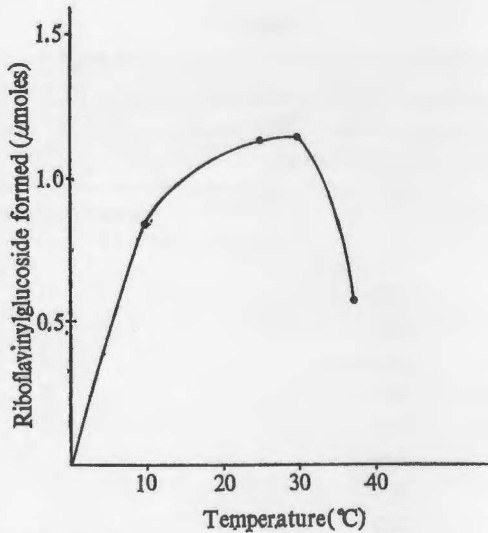


Fig. 2. Effect of pH

Reaction mixture con taining enzyme (0.5 ml), riboflavin (1.60 μmoles) and sucrose (200 μmoles) in acetate buffer (400 μmoles) was incubated for 6.5 hours at 25°C.



Fgi. 3. Effect of temperature
Reaction mixture containing enzyme (0.5 ml), riboflavin (1.60 μmoles) and sucrose (200 μmoles) in acetate buffer (400 μmoles, pH 5.3) was incubated for 6.5 hours.

TABLE 3

Thermostability of enzyme

The mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.60 μmoles) and acetate buffer (400 μmoles, pH 5.3) was preincubated at various temperatures, and thereafter, the reaction was allowed to proceed in the presence of sucrose (200 μmoles) at 25°C for 6.5 hours.

Preheating		Riboflavinylglucoside formed
Temperature	Time	
°C	min	μmoles
25	10	1.16
40	10	0.13
45	10	0
55	10	0

Effect of temperature. Relation between temperature and enzyme activity was observed by determining the amount of riboflavinylglucoside in the reaction mixture after 6.5 hours incubation at various temperatures. From the results shown in Figure 3, it was found that the enzyme revealed the maximum activity at 25—30°C.

Stability of enzyme at various temperatures. After the enzyme solution containing riboflavin and acetate buffer was treated at various temperatures for a requisite time, it was cooled instantly in an ice bath. The activity of the enzyme was observed with the reaction mixture kept at 25°C for 6.5 hours after the addition of sucrose. It will be seen in Table 3 that the

TABLE 4

Formation of riboflavinylglucoside from various sugars and riboflavin

Reaction mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.60 μmoles), acetate buffer (400 μmoles, pH 5.3) and sugar was incubated at 25°C for 6.5 hours.

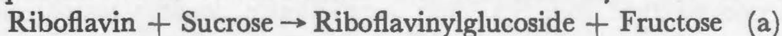
Glucosyl donor		Riboflavinylglucoside formed
	μmoles	μmoles
Glycerol	200	0
Glucose	200	0
Glucose+Fructose	100+100	0
Glucose-1-phosphate	200	0
α-Methylglucoside	200	0
Sucrose	200	1.14
Maltose	200	0
Lactose	200	0
Cellobiose	200	0
Melibiose	200	0
Raffinose	200	0
Soluble starch	70*	0
Dextran	70*	0

*: mg

enzyme was easily inactivated by the heat treatment. For example, by treatment of enzyme at 40°C for more than ten minutes at pH 5.3, a greater part of the enzyme activity was destroyed.

4. *Formation of Riboflavinylglucoside from Riboflavin and Various Sugars*

Table 4 shows the results of experiments with the reaction mixture composed of enzyme, riboflavin and various kinds of sugars. Sucrose was found to be the only active glucosyl donor, whereas glucose, glucose-1-phosphate and the other sugars were quite ineffective on the formation of riboflavinylglucoside. Therefore, the process of enzymic formation of riboflavinylglucoside by *Leuc. mesenteroides* was not a direct combination of monosaccharide to riboflavin, but a transfer reaction of the D-glucosyl group from sucrose to riboflavin. The reaction may be written as follows:



Riboflavin + Sucrose → Riboflavinylglucoside + Fructose (a)

It will be considered that the transglucosidase revealing high specificity on sucrose would catalyze reaction (a).

5. *Effect of Added Sugars on the Formation of Riboflavinylglucoside*

The mixed solutions (3 ml) composed of 0.15-0.50 ml of enzyme, 1.60 μmoles of riboflavin, 2 ml of 0.2 M acetate buffer (pH 5.3) and of added sugars, were preincubated at 25°C for 30 minutes. Thereafter, 200 μmoles

TABLE 5
Effect of added sugars

Sugar added		Relative activity for forming riboflavinylglucoside by	
		0.5 ml of enzyme	0.15 ml of enzyme
	μmoles	per cent	per cent
None		100	100
Maltose	200	45	30
	100		48
Lactose	200	98	76
Cellobiose	200	95	80
Melibiose	200	97	91
Glucose	200	99	86
Fructose	200	86	63
Glucose-1-phosphate	200	102	111
α-Methylglucoside	200	85	69
Raffinose	200	102	91
Dextran	70*	100	91

*: mg

of sucrose were added to each mixed solution and the reaction was allowed to proceed for 2-6.5 hours at 25°C. Appropriate control experiments were run at the same time. The results are summarized in Table 5. Scarcely any inhibitory effects were observed with glucose, glucose-1-phosphate, lactose, cellobiose, melibiose, raffinose or with dextran. Fructose and α-methylglucoside showed a little inhibitory effect, while maltose inhibited strongly the formation of riboflavinylglucoside by the extracellular enzyme of

TABLE 6
Effect of metals

Metal (final, 10^{-3} M)	Riboflavinylglucoside formed
	μmoles
None	1.16
$CaCl_2$	1.14
$CoCl_2$	1.04
$CuSO_4$	0.29
$FeCl_2$	0.18
$FeCl_3$	0.32
$FeSO_4$	0.30
$MgCl_2$	1.10
$MnCl_2$	1.00
$NiCl_2$	1.01
$ZnCl_2$	0.78

Leuc. mesenteroides.

6. *Effect of Metallic Ions on the Formation of Riboflavinylglucoside*

Various metallic ions were added to the mixed solutions composed of enzyme, riboflavin and acetate buffer (pH 5.3), and the solutions were preincubated at 25°C for 30 minutes. After the addition of sucrose, the reaction mixtures were incubated at 25°C for 6.5 hours for the determination of the enzyme activity. Among ten cations tested, Cu^{++}, Fe^{++} and Fe^{+++} had the pronounced inhibitory effect on the enzyme activity as is shown in Table 6.

SUMMARY

1. A strain of *Leuc. mesenteroides* was found to secrete an extracellular enzyme revealing formation of riboflavinylglucoside from sucrose and riboflavin.
2. The enzyme was isolated from the cell-free culture fluid of the bacterium grown on a sucrose medium by precipitation with ammonium sulfate and by reprecipitation with acetone.
3. Maximum activity of the enzyme was observed in the range of pH 5.0-5.6 and at 25°-30°C. The enzyme showed higher stability at 25°C and was found to be fairly sensitive to heat as it was destroyed in a few minutes at 40°C, even at pH 5.3.
4. It was found that the enzyme revealed high specificity on sucrose, since any other sugars failed to act as the glucosyl donor for the formation of riboflavinylglucoside. Moreover, the enzyme formation was observed only in a sucrose medium.
5. Maltose showed a strong inhibitory effect on the formation of riboflavinylglucoside and Cu^{++}, Fe^{++} and Fe^{+++} also acted as powerful inhibitors.

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