ON THE TRANSGLYCOSIDATION RELATING TO RIBOFLAVIN BY *ESCHERICHIA COLI*

VI. PRELIMINARY INVESTIGATIONS ON TRANSFRUCTOSIDATION

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In the previous papers (1—5), the authors reported the transglycosidations relating to B₂¹ by the catalytic action of transglucosidase or transgalactosidase of *Escherichia coli*, and clarified the reaction mechanism through which the B₂-compounds of oligosaccharides were synthesized. It was concluded that the interaction of transglycosidase on isoalloxazine of B₂ took place in the first step of the transglycosidation.

The present paper demonstrates the transglycosidation from sucrose to B₂ by the enzymic actions of the cell-free extract of *E. coli*, and the characteristics of the B₂-glycosides synthesized in such system. The results described below, confirm and extend the previous conclusion and lend support to the hypothesis that B₂ plays a significant role of glycosyl carrier in biological systems of oligosaccharide synthesis.

EXPERIMENTAL

Materials and Methods

Materials — All the materials employed were the commercial products.

Organism and Enzyme Preparation — *Escherichia coli neapolitanus* (B-25) was cultivated statically in a nutrient bouillon and harvested by Sharples centrifuge. The enzyme solution used in this study was the dialyzed cell-free extract prepared from the dried cells as previously reported (1).

Methods Microdetermination of B₂-compounds was carried out as previously described (2) after paper chromatographic separation. The natures of sugar constitutions of B₂-compounds were identified by partial acid hydrolysis, followed by paper chromatography (2, 4).

RESULTS

Transglycosidation in the Media Containing Sucrose and B₂ — A reaction mixture (2 l), containing 150 mg of B₂, 70 g of sucrose, 800 ml of 0.1 M glycine

¹ The following abbreviations are used: B₂, riboflavin, B₂⁻, riboflavinyl.

buffer (pH 7.0) and 500 ml of dialyzed extract, was incubated in the dark at 30° for 48 hr, followed by Crammer's procedure (6), and all the B₂-compounds were finally extracted with 40 ml of distilled water.

The examination of the paper chromatograms after two-dimensional development revealed not only B₂-glucoside and B₂-fructoside which were expected to exist, but also the other B₂-compounds of oligosaccharides, as shown in Fig. 1. These compounds migrated to the cathode in paper electrophoresis according to Siliprandi (7).

Separations and isolations of the new B₂-compounds were carried out by the procedure of multiple paper chromatography (1, 2), and final purification was achieved by reduction with hydrosulfite.

The *R_F* values and the amounts of the B₂-compounds were summarized in Table I.

FIG. 1 Two-Dimensional Paper Chromatography of the Reaction Products of Sucrose and B₂ by the Cell-free Extract of *E. Coli*.

Reaction mixture containing B₂, sucrose and cell-free extract of *E. coli* in glycine buffer (pH 7.0) was incubated at 37° for 48 hr. Each spot was detected under ultraviolet lamp. The chromatogram was developed at first with water saturated with isoamyl alcohol, and then with butanol-acetic acid-water (4:1:5).

TABLE I
R_F Values of B₂-Compounds of Oligosaccharides

B ₂ -compound	Amount of B ₂ -compound μM	<i>R_F</i> values by a solvent system ^a	
		B-Ac-W	W-isoAmyl
Comp. 1	16	0.23	0.51
2	26	0.20	0.53
3	9	0.17	0.52
4	7	0.14	0.54
5	12	0.13	0.57
6	7	0.10	0.62
7	3	0.08	0.70

^a Solvent systems used were the following:

B-Ac-W, butanol-acetic acid-water (4:1:5); W-isoAmyl, water saturated with isoamyl alcohol.

Characteristics of the B₂-Compounds—The purified preparation of each compound, which revealed chromatographically homogeneous, was partially hydrolyzed by 0.5 *N* HCl, and the products were tentatively identified by paper chromatography (Table II).

TABLE II
Products of Acid Hydrolysis, and Tentative Structures of the B₂-Oligosaccharides

B ₂ -compound of oligosaccharide	Hydrolysate	Tentative structure
Comp. 1	B ₂ , fructose.	B ₂ -fructoside
2	B ₂ , glucose.	B ₂ -glucoside
3	B ₂ , Comp. 1, fructose, (fructobiose).	(B ₂ -fructobioside)
4	B ₂ , Comp. 2, fructose, glucose, (disaccharide).	(B ₂ -disaccharide)
5	B ₂ , Comp. 2, glucose, isomaltose.	B ₂ -isomaltoside
6	B ₂ , Comp. 2, Comp. 5, glucose, isomaltose, dextrantriose.	B ₂ -dextrantrioside
7	B ₂ , Comp. 2, Comp. 5, Comp. 6, glucose, isomaltose, dextrantriose, dextrantetraose.	B ₂ -dextrantetraoside

Chemical Properties of B₂-Fructoside Synthesized by E. coli Further purification by ethanol recrystallization was applied to the Compound I described above, and about 1.5 mg of reddish yellow crystal was obtained. The ratio of optical densities, 375 m μ /450 m μ , was found to be 0.88, and its nitrogen content was 88 per cent of the theoretical value calculated for B₂-fructoside. Estimation of fructose was carried out colorimetrically using anthrone (8).

Chemical properties of this preparation were found to be identical with those of B₂-fructoside obtained by *Aspergillus oryzae* (9), as shown in Table III. It melted at about 250°, turning brown in color.

TABLE III
Chemical Properties of B₂-Fructoside

Source of enzyme	<i>E. coli</i> ^a	<i>Asp. oryzae</i>
R_F^b { B-Ac-W	0.23	0.25
P-W	0.39	0.39
W-isoAmyl	0.51	0.47
Ratio of { 260 m μ /450 m μ	2.22	2.20
O. D. { 275 m μ /450 m μ	2.11	2.10
375 m μ /450 m μ	0.88	0.87
mp °C (decomposition)	250	—

^a The values obtained in the present experiments.

^b R_F values were observed with Toyo filter paper No. 2 using following solvent systems: B-Ac-W, butanol, acetic acid and water (4:1:5); P-W, Na₂HPO₄·12 H₂O and water (5:100); W-isoAmyl, water saturated with isoamyl alcohol.

Effect of Various Saccharides on Transglycosidation — It was found that glucose, α -methylglucoside and α -glucose-1-phosphate revealed noticeable inhibition on the synthesis of B₂-glucoside, but hardly on the synthesis of B₂-fructoside. As regards the inhibition of α -glucose-1-phosphate on the

transglucosidation relating to B₂ by *E. coli*, the authors already reported in the previous papers (1, 3), and was again confirmed in the present experiment. Its inhibition was found to be attributable to a competitive effect on α -glucosyl group of sucrose molecule, while the synthesis of B₂-fructoside was preferably inhibited by the addition of fructose, as shown in Table IV.

TABLE IV

Effect of Sugars and Alcohols on B₂-Glycosides Synthesis

Reaction mixture (10 ml), containing 200 μ M B₂, 50 mM sucrose, 0.1 M glycine buffer (pH 7.0) and 4 ml of cell-free extract of *E. coli*, was incubated at 37° for 48 hr.

Addition	Concentration	Inhibition on the synthesis of	
		B ₂ -glucoside	B ₂ -fructoside
	mM	per cent	per cent
Glucose	25	8	2
Fructose	25	6	11
α -Methylglucoside	25	11	6
α -Glucose-1-phosphate	25	55	13
Methyl alcohol	50	3	5
Glycerol	50	2	0

Transfructosidation from Various Saccharides—As for the fructosyl donor, an experiment was carried out with various saccharides. Raffinose was found to replace sucrose in the synthesis of B₂-fructoside, although the relative rate of utilizability was fairly low. Fructose was hardly utilized as a fructosyl donor. The results were shown in Table V.

TABLE V

Synthesis of B₂-Fructoside from Various Saccharides

Reaction mixture (10 ml), containing B₂ saccharide and cell-free extract of *E. coli* in glycine buffer (pH 7.0), as described in Table IV, was incubated at 37° for 48 hr.

Fructosyl donor	B ₂ -fructoside synthesized	Ratio of utility ^a
	μ M	per cent
None	0	0
Fructose	0	0
Sucrose	18	100
Raffinose	4	22

^a Expressed as percentage of the action with sucrose.

DISCUSSION

So far mentioned above, B₂-glucoside, B₂-fructoside and several B₂-compounds of oligosaccharides were synthesized from sucrose and B₂ by the transglycosidase of *E. coli*.

It is, in general, accepted that two isodynamic transglycosidases exist; both enzymes can react with sucrose, but transglucosidase transfers the

glucosyl part of sucrose molecule, while transfructosidase transfers the fructosyl part. Honey transglycosidase (10) is regarded to belong to the former, and the invertase of yeasts, and moulds (11) are found to be the latter.

However, the results described above show that both enzymic activities exist together in the cells of *E. coli*, and catalyze independently glucosyl- and fructosyl-transfers from sucrose to B_2 . Glucose, α -methylglucoside and α -glucose-1-phosphate are found to inhibit the transglucosidation, while fructose inhibits the transfructosidation.

B_2 -glucoside and B_2 -fructoside synthesized in the first stage of the reaction, would react as second acceptors in the subsequent transglycosidation, then the synthesis of complex oligosaccharide compounds of B_2 , containing either glucose or fructose, would proceed from sucrose and B_2 by transglycosidases of *E. coli*.

It is, therefore, conceivable that the primary step in the formation of B_2 -oligosaccharides is due to the interaction of B_2 , especially on the part of its isoalloxazine, with transglycosidase and that the subsequent formation of glycosyl chain occurs by successive transglycosidations to the B_2 -enzyme complex.

On the other hand, the authors' previous studies on the transglucosidation (1, 3) showed that the purified transglucosidase reacted only on maltose as its glucosyl donor, but not on sucrose. The results of the present transglucosidase, capable of utilizing sucrose as its glucosyl donor, differ from that of the previous enzyme in their substrate specificity.

All the results described above are based on the experiment with the crude enzyme solution. The authors are now instituting a study on the isolations of the enzyme.

SUMMARY

1. An extract of *E. coli* catalyzed the successive transglycosidation between sucrose and B_2 , synthesizing various B_2 -compounds of oligosaccharides, containing either glucose or fructose.

2. Tentative structures of these B_2 -compounds were characterized by paper chromatographic isolations, followed by partial acid hydrolysis.

3. B_2 -fructoside synthesized by *E. coli*, was isolated in a crystalline form and its chemical properties were found to be identical with those of B_2 -fructoside synthesized by *Asp. oryzae*.

4. It was found that glucose, α -methylglucoside and α -glucose-1-phosphate inhibited the transglucosidation, while fructose preferably inhibited the transfructosidation.

5. Raffinose was capable of replacing sucrose as a fructosyl donor, but fructose was hardly utilized.

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