Enzymatic Transfer of β-fructofuranosyl Group from Sucrose to Various Acceptors with Brewer's Yeast Preparation

Part 1

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The various reports on general properties of enzyme which was prepared from brewer's yeast were published basing on many experimental results. Generally, the cleavage of glycosidic linkage was carried on in the form of hydrolysis. It was determined by many investigators with the act of hydrolase catalyzing hydrolytic reaction. However, on this paper, one new specific reactivity of the purified fraction obtained from dried yeast was studied on the transfer from sucrose on the assumption that the enzyme was one kind of transferase catalyzing atomic group transfer reaction. The products reacted enzymically, methyl-, ethyl-, n-propyl-, n-butyl-, and iso-butyl β-fructofuranosides were detected with chromatography. And ethylene glycol-, and erythritol-β-fructofuranosides were produced by this transferase under the condition that primary alcohol reacted as an acceptor in existence of sucrose. The methyl-β-fructofuranoside was prepared as one standard compound. The physical property, $[\alpha]_D$ was $-50.7°$ (c, 1.102, H_2O).

Also, the transfer reaction in enzymatic process was determined chemically with reducing power change. And the order of reaction of transfer reaction was assumed to so-called first order reaction in over all reaction as such a general hydrolysis reaction.

On the other hand, hydrolase of sucrose exists widely in higher and lower plants. And it exists in intestine of higher animal. The activity of the enzyme produced from dried yeast or mould is strong relatively. Then, the hydrolysis of sucrose was detected as a blank test. Moreover, Does it act on the glycoside as transferase or not? What kind of sugar and alcohol can be acceptor or not? The two problems are detecting parallely.

EXPERIMENTAL AND RESULTS

Enzyme fraction (I)

2 g of dried yeast was ground in porcelain motor. 100 ml of water was made to alkaline with one drop of 7 % ammonium hydroxide. This alkaline solution was added into the motor to make complete syrupy state gradually and carefully with grounding for 30 min. The syrupy solution replaced in glass ware was stirred with magnetic stirrer keeping constant temperature

for 40 min. At that time, pH of solution had to hold at 8.5 to 9.0 with 7 % ammonium hydroxide for keeping most effective extraction. After except of insoluble fraction, the extract that two or three drops of toluene were added was dialyzed for two days with tapping water. After dialysis, the insoluble material was excepted. And the extract was filtered with filter paper. It designated as enzymatic original solution.

Reducing power determination (I)

The reducing power was determined with the method of Schffer-Hartman[1]. 25 g of anhydrous carbonate and 25 g of Rochelle salt were dissolved in 500 ml of distilled water without heating. 75 ml of 10 % copper sulfate was added into the carbonate and tartrate solution to avoid losing carbon dioxide. Then 20 g of sodium bicarbonate and 5 g of potassium iodide and 25 ml of 0.1 N potassium iodate which was 3.567 g of potassium iodate per liter and these reagents were diluted to one liter and mixed entirely. The 0.5 ml of sample solution was titrated with 0.005 N thiosulfate.

Indicator, starch solution, was prepared with procedure that 2.5 g of soluble starch was added into about 8 ml of water. Its solution made to homogeneous one. And 4 ml of hot water was added into it. After cool, 50 ml of saturated sodium chloride solution was added. The solution was centrifugalized to obtain a clear starch solution.

Buffer solution

0.2 M acetic acid was mixed with 0.2 M sodium acetate to prepare four buffer solutions as showed in Table 1.

Table 1.
Composition of buffer solution at 18°C

pH	0.2 M acetic acid (ml)	0.2 M sodium acetate (ml)
3.8	8.80	1.20
4.8	4.10	5.90
5.2	2.10	7.90
5.8	0.60	9.40

These buffer solutions were used at 30°C neglecting temperature dependence.

Enzymatic reaction, hydrolytic reaction

1 ml of enzyme original solution was diluted to 4 ml with water. This solution was the first enzymatic diluted solution. The enzymatic reaction was carried out at 30° C. 1 ml of substrate solution, 0.048 M sucrose solution, was reacted with 2 ml of diluted enzymatic solution in 1 ml of acetate buffer (pH 3.8).

0.5 ml of reacted solution was added into 5 ml of Somogyi's reagent and 4.5 ml of water at constant time. The reaction was performed in boiling water for 15 min. After cooling with

tapping water, 5 ml of 1 N sulfuric acid was added in to it. The solution was determined with thiosulfate. The reaction time with enzyme was 15 min, 30 min, and 45 min.

Also, each blank test of reagent or enzymatic solution was carried on by the components of 5 ml of water, 5 ml of Somogyi's reagent, and 5 ml of 1 N sulfuric acid or 4.75 ml of water, 5 ml of Somogyi's reagent, 0.25 ml of diluted enzymatic solution, and 5 ml of 1 N sulfuric acid.

The results obtained from hydrolytic reaction are summarized in Table 2.

Table 2.
Hydrolytic reaction at 30°C under the conditions of several pH values, and several reaction times

Reaction time (min)	pH value	titration amount (ml)	consumed amount (ml)	hydrolytic percentage
15	3.8	7.5	12.3	65.5
30	3.8	3.9	15.9	84.1
45	3.8	3.0	16.8	88.4
15	4.8	7.0	12.8	68.1
30	4.8	2.9	16.9	88.9
45	4.8	2.6	17.2	90.3
15	5.2	7.8	12.0	63.9
30	5.2	3.0	16.8	88.4
45	5.2	1.3	18.5	–
15	5.8	10.8	9.0	48.4
30	5.8	5.0	14.8	78.2
45	5.8	2.8	17.0	89.4

At pH 3.8, the degree of hydrolysis was considerably high. However, at pH 4.8, the degree of hydrolysis was higher than that of hydrolysis at pH 3.8 and 5.8.

At last, the suitable experimental condition was decided at pH 4.8 under the present experimental condition.

The more proceeding purification of enzyme might be required at the next step to obtain the crystal and to decide the absolute structure which was a final objective point.

The results from hydrolytic reaction are shown in Fig. 1 and Fig. 2.

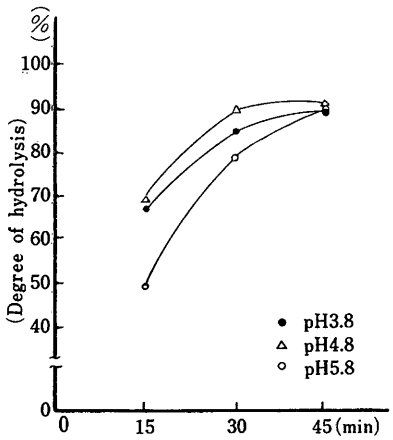


Fig. 1. Hydrolytic reaction at pH 3.8, 4.8, 5.8.

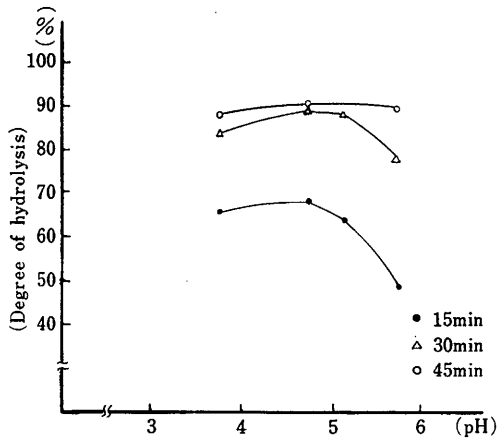


Fig. 2. pH dependence curve.

Transfer reaction (I)

1) 2 ml of the first enzymatic diluted solution was reacted with 1 ml of sucrose solution, 1 ml of acetate buffer solution at 30°C for 60 min.

2) 4 ml of the second enzymatic diluted solution (half concentration of enzymatic original solution) was reacted with 2 ml of 30 % sucrose solution, 2 ml of acetate buffer solution and 1 ml of methyl alcohol solution at 30°C. The reaction time was 40 min, 60 min, and 90 min. This methyl alcohol took the place of ethyl alcohol, n-propyl alcohol, iso-propyl alcohol, n-butyl alcohol, iso-butyl alcohol and glycerol.

Paper chromatography (I)

The spotted papers were developed ascending n-butyl alcohol : acetic acid : water (4 : 1 : 2, v/v) for 15 hours to 24 hours at room temperature.

Spray reagents, resorsin solution, which was prepared with 10 ml of 1 % resorsin alcohol solution and 90 ml of 2 N hydrochloric acid, benzidine solution which was prepared with 0.5 g of benzidine dissolved in 100 ml of water, 10 ml of 40 % trichloroacetic acid, and 80 ml of ethyl alcohol, silver nitrate solution which was prepared with 1 ml of saturated silver nitrate, 200 ml of acetone and a small amount of water to dissolve the deposit of silver nitrate, alkaline alcohol solution which was prepared with 4 g of sodium hydroxide, 25 ml of water and 75 ml of ethyl alcohol, were used to detect the spots on the paper developed.

The results of paper chromatography are shown in Fig. 3.

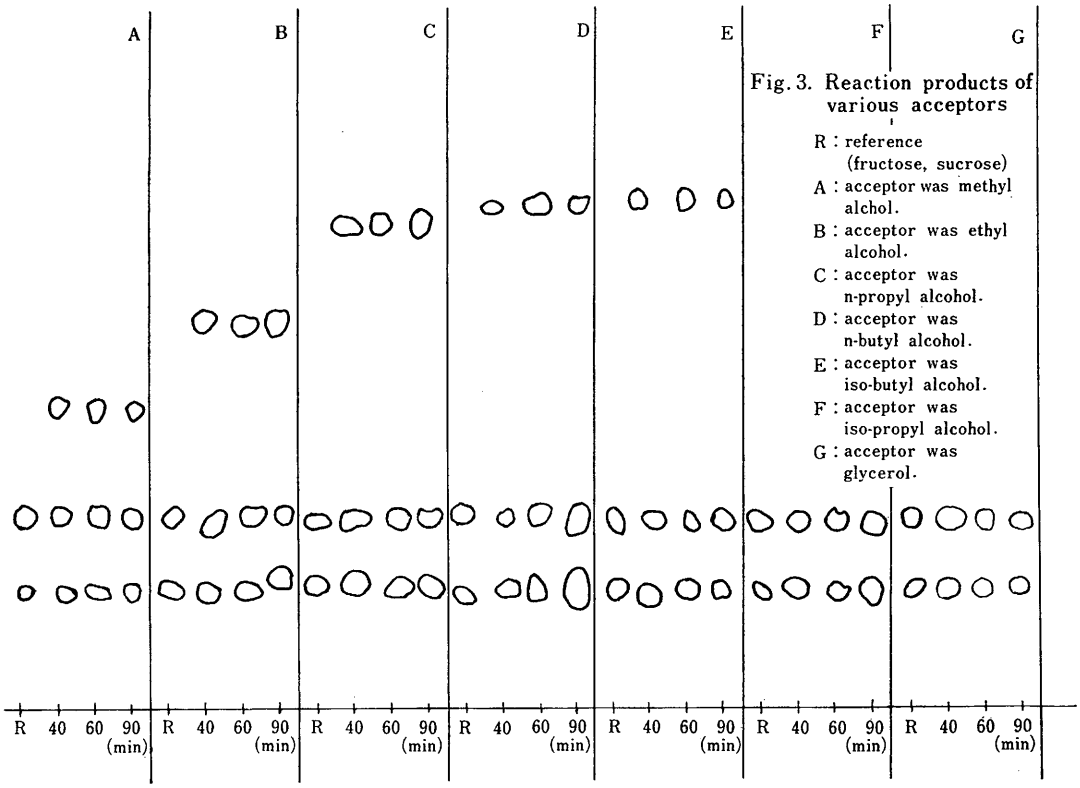


Fig. 3. Reaction products of various acceptors

Enzyme Fraction (II)

1 g of dried yeast was ground in porcelain motor with a small amount of water that total amount was 99 ml. After the syrupy solution was stirred for about one hour, it was replaced into a glass ware. Then, the inside of glass ware was washed completely with the rest amount of water. The extract was stirred for about three hours keeping at pH 7.8 to 8.8 with 7 % ammonium hydroxide. And it was dialyzed for about two days with tapping water and was filtered with filter paper. pH of the filtrate was adjusted to 4.2 with 5% acetic acid. After it was allowed to stand for about half an hour to except insoluble material, its solution was filtered with filter paper again. Then, pH of the filtrate was adjusted to 6.8 with 7 % ammonium hydroxide. And the enzyme fraction that two to three drops of toluene were added was kept in ice box.

Enzymatic reaction (II)

1 ml of 12 % sucrose, 0.5 ml of acetate buffer solution (pH 4.8) and 0.5 ml of water were hold at 30°C. And the substrate buffer solution was reacted with 2 ml of enzyme fraction at 30°C. 0.1 ml or 0.5 ml of reacted solution was sampled at setting times during 23 hours. After the former to determine the reducing power was rapidly heated with 5 ml of Schaffer's reagent and 4.9 ml of water in boiling water bath for 15 min, 5 ml of 1 N sulfuric acid was added into the solution. After 3 min, the solution was titrated with N/200 thiosulfate using

2 % soluble starch indicater. The test that the sampling volume was 0.1 ml was designated as blank test. After the latter to detect the components of reacted product was rapidly heated in boiling water bath for 5 min, it was kept in ice box with a drop of toluene after cooling.

(a) Blank test
the above-described procedure

(b) Glycerol acceptor
0.5 ml of water in blank test was replaced by 0.5 g of glycerol.

(c) Ethylene glycol acceptor
0.5 ml of water in blank test was replaced by 0.5 ml of ethylene glycol.

(d) Mannitol acceptor
0.5 ml of water in blank test was replaced by 0.5 g of mannitol dissolved in 0.5 ml of water.

(e) Erythritol acceptor
0.5 ml of water in blank test was replaced by 0.5 g of erythritol dissolved in 0.5 ml of water.

(f) Ethyl alcohol acceptor
0.5 ml of water in blank test was replaced by 0.5 ml of ethyl alcohol.

(g) Mehyl alcohol acceptor
0.5 ml of water in blank test was replaced by 0.5 ml of methyl alcohol.

The consumed amount of thiosulfate and the degree of hydrolysis were summarized in Table 3.

Table 3.
Consumed amount (ml) of N/200 thiosulfate
and degree of hydrolysis after 23 hours at 30°C

	(a)	(b)	(c)	(d)	(e)	(f)	(g)
Titrated Amount	20.5	18.5	12.5	10.0	17.0	12.0	12.5
Consumed Amount	–	2.0	8.0	10.5	3.5	8.5	8.0
Hydrolysis (%)	–	13.5	46.8	60.5	21.8	49.5	46.8

(a), (b), (c), (d), (e), (f), (g): each one was shown in Page 24.

Paper chromatography (II)

The each sample solution was spotted on paper. The paper was developed with solvent vapor, n-butyl alcohol:acetic acid:water (4:1:2, v/v), for 15 hours to 24 hours at room temperature. These spots could be made visible by first dry and then spraying the paper with resorsin spray reagent or alkaline silver nitrate solution.

Resorsin spray reagent was the solution that 10 ml of 1 % resorsin ethyl alcohol solution was added into 90 ml of 2 N hydrochloric acid. After resorsin spray reagent was sprayed on the paper, the sprayed paper was heated to appear the visible spots.

Alkaline silver nitrate spray reagent required three solutions.

First solution : minimum volume of water was added to dissolve the silver nitrate aqueous solution.

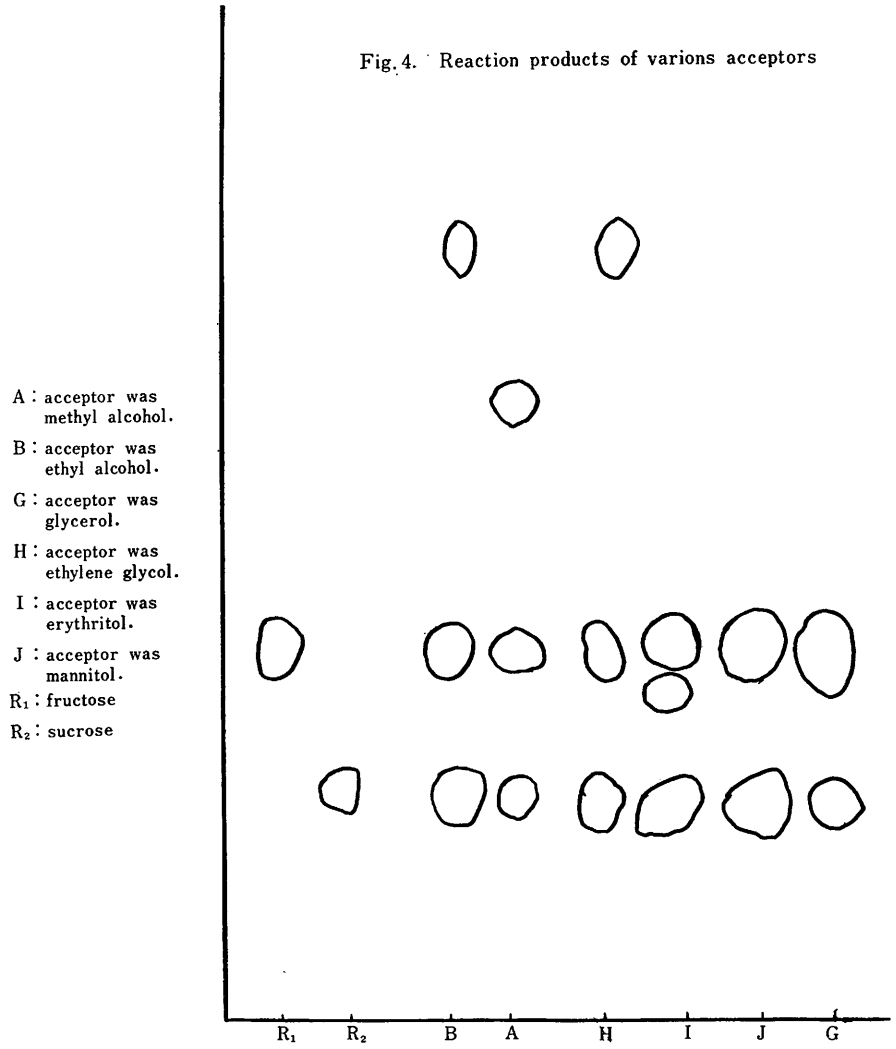
Second solution : After 4 g of sodium hydroxide was dissolved in 5 ml of water, 200 ml of ethyl alcohol was added more.

Third solution : 30 g of sodium thiosulfate was dissolved in 200 ml of water.

When alkaline silver nitrate was used, the paper was immersed in first solution at first and was dried. At next step, the dried paper was immersed in second solution and was dried again. At last step, the dried paper was immersed in third solution and was dried again. Then, the dried paper was washed with running water.

The results by paper chromatography are shown in Fig. 4.

Fig. 4. Reaction products of varions acceptors



Methyl β-fructofuranoside

3 g of sucrose, 5 ml of methyl alcohol, and 20 ml of enzyme solution which was prepared from yeast, were reacted in pH 5.6 acetate buffer solution at 30°C. As a reference experiment, another reaction was made with yeast β-fructofuranosidase preparation that was known to form methyl β-D-fructofuranoside under the same condition. After reaction at 30°C for appropriate periods, the reaction mixture was heated in boiling water and filtered.

The filtrate was condensed and chromatographed on many sheets of filter paper with a solvent mixture, n-butyl alcohol, glacial acetic acid, water, with ascending method. The solution was condensed in vacuo to a syrup and dissolved in a small amount of water.

The product did not reduce an alkaline copper solution and when it was heated in boiling water bath with 0.05 N hydrochloric acid it gave free fructose as detected by paper chromatography. And the optical rotatory was levorotatory and the specific rotation was calculated from the amount of fructose produced upon hydrolysis. The value of specific rotation obtained agreed well with that of methyl β-fructofuranoside ($[\alpha]_D=-50.7°$) which was prepared with the method of Schlubach and Bartels. The results are summarized in Table 4.

Table 4.
Specific rotation of methyl fructoside obtained by transfructosylation of enzyme from yeast

Source of enzyme	$[\alpha]_D$ (in H_2O)
Yeast	−50.7° (c, 1.102)

The methyl β-fructofuranoside was synthesized by enzyme which was prepared by the above described method. And, methyl-, ethyl-, propyl-, n-butyl-, and isobutyl- β-fructofuranosides were synthesized by enzyme fraction (I). Also, methyl-, ethyl-, ethylene glycol-, and erythritol-β-fructofuranosides were synthesized by enzyme fraction (II).

Then, these enzyme fractions contained the enzyme which had the same enzymatic action.

However, this enzyme was not yet crystalline. So, this try to crystallize are attempted by repeating purification step by step.

The R_f values of authentic compounds, glucose, fructose, sucrose, methyl β-fructofuranoside were 0.26, 0.32, 0.19, 0.56, respectively. When the R_f of sucrose was converted into 1.00, R_f of each sample was calculated.

The comparative R_f values of samples are summarized in Table 5.

Table 5.
The comparative R_f values of various compounds
(R_f sucrose = 1.00)

Sucrose	glucose	fructose	methyl-β-fructofuranoside	(b)
1.00	1.39	1.69	2.95	–
(c)	(d)	(e)	(f)	(g)
3.55	–	1.51	3.55	2.95

(b), (c), (d), (e), (f), (g): each one was shown in Table 3.

SUMMARY

The enzymatic preparation which was extracted from dried brewer's yeast catalyzed the transfer of atomic group under the condition that primary alcohol reacted as an acceptor. The products which were reacted with sucrose by this transfer were detected and were methyl β-fructofuranoside, ethylene glycol- β-fructofuranoside, and crythritol- β-fructo-furanoside.

The methyl-β-fructofuranoside was prepared as one standard compound. The physical property, $[\alpha]_D$ was −50.7° (C, 1.102, H_2O).

Iso-propyl alcohol, mannitol and glycerol did not produce the β-fructofuranoside respectively. So it was thought that these compounds did not react as acceptors. Then, it is concluded that primary alcohol may be suitable compound as an acceptor.

And, other primary alcohols and compounds are trying to detect a formation of β-fructofuranoside.

REFERENCES

1) Schaffer, P. A., M. J. Somogyi: Biol. Chem., 100, 695 (1933)

2) Schlubach, H.H., H.E. Bartels: Ann. d. Chem., 54, 76 (1939).

〔内容抄録〕 ビール酵母標品による庶糖から各種受容体へのβ-果糖配糖体に関する酵素的転移 (第1報)

高野克夫　堀津圭佑　岡田留美子

乾燥ビール酵母から，受容体として一級アルコールが反応する転移に関する酵素を分離した。その酵素によりメチル-β-果糖配糖体を分離精製した。〔α〕D = −50.7°(水溶液，濃度1.102)。このメチルアルコールの他に庶糖からの受容体として，エチル，n-プロピル，ブチル，イソブチルーアルコール，エチレングライコール，エリスリトールはそれぞれβ果糖配糖体を生成したが，イソプロピルアルコール，グリセロール，マンニトールはβ果糖配糖体を生成しなかった。