ON THE TRANSGLYCOSIDATION RELATING TO RIBOFLAVIN BY *ESCHERICHIA COLI* IV. FORMATION OF GALACTOSYL COMPOUNDS OF RIBOFLAVIN

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The action of transglucosidase relating to B₂ of *Escherichia coli* has been described in detail by the authors (1, 2, 3). In this paper the experiments on transgalactosidation will be reported.

B₂-galactoside has first been produced and isolated by the authors from both *Aspergillus oryzae* (4) and *E. coli* through transgalactosidation between B₂¹ and lactose, and its chemical structure was established as 5'-D-riboflavin-β-D-galactoside. The reaction takes place as follows:

Since successive transfer reaction was also observed in the transgalactosidation relating to B₂, producing galactosyl oligosaccharides of B₂, it was assumed that this synthetic process might be one of the actual mechanisms of forming 1→6 linked, galactosyl oligosaccharides which occur in bacteria.

The present paper describes the experiments on the isolation and identification of B₂-galactoside, -galactobioside and -galactotrioside produced by *E. coli* to clarify the reaction mechanism, through which oligosaccharide formation proceeds in bacterial cells.

EXPERIMENTAL

Materials and Methods

Materials — B₂-galactoside was prepared enzymatically, as will be described later in this paper. The other materials were the commercial products.

Organism — *Escherichia coli neapolitanus* (B-25) was used. The bacterium was cultivated statically in a nutrient bouillon and harvested by Sharples centrifuge. The sedimented cells were dried by electric fan at room temperature and kept in a desiccator over CaCl₂ under reduced pressure.

The crude enzyme was prepared from the dried cells as described previously (1), and used for the experiment on decomposition of B₂-galactoside.

Methods — The assay of B₂-compounds was carried out in the same manner as described in the previous papers (1, 2). The method of Pazur (5) was used

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

to identify the galactosyl oligosaccharides separated by multiplied paper chromatography, and aniline phthalate was used to locate the reducing saccharides (6).

RESULTS

1. Formation of Galactosyl Compounds of B_2

The reaction mixture (100 ml), containing 40 mg of B_2 , 2 g of lactose and 2 g of the dried cells suspended in 40 ml of glycine buffer (pH 7.3), was incubated in the dark at 30°. Aliquots of 10 ml each of the reaction solution were removed at intervals to be tested by Crammer's procedure. Examination of the chromatogram by ultra-violet lamp, there appeared apparently three new B_2 -compounds, the formation of which with time was illustrated in Fig. 1. The R_F values of the three compounds obtained with a solvent system of butanol-acetic acid-water (4:1:5 by volume) were 0.18, 0.11 and 0.05, respectively, whereas that of B_2 was 0.30. Compound I which was formed in the earlier stage of the reaction, decreased gradually after 14 hr, while the formation of Compound II rose during 5 to 20 hr of incubation and then fell gradually. However, Compound III was formed insignificantly throughout the whole reaction period.

Isolation of the new products was carried out by paper chromatographic procedure described previously (1, 2) using 200 ml of the reaction solution after 18-hr incubation. Final purification was achieved by reduction with hydrosulfite and recrystallization from ethanol.

2. B_2 -galactoside

About 2 mg of reddish yellow crystalline powder of Compound I was obtained by the above treatment. The ratio of the optical densities (375 m μ /450 m μ) of this compound was found to be 0.87, and its nitrogen content corresponded to 90 per cent of the theoretical value calculated for B_2 -galactoside.

It was found to coincide with the B_2 -galactoside preparation (4) obtained with *Asp. oryzae* in absorption spectra and paper chromatographic behaviours (Table I). It melted at about 250°, turning brown in color. The presence of galactose was confirmed by paper chromatography after hydrolysis according to Whitby's method (7). Estimation of galactose was carried out colorimetrically using anthrone (8).

FIG. 1 Formation of galactosyl compounds of B_2 by dried cells of *E. coli*.

The reaction mixture (100 ml), containing 40 mg of B_2 , 2 g of lactose and 2 g of dried cells of *E. coli* suspended in 40 ml of glycine buffer (pH 7.3) was incubated at 30°. Curve 1, Compound I. Curve 2, Compound II. Curve 3, Compound III.

TABLE I
Chemical Properties of B_2 -Galactoside

Source of enzyme	<i>E. coli</i> ^a	<i>Asp. oryzae</i>
R_F ^b { B:Ac:W	0.18	0.18
P:W	0.42	0.43
W:isoAmyl	0.49	0.49
Ratio of optical densities { 260 m μ /450 m μ	2.28	2.24
275 m μ /450 m μ	2.03	2.03
375 m μ /450 m μ	0.87	0.86
mp in °C (decomposed)	249	250-251

^a The values obtained in the present experiment

^b R_F values were observed with Toyo filter paper No. 2 using following solvent systems: B:Ac:W, butanol-acetic acid-water (4:1:5); P:W, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ -water (5:100); W:isoAmyl, water saturated with isoamyl alcohol.

3. B_2 -Compounds of Galactosyl Oligosaccharides

Attempts to crystallize Compounds II and III have not yet been successful. However, the whitish yellow powders of the compounds were obtained after reprecipitation from ethanol at -5° . After complete hydrolysis with 2 *N* HCl for 2 hr at 100° , it was observed that Compound II was composed of B_2 and

galactose residues. From the result of quantitative estimation of galactose of Compound II, the molar ratio of B_2 to galactose appeared to be 1:2. Further, by partial hydrolysis of Compound II with 0.2 *N* HCl, galactobiose was obtained. The structure of Compound II is therefore assumed to be B_2 -galactobioside. The purity of powdered preparation of Compound II was found to be 42 per cent by spectrophotometric analysis.

The yield of Compound III was observed to be markedly less than the other two compounds I and II. By partial acid hydrolysis of Compound III, a small amount of galactotriose was obtained, so that the structure of this compound is established to be B_2 -galactotrioside.

4. Enzymatic Decomposition of B_2 -Galactoside

B_2 -galactoside was decomposed rapidly into B_2 and galactose by galac-

FIG. 2 Decomposition of B_2 -Galactoside by the Extract of *E. coli*

The reaction mixture (50 ml) containing 50 μM B_2 -galactoside, 0.1 *M* glycine buffer (pH 7.3) and 20 ml of the extract, was incubated at 30° .

Curve 1, B_2 -galactoside. Curve 2, B_2 .
Curve 3, B_2 -galactobioside.

tosidase of the extract of *E. coli*, whereby the formation of B₂-galactobioside was ascertained as shown in Fig. 2.

The reactions are considered to proceed through both of the following routes.

The rate of B₂ formation fell slightly in the presence of 50 mM galactose. On the other hand, after addition of lactose, rapid formation of B₂-galactobioside was observed, as can be expected from the above results showing successive transfer of galactosyl group to B₂.

DISCUSSION

There is a marked similarity between the mode of transglucosidation relating to B₂ reported previously (1, 2), and that of the transferring reaction of galactosyl groups. In the former case, glucosyl group of maltose is transferred successively to B₂, whereas in the latter case, galactosyl group of lactose is transferred.

Owing to the difficulty of purification and of preparing sufficient quantities of all the B₂ compounds of galactosyl oligosaccharide formed in the present reaction, their structural analysis by classical methods was not attempted. However, from the chromatographic examination of the partial acid hydrolysate, the structures of these oligogalactosides of B₂ were assigned as described above.

Thus the mechanism of transgalactosidation relating to B₂ may be formulated as follows:

The works of many authors, *e.g.*, Pazur (5), Wallenfels (9), and Aronson (10), on the reaction of galactosyl transfer have shown the formation of galactosyl oligosaccharides from lactose with certain lactase and β -galactosidases, although the actual significance of the reaction was almost uncertain.

The transfer system relating to B₂ is revealed to be the successive formation of oligosaccharides, and differs from the above reactions in such point as it proceeds, to an appreciable extent, at a considerably low concentration of acceptor B₂, as previously described (2).

SUMMARY

1. Successive transgalactosidation from lactose to B₂ took place by the action of *E. coli*, forming galactosyl oligosaccharide compounds of B₂.

2. By preparative chromatography of the reaction products three B₂ compounds were separated and identified as B₂-galactoside, B₂-galactobioside and B₂-galactotrioside, respectively.

3. B₂-galactoside produced by *E. coli* was isolated in a crystalline form and compared with the preparation obtained by *Asp. oryzae*. Both were found to

be quite identical in chemical properties.

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