FORMATION OF RIBOFLAVINYLGLUCOSIDE BY INTRACELLULAR ENZYME OF *LEUCONOSTOC MESENTEROIDES* GROWN ON SUCROSE

Yukio SUZUKI

Whitby (10, 11) observed that riboflavin was transformed by rat liver preparations into a new flavin derivative which was more soluble in water than riboflavin. The compound was identified as 5′-D-riboflavin-α-D-glucopyranoside and thought to arise by transglucosidation from maltose or glycogen. The reaction was presented by equation (a).

Riboflavin + Maltose ⟶ Riboflavinylglucoside + Glucose (a)

The significance of riboflavinylglucoside formation in animal tissues remains to be assessed, because its natural occurrence has not been demonstrated. While Katagiri, Tachibana and Yamada obtained not only riboflavinylglucoside but various kinds of sugar compounds of riboflavin with enzyme preparations from a mutant of *Aspergillus oryzae* (2, 6), Escherichia coli (1, 4) and from *Clostridium acetobutyricum* (5), and it was suggested by them that riboflavin and riboflavinylglucoside played a role of glucosyl carrier in the enzymic synthesis of oligosaccharides and further the transglycosidation relating to riboflavin might be one of the synthetic processes of polysaccharides in microorganisms (3), but, again, the significance of the reaction under normal cultural conditions is uncertain.

In the previous paper (8), it was found that *Leuconostoc mesenteroides* produced a remarkable amount of riboflavinylglucoside from sucrose and riboflavin. From the results of experiments with various strains of *Leuc. mesenteroides* (9), it was also pointed out that strains producing dextrans with a high ratio of branching chain revealed higher activities of glucosyl compounds of riboflavin production than strains producing dextrans of linear chain; α-D-l, 6-linked polymers of D-glucose.

As a step to obtaining the preliminary information on the physiological significance of riboflavinylglucoside formation in bacterial cells, we have examined the intracellular activity of riboflavinylglucoside formation of *Leuc. mesenteroides* grown on sucrose.

METHODS

Four strains of *Leuc. mesenteroides*, NRRL B-512, NRRL B-1299 (=IAM 1151), L. β and L. 301, were used. Analytical procedure was performed by the methods mentioned in the previous papers (7, 9).

RESULTS

1. *Formation of Riboflavinylglucoside by Cell-free Extracts of Various Strains of Leuc. mesenteroides*

Leuc. mesenteroides was grown at 25°C in a seed medium containing, in g per 100 ml of tap water, sucrose, 3; yeast extract, 1; bonito extract, 0.1; KH_2PO_4, 0.5; K_2HPO_4, 1; NaCl, 0.5 and tomato juice, 4, and 10 ml of an 1—2 days' seed culture was used as an inoculum into a 300 ml Erlenmeyer flask containing 250 ml of the following medium, in g per 100 ml of tap water: sucrose, 2; yeast extract, 0.3; KH_2PO_4, 0.75; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 0.02; NaCl, 0.002 and $MnSO_4$, 0.002. After inoculation, the medium (one liter) was incubated at 25°C for 24 hours, by which time the pH of culture dropped to 5.3 from 7.2 and the cell-free culture fluid revealed an appreciable amount of riboflavinylglucoside-forming activity. The bacterial cells were harvested by centrifugation for 30 minutes at 10,000×g and washed three times by shaking with 0.01 M acetate buffer (pH 5.3) for 10 minutes and by recentrifuging. The washed cells were suspended in 20 ml of the same buffer. The suspension was subjected to the action of Kubota's 10 kc oscillator for 30 minutes and the sonicate was centrifuged at 10,000×g for 30 minutes. The supernatant was diluted with the buffer to contain approximately 8—10 mg of protein per ml.

The resulting cell-free extract was added to the reaction mixture containing sucrose, riboflavin and acetate buffer, and the mixture was incubated at 25°C. From the results given in Table 1, it will be seen that cell-free extracts of

TABLE 1

Formation of riboflavinylglucoside by cell-free extracts of various strains of *Leuc. mesenteroides*

Strain No.	Protein	Time of incubation	Riboflavinylglucoside formed	Riboflavinyloligosaccharides formed
	mg	hr	μmoles	μmoles
L. β	7.6	5	0	0
		16	0	0
NRRL B-512	8.5	5	0	0
		16	0	0
		48	+	0
L. 301	8.7	5	0.367	+
		16	0.681	+
NRRL B-1299 (= IAM 1151)	3.9	5	0.697	0.388
		16	0.755	0.452

Reaction mixture (3 ml) containing the sonicated cell-free extract, acetate buffer (400 μmoles, pH 5.3), riboflavin (1.600 μmoles) and sucrose (200 μmoles) was incubated at 25°C.

strains NRRL B-512 and L. β were scarcely able to form riboflavinylglucoside from sucrose and riboflavin, whereas a large quantity of riboflavinylglucoside was

formed by extracts of strains L. 301 and NRRL B-1299 (=IAM 1151). The activity of strain NRRL B-1299 (=IAM 1151) was observed to be higher than that of strain L. 301.

Moreover, from the results of experiments with the reaction mixture containing the cell-free extract of strain NRRL B-1299 (=IAM 1151) dialyzed against distilled water, riboflavin and various kinds of sugars given in Table 2, sucrose

TABLE 2
Formation of riboflavinylglucoside from various sugars and riboflavin

Glucosyl donor	pH	Riboflavinylglucoside formed	Riboflavinyloligosaccharides formed
		μmoles	μmoles
Sucrose	5.3#	0	0
	5.3	0.559	0.942
	7.0#	0	0
	7.0	0.128	0
Maltose	5.3	0	0
	7.0	0	0
Lactose	5.3	0	0
	7.0	0	0
Glucose	5.3	0	0
	7.0	0	0
Fructose	5.3	0	0
	7.0	0	0
Galactose	5.3	0	0
	7.0	0	0

#: Bacterial extracts were boiled for 5 minutes.
Reaction mixture (3 ml) containing dialyzed cell-free extract (1.0 ml). riboflavin (1.600 μmoles), sugar (200 μmoles) and acetate buffer (400 μmoles, pH 5.3) or McIlvaine's buffer (400 μmoles, pH 7.0) was incubated at 25°C for 5 hours.

was found to be the only effective glucosyl donor, whereas glucose itself and the other sugars were ineffective on the formation of riboflavinylglucoside.

2. *Isolation and Identification of Riboflavinylglucoside*

Isolation and purification of the riboflavin compound, corresponding to riboflavinylglucoside on paper chromatograms, formed by the cell-free extract of strain NRRL B-1299 (= IAM 1151) were carried out in the similar way as described in the previous paper (8). The Rf values of its purified preparation on paper chromatograms developed with various solvent systems were found to be consistent with those of riboflavinylglucoside formed by the extracellular enzyme of strain NRRL B-1299 (= IAM 1151). In the acid hydrolysate with N hydrochloric acid at 100°C for 2.5 hours, glucose and riboflavin were found to be present in the molar ratio of 1 to 1 by colorimetric determination. Glucose was detected as a single reducing product of hydrolysis on paper chromatograms with the aniline hydrogen

phthalate spray and further identified by the enzyme glucose oxidase (Nagase Sangyo Co., 3×10^4 units/g). The acid hydrolysate of this riboflavin compound was neutralized and incubated with or without glucose oxidase in the presence of phosphate buffer (0.2 M, pH 6.0). After incubation with glucose oxidase, the complete disappearance of glucose in hydrolysate was proved by paper chromatography. Thus, the purified riboflavin compound was confirmed to be riboflavinylglucoside.

3. *Some Properties of Crude Intracellular Enzyme of Strain NRRL B-1299 (= IAM 1151)*

Solid ammonium sulfate was added to the sonic extract (300 ml) containing 10 mg of protein per ml, and fractions corresponding to zero to 0.30, 0.30 to 0.50, 0.50 to 0.75 and 0.75 to 0.90 saturation were collected. Usually the fraction 0.30 to 0.50 contained about 70 per cent of the activity of riboflavinylglucoside formation. The fraction was dissolved in a volume of 0.05 M acetate buffer (pH 5.3) equivalent to one-tenths the initial volume of extract and dialyzed for 24 hours at 4°C against 0.05 M acetate buffer (pH 5.3). The dialyzed solution was centrifuged and the resulting supernatant was used in the following experiments to investigate some fundamental conditions on the formation of riboflavinylglucoside by the intracellular enzyme of *Leuc. mesenteroides* NRRL B-1299 (= IAM 1151).

Fluctuation of the Amounts of Riboflavin and its Sugar Compounds During Incubation — The reaction mixture containing the enzyme preparation, sucrose, riboflavin and acetate buffer was incubated in darkness at 25°C. Aliquots were removed at intervals, and the amounts of riboflavin and its sugar compounds were examined. The fate of riboflavin compounds is shown in Figure 1. The

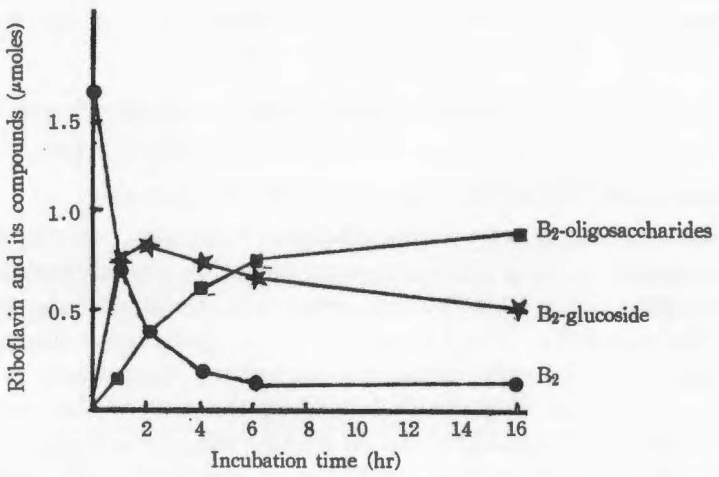

Fig. 1. Fluctuation of riboflavin and its compounds. Reaction mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.600 μmoles), sucrose (200 μmoles) and acetate buffer (400 μmoles, pH 5.3) was incubated at 25°C.

synthesis of riboflavinylglucoside reached the maximum state in the earlier stage of incubation, and thereafter riboflavinyloligosaccharides formation from riboflavinylglucoside were successively increased by receiving the glucosyl group from sucrose. This problem will be reported in the following paper.

Effect of Temperature —— The reaction mixtures were incubated at various temperatures (15—45°C), and the amount of riboflavinylglucoside formed after one hour was measured. The results (Figure 2) showed that the enzyme possessed the optimum activity at about 30°C.

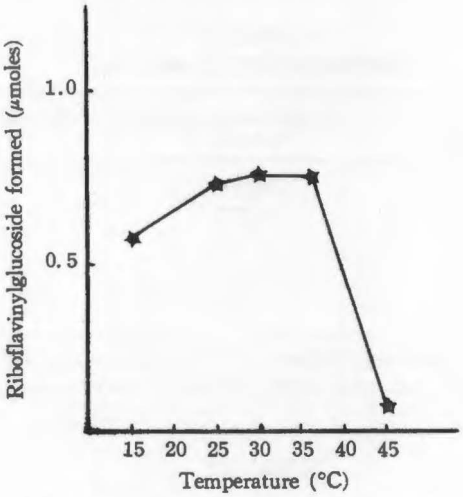

Fig. 2. Effect of temperature. Reaction mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.600 μmoles), sucrose (200 μmoles) in acetate buffer (400 μmoles, pH 5.3) was incubated for one hour.

Effect of pH —— The enzyme was incubated for one hour with the reaction mixture, whose pH had been adjusted to 4.0—6.6. It will be seen in Figure 3

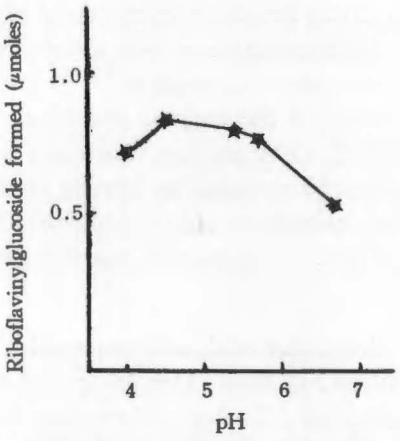

Fig. 3. Effect of pH. Reaction mixture (3 ml) containing enzyme (0.5 ml), riboflavin (1.600 μmoles) and sucrose (200 μmoles) inac etate buffer (400 μmoles) was incubated for one hour.

that the enzyme was the most active in the range of pH 4.5—5.3.

Stability of Enzyme at Various Temperatures — After the enzyme solution in 0.05 M acetate buffer (pH 5.3) was treated at various temperatures for 10 minutes, it was cooled instantly in an ice-bath. For the determination of residual enzyme activity the reaction mixture was allowed to stand at 25°C for one hour. As shown in Table 3, the enzyme was easily inactivated by heat treatment: its activity was lost almost completely by the treatment of the enzyme at 45°C for 10 minutes.

TABLE 3
Thermostability of enzyme

Preheating		Riboflavinylglucoside formed	Riboflavinyloligosaccharides formed
Temperature	Time		
°C	min	μmoles	μmoles
25	10	0.772	0.371
45	10	+	0
55	10	0	0
65	10	0	0

Reaction mixture (3 ml) containing riboflavin (1.600 μmoles), sucrose (200 μmoles), acetate buffer (400 μmoles, pH 5.3) and preheated enzyme (0.5 ml) at various temperatures, was incubated at 25°C for one hour.

SUMMARY

1. Cell-free extracts prepared from strains of *Leuc. mesenteroides* grown on sucrose were found to reveal the formation of riboflavinylglucoside from sucrose and riboflavin. Moreover, it was found that the extracts utilized only sucrose as a glucosyl donor in the formation of riboflavinylglucoside, while glucose itself was quite ineffective.

2. The intracellular enzyme revealing formation of riboflavinylglucoside was separated by ammonium sulfate precipitation from the cell-free extract of the strain NRRL B-1299 (= IAM 1151) grown on sucrose.

3. The maximum activity of the enzyme was observed in the range of pH 4.5—5.3 and around at 30°C. The enzyme was found to be fairly sensitive to heat as it was almost completely inactivated by heating at 45°C for 10 minutes.

4. By the action of the enzyme, riboflavinyloligosaccharides were observed to be formed through the successive transfer of glucosyl group between riboflavin compounds and sucrose.

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