Studies on α-Galactosidases (III)

The Isolation and Identification of the Products of the Transgalactosylation Reaction of α-Galactosidase from Bottom Yeast

By

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FRENCH has demonstrated, using paper chromatography, the formation by transglycosylation of a trisaccharide which seemed to be manninotriose, α-galactosyl-(1→6)-α-galactosyl-(1→6)-glucose[1]. In addition, he could isolate in crystalline form a galactobiose and a galactotriose, which were formed in a mixture of galactose and enzyme by reversal of the hydrolysis reaction.

During the hydrolysis of melibiose by the enzyme of bottom yeast, BLANCHARD and ALBON[2] have also recognized by paper chromatography the production of two unknown oligosaccharides, the Rf values of which were smaller than that of melibiose. They assumed that one of the products is manninotriose, although the substance was not examined in detail.

No further information on products of this kind seems to have appeared in later literature.

In the preceding paper of this series[3], the author demonstrated the appearance on paper chromatograms of several oligosaccharides which appeared to be produced by the action of the α-galactosidase of bottom yeast when both highly purified and crude enzyme preparations were used. However, since no examination was made at that time of the nature of the transfer products, a detailed study has now been undertaken. The author has been able to prepare the products in a pure state and identify them by physical and chemical methods. The present paper deals with the results of these experiments.

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Materials and Methods

1. *Preparation of enzyme and the measurement of enzyme activity*

A crude extract was prepared from bottom yeast almost exactly as in the preceding paper[3] and used as enzyme solution without any purification throughout this work.

The total volume of the reaction mixture for the assay of enzyme activity was 2.5 ml. made up of *p*-nitrophenyl α-galactoside (0.1536 M), acetate buffer (pH 4.8, 0.04 M), and 1 ml. of enzyme solution. Enzyme activity was estimated from the amount of *p*-nitrophenol liberated which was measured by the absorption at 400 mμ.

2. *Substrates and acceptors*

Substrates :

p-Nitrophenyl α-D-galactoside (m. p. 167~168°C, $[\alpha]_D^{15} = +222.0°$)

p-Nitrophenyl β-D-galactoside (m. p. 177~178°C, $[\alpha]_D^{18} = -74.7°$)

p-Nitrophenyl β-D-glucoside (m. p. 164~165°C, $[\alpha]_D^{20} = -98.7°$)

Melibiose (commercial, superior quality)

Acceptors :

Methanol

Galactose

3. *Paper chromatography and elution of products from the paper chromatogram*

The enzyme reaction for the preliminary test for transfer products was carried out at 30°C in 5 ml. of a reaction mixture composed of substrate (0.1536 M *p*-nitrophenyl α-galactoside or melibiose), acetate buffer (0.04 M, pH 4.8) and enzyme solution with or without acceptor (5 M). 0.5 ml. aliquots of the incubation mixture were withdrawn at intervals, quickly heated in a boiling water bath for 5 minutes, and centrifuged; by this procedure the enzyme was completely inactivated. 0.01 ml. aliquot of the each supernatant was then applied to a sheet of Toyo filter paper No. 52 near one end. The chromatographic run made use of either the one-dimensional ascending or the descending technique at room temperature with the solvent system, *n*-butanol-pyridine-water (6 : 4 : 3, v/v). To detect of sugar and *p*-nitrophenol, the acetone-silver nitrate method and treatment with ammonia vapor were

used respectively exactly as described in the preceding paper[3].

The following procedure was applied for eluting the transfer products from the paper chromatogram. 2.0 ml. of 0.1 M acetate buffer of pH 4.8, containing 231.2 mg. of *p*-nitrophenyl α-galactoside was mixed with 1.0 ml. of 25 M methanol and 2.0 ml. of enzyme solution. After incubation for 420 minutes, the mixture was heated and centrifuged for 5 minutes. The supernatant was then concentrated to about 2 ml. under reduced pressure and applied as a lateral band to Toyo filter paper No. 52 (6 × 60 cm.) near one end. The descending technique was applied using the same solvent system as above. After any transfer product had been located on guide strips, a corresponding lateral strip containing the product was cut across the initial paper. The strips were then boiled with 50 per cent ethanol under reflux for 120 minutes. These extracts were concentrated under reduced pressure to about 2 ml. for characterization of the sugar components of the products.

4. *Isolation of transfer products by carbon column chromatography*

4.69 g. melibiose, 0.58 g. galactose and 34 ml. of 0.1 M acetate buffer, pH 4.8, were incubated at 30°C with 34 ml. of enzyme solution. After 1380 minutes the reaction mixture was heated in a boiling water bath for 5 minutes to stop the enzyme action and filtered.

The filtrate was concentrated under reduced pressure and purified by treatment with alcohols to remove high molecular-weight contaminants.

The partially purified transfer products were then applied to a charcoal column, a glass tube (4.2 × 24 cm.) packed with a charcoal slurry. The column was eluted first with water and then with aqueous ethanol of increasing strength. The transfer products were isolated from the appropriate eluates by the addition of ethanol and purified.

5. *Hydrolysis of transfer products*

i) *With dilute sulfuric acid solution*

The solution of a transfer product obtained by elution with aqueous ethanol from paper chromatograms was added to an equal volume of 0.2 N sulfuric acid and heated in a boiling water bath for 120 minutes. After neutralizing with 0.2 N sodium hydroxide, the hydrolyzates were analyzed for hexoses which were then further characterized.

ii) *With an enzyme solution from brewer's bottom yeast*

A 1 ml. aliquot of the solution of a transfer product was mixed with an

equal volume of 0.2 M acetate buffer, pH 4.8, and 2 volumes of enzyme solution. The reaction mixture was incubated at 30°C for 10~12 days. The products formed by the enzymic hydrolysis were then analyzed and identified by the standard procedure.

The transfer products from *p*-nitrophenyl α-galactoside were always hydrolyzed by acid. However, since hydrolysis by acid often led to slight loss of hexose, while hydrolysis by enzyme always gave theoretical values, the latter method was used to identify the transfer products obtained by column chromatography.

Since very small amounts of the transfer products obtained by column chromatography had to be used when their optical rotations were determined, the concentration of these products was determined from the amount of hexoses formed by enzymic hydrolysis.

6. *Determination of reducing sugar and p-nitrophenol*

Reducing mono- and oligosaccharides were determined by SHAFFER-HARTMANN-SOMOGYI's[4] method, unless otherwise indicated. *p*-Nitrophenol was determined colorimetrically from the absorption at 400 mμ.

7. *Periodate oxidation*

i) *Conditions*

20 ml. of the solution of a transfer product was mixed with an equal volume of 0.064 M sodium periodate solution in a glass-stoppered bottle and the mixture kept in the dark at 2°C for a period which has been noted for each experiment.

ii) *Analysis*

Formic acid: 2.0 ml. aliquots of the oxidation mixture were added to a glass-stoppered bottle at intervals along with 0.6 ml. of ethylene glycol, and the mixture allowed to stand in the dark at room temperature for 60 minutes to reduce excess periodate. The formic acid that had been produced was then determined with 0.01 N barium hydroxide using methyl red as indicator.

Reduced periodate: Following FLEURY and LANGE[6], a 2 ml. aliquot of the oxidation mixture was added to 2 ml. of saturated sodium bicarbonate solution and 2 ml. of 20 per cent potassium iodate and 4 ml. of 0.1 N sodium arsenite were immediately added. After standing at room temperature for at least 15 minutes, the mixture was titrated rapidly with 0.01 M iodine solution with

the addition of a little starch solution to indicate the end point. Control experiments were made in the same way with an equal volume of distilled water in place of the samples.

Results

1. *α-Galactosidase activity of the extract from brewer's bottom yeast*

The hydrolytic activities of the crude extract towards *p*-nitrophenyl α- and β-galactosides and β-glucoside are presented in Table 1. The enzyme solution was practically free of β-galactosidase and β-glucosidase activities.

Table 1. Hydrolytic activity of the crude extract from bottom yeast.

Substrate	Incubation time	Hydrolysis
	(min.)	(%)
p-Nitrophenyl α-galactoside	60	19.5
	240	52.1
	345	60.0
	420	68.0
	800	90.0
p-Nitrophenyl β-galactoside	60	0.0
	800	0.0
p-Nitrophenyl β-glucoside	60	0.0
	800	0.0

2. *Paper chromatography of the enzymic transfer products from p-nitrophenyl α-galactoside*

The hydrolysis of *p*-nitrophenyl α-galactoside by enzyme solution was carried out in the presence of methanol in order to examine transfer products. In the course of the hydrolysis, it was found by paper chromatography that four products (A, B, C and D) with smaller Rf than the substrate were formed besides the split products, as shown in Fig. 1. In the preceding work[3], however, three products only were found when the hydrolysis was made in the presence of previously added galactose.

Of the four, spots A and C both appeared in the early stages of incubation while the other two appeared in the late ones. These spots increased in size and deepened in their colour intensity as the incubation time increased, but they disappeared after prolonged incubation. The products were possibly

hydrolyzed again by the same enzyme.

Since it had been established by other tests that no such products as A to D were formed from a mixture of galactose, methanol, *p*-nitrophenol and enzyme, and also that no reducing sugars were to be found in the enzyme solution, these products must be formed by transglycosylation by the α-galactosidase.

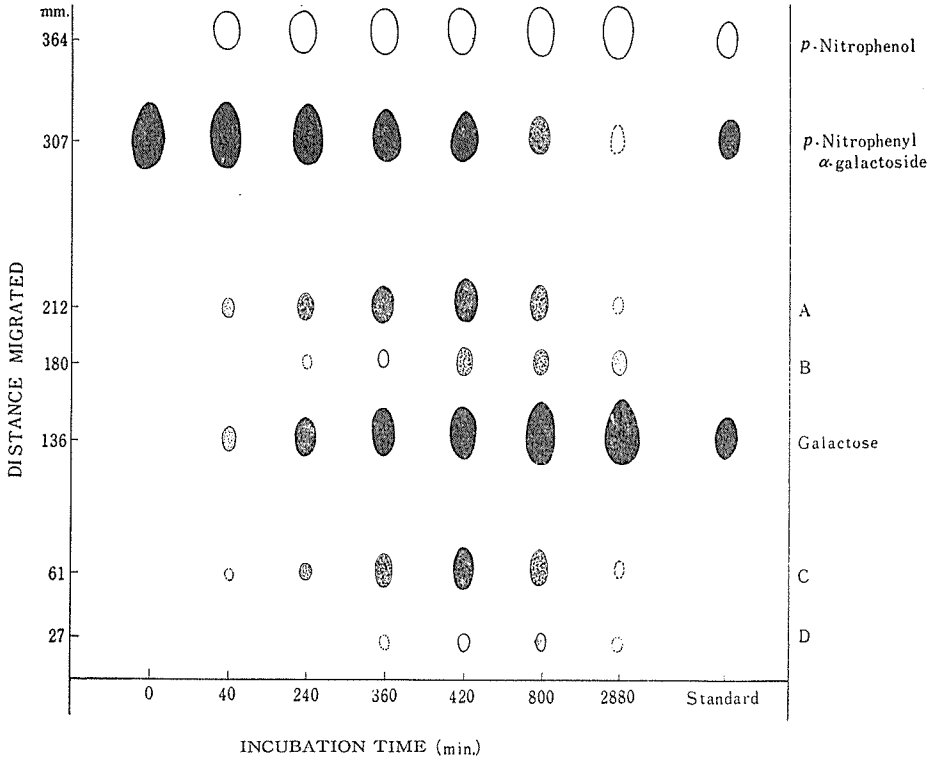

Fig. 1. Drawing of a paper chromatogram showing the products formed during the enzymic hydrolysis of *p*-nitrophenyl α-galactoside. The descending technique was used.

3. *Isolation of the transfer products by paper chromatography*

Concentrates (2 ml.) of the eluates from paper strips, each containing one of the products A, B, C and D, were first examined for reducing powers and chromatographic mobility and then hydrolyzed with acid. The sugars produced were identified chromatographically and the following results were obtained.

Product A: The product was nonreducing. Galactose and *p*-nitrophenol were the products of hydrolysis, in amounts shown in Table 2. It is clearly seen that the molar ratio of *p*-nitrophenol to galactose was 1 : 2. Product A may accordingly be considered a hetero-oligosaccharide, *p*-nitrophenyl galactosyl galactoside, composed of one mole of *p*-nitrophenol and two moles of galactose.

Table 2. Molar ratio of *p*-nitrophenol to galactose liberated by hydrolysis from an 0.056 per cent solution of product A.

Product liberated	Amount of products in 1 ml. of hydrolyzate	Molar number	Molar ratio
	(mg.)	($\times 10^6$)	
p-Nitrophenol	0.1668	1.20	1.0
Galactose (as glucose)	0.421	2.34	1.95

Product B: This was a nonreducing saccharide. It showed almost the same Rf value, 0.37, as authentic methyl α-galactoside previously synthesized chemically[6] and gave galactose but no *p*-nitrophenol on hydrolysis. It was also , like methyl α-galactoside, quite resistant to yeast α-galactosidase. These facts suggest that product B is identical with methyl α-galactoside.

Product C: The product was reducing, and the reducing power was doubled by hydrolysis, as shown in Table 3. Hydrolysis and paper chromatography demonstrated the presence of galactose but not of an aglycon such as *p*-nitrophenol or methanol. Product C, therefore, seemed to be a disaccharide, galactosyl galactose.

Table 3. Ratio of reducing power, before and after hydrolysis, of an 0.048 per cent solution of product C.

Hydrolysis	Volume of 1/200 N thiosulfate for 2 ml. hydrolyzate	Amount of hexose (as glucose)	Molar number	Molar ratio
	(ml.)	(mg.)	($\times 10^6$)	
Before	3.20	0.507	2.82	1.0
After	6.71	0.970	5.39	1.91

Product D: As the amount was too small, no attempt was made to separate this product. However, it seemed likely to be a reducing trisaccha-

ride composed of galactosyl residue on the basis of its reducing power and Rf value.

4. *Paper chromatography of the enzymic transfer products from melibiose*

Melibiose was incubated with the enzyme solution and the transfer products in the incubation mixture were examined by paper chromatography. The results are illustrated in Fig. 2.

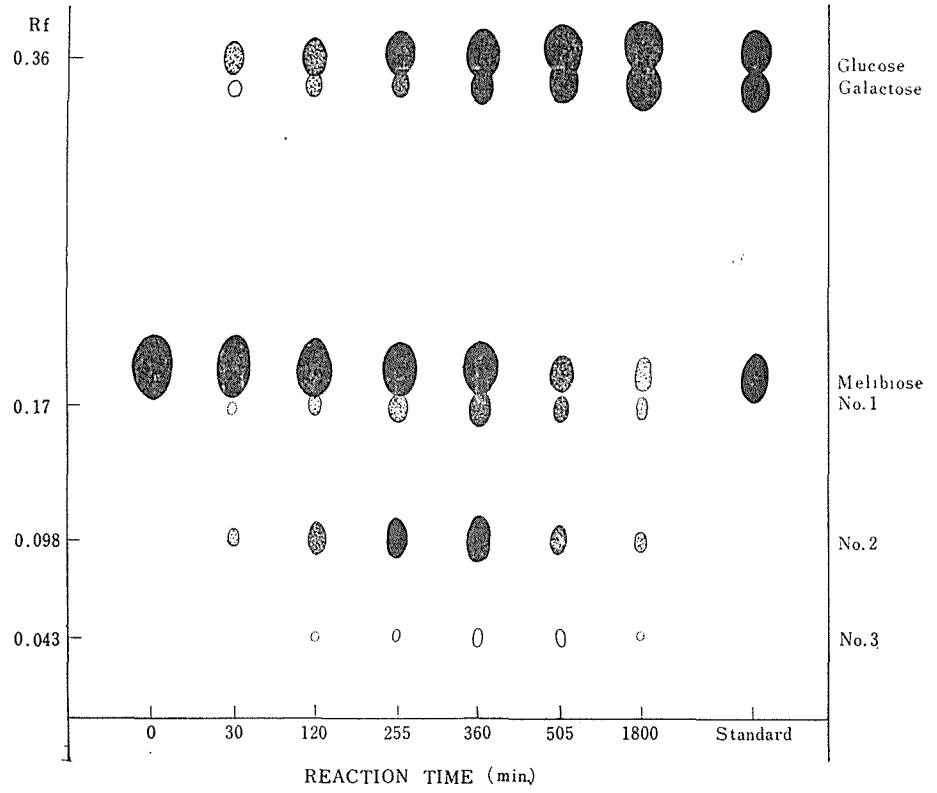

Fig. 2. Drawing of a paper chromatogram showing the products formed during the enzymic hydrolysis of melibiose. The ascending technique was used.

Besides glucose (Rf 0.36), galactose (Rf 0.32) and melibiose (Rf 0.19), three spots (No. 1, No. 2 and No. 3) with Rf values lower than that of melibiose appeared with increasing incubation time. Spot No. 2 appeared first and No. 1 and No. 3 followed in this order. Spot No. 3 was lightest in colour. However,

they all faded gradually as the incubation advanced, probably being hydrolyzed again by the same enzyme. Their Rf values were 0.17 (No. 1), 0.098 (No. 2) and 0.043 (No. 3).

5. *Isolation of transfer products by carbon column chromatography*

The filtrate of an incubation mixture containing transfer products from melibiose was treated with alcohols according to the procedure summarized in Fig. 3. Preparation F4 was shown by paper chromatography to contain all those transfer products which had been present in the initial incubation mixture and to be practically free of high molecular-weight contaminants.

After F4 had been applied to a glass column, this was washed first with 1.4 *l.* of water, then with 1.5 *l.* of 5 per cent, 1.7 *l.* of 10 per cent and 0.75 *l.* of 20 per cent aqueous ethanol solution in succession. No anthrone reaction[7] was observed at the end of each elution.

Those fractions that contained the same sugar were combined after identification by paper chromatography and concentrated under reduced pressure to a syrup. Each syrup was then purified with ethanol to a white crystalline powder. Table 4 shows the results of the fractionation.

Fig. 3. Procedure for the purification of transfer products in the incubation mixture.

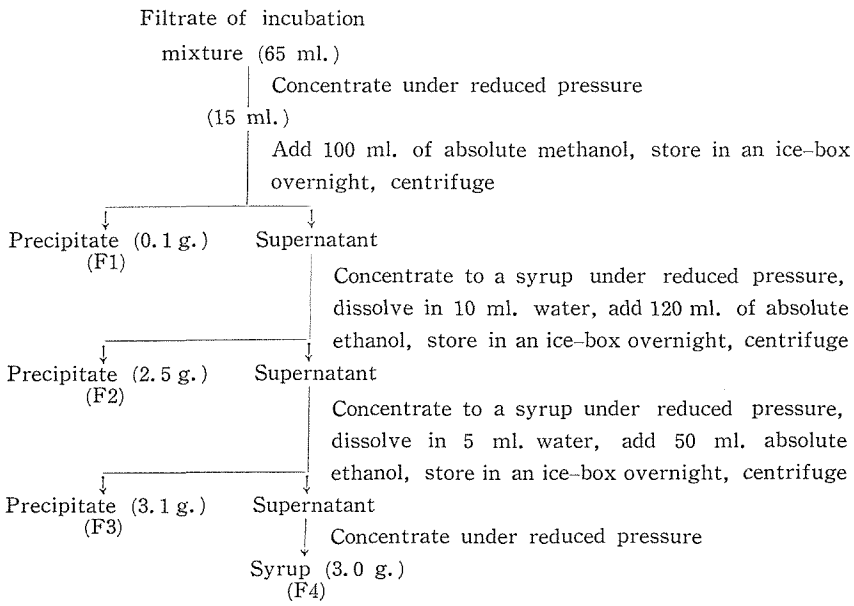

Table 4. Fractionation of product F4 with aqueous ethanol solutions of increasing concentration by carbon column chromatography.

Fraction number	Sugar	Concentration of ethanol	Yield
		(%)	(mg.)
1	{Glucose Galactose	0	—
2	No. 1	5	158
3	Melibiose	5	—
4	No. 2	10	269
5	No. 3	20	43

6. *Identification of the transfer products*

i) *Product* No. 1

The saccharide was reducing. It was completely hydrolyzed by the bottom yeast enzyme to give only galactose. As shown in Table 5, the ratio of the reducing power before and after hydrolysis, was close to 1 : 2. This may indicate that product No. 1 was a disaccharide, galactosyl galactose, $[\alpha]_D^8 = +154.7°$ ($+1.64 \times 100/2 \times 0.53$, in water) *. The value is in good agreement with the one for galactobiose appearing in the literature[1], *i.e.* + 158°.

Table 5. Ratio of the reducing power, before and after hydrolysis, of an 0.105 per cent solution of product No. 1.

Incubation time	Volume of 1/200 N thiosulfate for 1 ml. of hydrolyzate	Amount of hexose (as glucose)	Molar number	Molar ratio
(hr.)	(ml.)	(mg.)	($\times 10^6$)	
0	3.20	0.507	2.82	1.0
240	7.81	1.117	6.21	2.20
288	7.72	1.105	6.14	2.18

ii) *Product* No. 2

The product was reducing, and the reducing power was increased approximately three times by enzymic hydrolysis (Table 6). Galactose and glucose

* The concentration was estimated by the reducing power after enzymic hydrolysis. BERTRAND's method was used for the determination.

were detected as products by paper chromatography. The ratio of these hexoses was estimated as being close to 2 : 1 after elution from the paper chromatogram, as shown in Table 7. Product No. 2 may be, therefore, a trisaccharide composed of one mole of glucose and two moles of galactose, $[\alpha]_D^8 = +170.0°$ ($+9.62 \times 100/2 \times 2.83$, in water)*. According to TANRET[8] and ONUKI[9] respectively, the specific rotation of manninotriose, α-galactosyl-(1→6)-α-galactosyl-(1→6)-glucose, is +167° or +174.6°. These results suggest that product No. 2 is identical with manninotriose.

Table 6. Ratio of the reducing power, before and after the hydrolysis, of an 0.223 per cent solution of product No. 2.

Incubation time	Volume of 1/200 N thiosulfate for 0.5 ml. of hydrolyzate	Amount of hexoses (as glucose)	Molar number	Molar ratio
(hr.)	(ml.)	(mg.)	($\times 10^6$)	
0	2.30	0.382	2.12	1.0
240	8.60	1.224	6.81	3.20
288	8.41	1.199	6.66	3.14

Table 7. Molar ratio of glucose to galactose in the enzymic hydrolyzate of an 0.71 per cent solution of product No. 2.

Product liberated	Volume of 1/200 N thiosulfate for 5 ml. of eluate	Amount of hexoses (as glucose)	Molar number	Molar ratio
	(ml.)	(mg.)	($\times 10^6$)	
Glucose	2.75	0.440	2.44	1.0
Galactose	5.80	0.852	4.73	1.94

iii) *Product* No. 3

Product No. 3 was also reducing and was readily hydrolyzed by bottom yeast extract to give glucose and galactose. The reducing power increased approximately four times after hydrolysis as shown in Table 8. The ratio of glucose to galactose in the digest was found to be 1 : 3 in the same way as for product No. 2, as shown in Table 9. Product No. 3 may, therefore, be considered a tetrasaccharide composed of one mole of glucose and three moles

* The concentration was determined in the same way as for product No. 1.

of galactose. $[\alpha]_D^8 = +187.2°$ ($+2.86 \times 100/2 \times 0.764$, in water)*. Verbascotetraose, α-galactosyl-(1→6)-α-galactosyl-(1→6)-α-galactosyl-(1→6)-glucose, has $[\alpha]_D = +191.4°$[10]. These facts suggest that product No. 3 is identical with this tetrasaccharide.

Table 8. Ratio of reducing power, before and after the hydrolysis, of an 0.262 per cent solution of product No. 3.

Incubation time	Volume of 1/200 N thiosulfate for 0.5 ml. of hydrolyzate	Amount of hexoses (as glucose)	Molar number	Molar ratio
(hr.)	(ml.)	(mg.)	($\times 10^6$)	
0	2.11	0.358	1.99	1.0
240	10.05	1.418	7.88	3.96

Table 9. Molar ratio of glucose to galactose in the enzymic hydrolyzate of an 0.764 per cent solution of product No. 3.

product liberated	Volume of 1/200 N thiosulfate for 5 ml. of eluate	Amount of hexoses (as glucose)	Molar number	Molar ratio
	(ml.)	(mg.)	($\times 10^6$)	
Glucose	2.42	0.398	2.21	1.0
Galactose	8.71	1.235	6.86	3.1

7. *Periodate oxidation of transfer products*

To corroborate the above observations, products No. 1, 2 and 3, together with melibiose, were subjected to periodate oxidation and the periodate consumed and formic acid liberated were measured under standard conditions. The results are shown in Figs. 4 and 5.

As Fig. 4 shows, the amount of oxidation increased rapidly to an initial plateau, after which it again increased, but very slowly. From the shape of curves it may be deduced that 5 moles of periodate were consumed by both product No. 1 and melibiose. This value shows a theoretical consumption of oxidant by melibiose under mild conditions. However, nearly 7 and 9 moles of periodate were reduced by products No. 2 and No. 3, respectively. These values are those for a theoretical consumption of oxidant by No. 2 and No. 3

* The concentration was determined in the same way as for product No. 1.

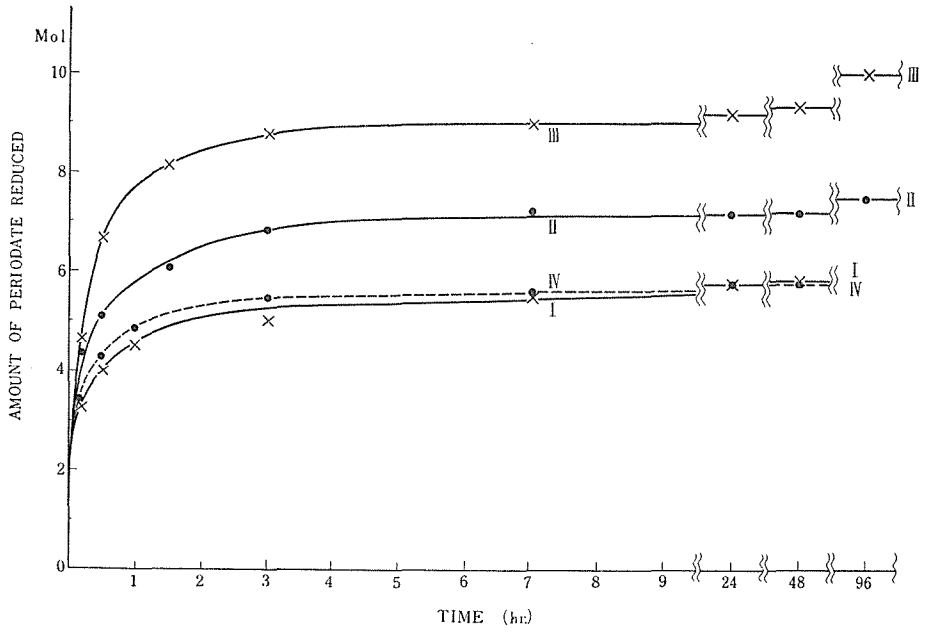

Fig. 4. Uptake of periodate in the course of the periodate oxidation of one mole of transfer products.
Curve I: No. 1 (as 1,6-galactosyl galactose).
Curve II: No. 2 (as manninotriose).
Curve III: No. 3 (as verbascotetraose).
Curve IV: Authentic melibiose.

under mild conditions, if they are assumed to be composed respectively of 3 and 4 hexopyranose residues having only 1,6 linkages.

The same conclusion could also be drawn from the figures for the release of formic acid. Under mild conditions, melibiose should theoretically release 3 moles of formic acid on periodate oxidation. As Fig. 5 shows clearly, 3 moles of formic acid were produced from melibiose and product No. 1 in the early stages of oxidation, but no noteworthy formation of formic acid occurred after this for either sugar. It may be similarly deduced that products No. 2 and No. 3 release 4 and 5 moles of formic acid, respectively. These amounts represent a theoretical release under mild conditions if they were assumed to be a reducing tri- and tetrasaccharide, respectively, composed of hexopyranose residues having only 1,6 linkages.

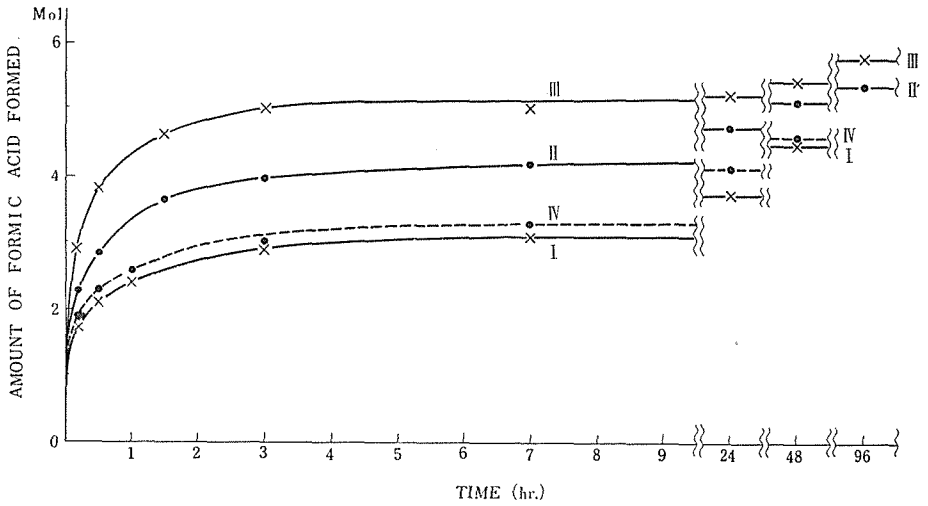

Fig. 5. Release of formic acid in the course of the periodate oxidation of one mole of transfer products.
Curve I: No. 1 (as 1, 6-*galactosyl galactose*).
Curve II: No. 2 (as manninotriose).
Curve III: No. 3 (as verbascotetraose).
Curve IV: Authentic melibiose.

Discussion

Three oligosaccharides were isolated as transfer products from an incubation mixture of melibiose and an extract of bottom yeast free of β-galactosidase and β-glucosidase activities. No other oligosaccharides were found under the present conditions.

These oligosaccharides, however, were completely hydrolyzed again by the same enzyme preparation after prolonged incubation. By paper chromatography, one of them was found to be galactosyl galactose and the other two to be di- and trigalactosyl glucose. Their specific optical rotations were in agreement with those of galactobiose, obtained by FRENCH[1], manninotriose studied by TANRET[8] and ONUKI[9] and verbascotetraose prepared by MURAKAMI[10] from verbascose, respectively.

This agreement was further confirmed by periodate oxidation, all the linkages of these oligosaccharides being demonstrated to be of the 1, 6 type.

All these findings suggest that bottom yeast α-galactosidase transfers the α-galactosyl residue of melibiose exclusively to the 6th carbon atom of free

galactose, the galactosyl residue of melibiose or the terminal galactosyl residue of manninotriose.

It is known that in general the transglycosylation of glycosyl residues from aryl glycosides or oligosaccharides occurs most easily at the 6th carbon atom of the terminal nonreducing residue when saccharides are used as acceptors[11][12][13]. Transglycosylation to other carbon atoms has been found to take place at the 4th, 3rd and 2nd ones in this order of ease[13]. Transglycosylations to the 4th or 3rd carbon atoms can therefore often be observed in addition to that to the 6th. However, the transfer products produced by the α-galactosidase of bottom yeast from melibiose had only 1,6 linkages and no other types of oligosaccharides were found chromatographically in the incubation mixture. Therefore, α-galactosidase may be specific for such a type of transglycosylation.

It should also be taken into consideration that galactobiose and *p*-nitrophenyl galactobioside were formed even when the α-galactosidase of bottom yeast was mixed with *p*-nitrophenyl α-galactoside and methanol, although the linkage type of the holosidic bond of the bioside has not been determined in the present work.

It is also significant that the α-galactosidase of bottom yeast hydrolyzed melibiose considerably faster than most aryl α-galactosides while the reverse was the case for other several enzyme sources[6].

Summary

Three reducing oligosaccharides were formed as transfer products when melibiose was incubated with an α-galactosidase preparation from bottom yeast in the presence of a little galactose. No other products could be found. These products were isolated by column chromatography and identified as α-1, 6-galactobiose, manninotriose and verbascotetraose by means of paper chromatography and periodate oxidation. Their specific optical rotations were in accord with those reported in the literature.

Even when the same enzyme preparation was incubated with *p*-nitrophenyl α-galactoside and methanol, galactobiose and *p*-nitrophenyl galactobioside were formed as predominant transfer products alongside methyl α-galactoside.

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References

(1) FRENCH, D., *Advances in Carbohydrate Chem.*, **9**, 149 (1954)
(2) BLANCHARD, P. H., and ALBON, N., *Arch. Biochem.*, **29**, 220 (1950)
(3) WAKABAYASHI, K., "Studies on α-Galactosidases (II). Transgalactosylation by α-Galactosidase Preparations from Brewer's Bottom Yeast.," *J. Faculty of Eng. Shinshu Univ.*, No. **10**, 1 (1960)
(4) SHAFFER, P. A., and SOMOGYI, M., *J. Biol. Chem.*, **100**, 695 (1933)
(5) FLEURY, P. P., and LANGE, J., *J. Pharm. Chim.*, **17**, 107 (1933)
(6) WAKABAYASHI, K., and NISIZAWA, K., *J. Jap. Biochem. Soc.*, **27**, 662 (1955) (in Japanese)
(7) TREVELYAN, W. E., and HARRISON, J. S., *Biochem. J.*, **50**, 298 (1952)
(8) TANRET, C., *Bull. Soc. Chim. France*, [3] **27**, 947 (1902)
(9) ONUKI, M., *Proc. Imp. Acad.* (Tokyo), 8, 496 (1932)
(10) MURAKAMI, S., *Acta Phytochim.* **11**, 213 (1940)
(11) PAZUR, J. H., and FRENCH, D., *J. Biol. Chem.*, **196**, 265 (1952)
(12) PAZUR, J. H., *J. Biol. Chem.*, **208**, 439 (1954)
(13) CROOK, E. M., and STONE, B. A., *Biochem. J.*, **65**, 1 (1957)