ACTA CHEMICA SCANDINAVICA 23 (1969) 2367–2374

The Transferring Activity of β-Fructofuranosidase from Yeast: A Quantitative Description

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Qualitative and quantitative analyses of a reaction mixture containing 10 % sucrose and β-fructofuranosidase from yeast are described. The percentage compositions of mono-, di-, tri-, and tetra-saccharides are given as functions of the degree of reaction. The concentration of free fructose is significantly lower than that of free glucose for the whole reaction; a discussion of the different methods of determining the degree of reaction is given.

The qualitative and quantitative results for the intermediate di-, tri-, and tetra-saccharides confirm the hypothesis that the enzyme is a transferase causing hydrolysis during a two-step reaction in which the first step is the formation of an active enzyme-fructose complex.

Yeast invertase (β-fructofuranosidase), first described in 1860 by Berthelot,[1] was for nearly 100 years thought to be a hydrolase which performed a simple hydrolysis of sucrose to glucose and fructose. This was probably due to the analogy with the acid catalyzed hydrolysis of sucrose, known from 1850[2] to be a simple hydrolysis following a first order kinetics. However in 1950 it was shown simultaneously by Bacon and Edelman[3] and Blanchard and Albon,[4] who described a fraction containing trisaccharides, that glucose and fructose were not the only products observed during the enzyme catalyzed process. Since crude enzyme extracts were used the possibility existed that contaminating transferases were responsible for the oligosaccharide formation, but Fischer *et al.*[5] in 1951 used the highest purified invertase preparation of that time and still obtained 3 oligosaccharides in their reaction mixture. Consequently, Fischer *et al.* put forward the hypothesis that the hydrolysis is a two-step transfer reaction in which the first step is formation of an enzyme-fructose complex and liberation of glucose. In the next step the active complex could transfer the fructosyl group to water or to sucrose, forming a trisaccharide. Another explanation, given by Edelman and Bacon,[6] suggested that the first stage was formation of an enzyme-sucrose complex and the next a liberation of glucose and a simultaneous transfer of the fructosyl group to water or to sucrose.

Further investigation were made by Bacon [7] who in the reaction mixture found 3 trisaccharides and proved that they were formed by fructosyl transfer to the 3 primary alcohol groups in sucrose. In addition he found the reducing disaccharide 6-fructofuranosylglucose. Bell and Edelman [8] demonstrated that the formation of this disaccharide could be enhanced by additional glucose in the reaction mixture. They also found small amounts of two difructosides and from analyses of the methylated products proved that these were 1-fructofuranosyl-fructose and 6-fructofuranosyl-fructose. Whelan,[9] using β-methyl-fructoside, obtained transfer to simple primary alcohols but no transfer to secondary alcohols. The enzyme-fructose complex hypothesis was supported by the observation [10] that fructose could act as donor and acceptor for the transfer at the same time. By incubating 5 % fructose solution with enzyme the two difructosides were obtained. The enzyme used was of very high purity.[11]

The present work presents a quantitative analysis of the formation of oligosaccharides during the hydrolysis of sucrose. From one reaction mixture samples were taken at different times. Each sample was passed through a Sephadex G-10 column of a length sufficient to obtain total separation between mono-, di-, tri- and tetra-saccharides. A table of the concentrations of the different saccharides as functions of the degree of reaction is given. Since the tetra-saccharides have not been described previously as reaction products 10 mg were collected and, after rechromatography, their composition was examined.

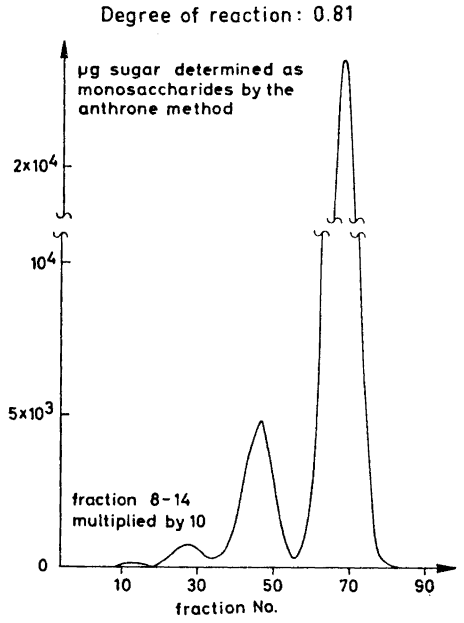

Fig. 1. Separation of tetra-, tri-, di-, and mono-saccharides on Sephades G-10. Fraction 8—14: tetrasaccharides; 14—34: trisaccharides; 34—55: disaccharides and 56—82: monosaccharides.

EXPERIMENTAL

Substrate. Acetate buffer 0.005 M containing 10.00 % sucrose. pH 4.75; Temperature 25.0°C.

Enzyme. β-Fructofuranosidase.[11]
Nitrogen content 11.9 %. Activity: 2630 U/mg [μmol sucrose/min/mg]$_{25°}$.
Crude enzyme preparations and partly purified preparations were tested; differences of oligosaccharide production were not observed.

Procedure. The reaction was followed in a polarimeter which gave an approximate estimate of the degree of reaction at any time. 2 ml samples were extracted at the desired reaction times. Accurate determination of the degree of reaction in any sample was made, as described later, from the content of free glucose.

Gelfiltration. Each sample was concentrated at a temperature of 20°C to 0.5 ml and then passed through a column of Sephadex G-10 equilibrated with ion-exchanged distilled water saturated with toluene to avoid interference from micro-organisms. To obtain the desired separation a total length of 4.5 m was necessary. Three pieces of QVF pipe,

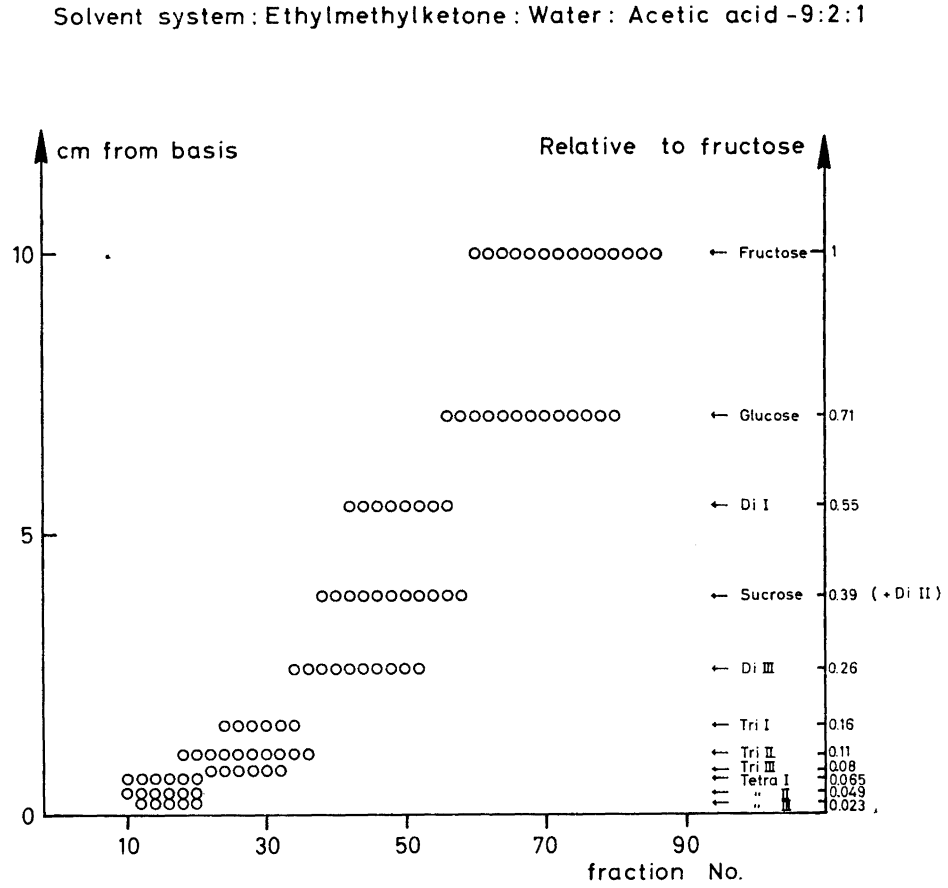

Fig. 2. Paper chromatogram showing the separation of the different saccharides.

Table 1. In the table is given the value of the particular saccharide in percent of the total saccharide content — both calculated as monosaccharide.

a. The degree of reaction, calculated from the amount of free glucose as described in the text.
b. The amount of free glucose determined by the glucoseoxidase method.
d., g., h. and i. are the total amounts of the different saccharides, determined by the anthrone method.
f. is the amount of reducing disaccharides determined by the method of Somogyi.
c. is calculated as the difference between i. and b.
e. is calculated as the difference between d. and f.

	Min	0.75	3.0	8.0	17.5	27.5	35.5	44.5	57.5	77.5	180	300	1440
a	Degree of reaction	6.6	10.0	22.2	37.2	54.4	62.8	74.6	86.2	94.4	—	99.0	100.0
b	Free glucose	3.3	5.0	11.1	18.6	27.2	31.4	37.3	43.1	47.7	—	49.5	50.0
c	Free fructose	1.0	3.9	5.6	14.1	22.8	23.7	30.0	37.9	39.3	—	49.0	50.0
d	Total disaccharide	95.1	90.2	81.8	65.1	47.2	41.0	29.8	16.7	10.5	2.0	0.9	—
e	Free sucrose	94.8	89.6	81.2	64.2	45.7	37.8	26.9	13.5	6.1	0	0	0
f	Reducing disaccharides	0.3	0.6	0.6	0.9	1.5	3.2	2.9	3.2	4.4	2.0	0.9	0
g	Total trisaccharides	0.6	0.9	1.5	2.3	2.8	3.8	3.2	2.3	2.4	1.2	0.5	0
h	Total tetrasaccharides	—	—	—	—	—	traces	0.03	0.02	0.09	0.2	traces	0
i	Total monosaccharides	4.3	8.9	16.7	32.7	50.0	55.1	67.3	81.0	87.0	96.4	98.5	100

each with a length of 1.5 m and an internal diameter of 15 mm, were connected by polyethylene tubes of internal diameter 1 mm. The velocity of elution was 13 ml/h and the volume of each fraction was 1.37 ± 0.05 ml. In Fig. 1 a graphical representation of the separation of the sample with a degree of reaction of 0.81 is given.

Paper chromatography. Whatman No. 1 paper was used. 20 h elution in a 9:2:1 2-butanone, water, acetic acid mixture gave a good separation. Development was performed by spraying with benzidine-CCl_3COOH.

20 μl from each sample were chromatographed, giving a picture of the distribution of the different saccharides for each degree of reaction. In Fig. 2 is shown the composition for the sample with a degree of reaction of 0.81.

Quantitative analysis. In each fraction the total amount of carbohydrate, the amount of free and bound glucose and the amount of reducing sugar were determined.

Total carbohydrate. The anthrone method [12,13] was used. The standard was a 1:1 glucose-fructose mixture. Spectrophotometeric measurements were made at 625 nm.

Free and bound glucose. Free glucose was determined by the glucose-oxidase-peroxidase method (GOD—POD).[14] *o*-Dianisidine was used as chromogen. After hydrolysis for 5 min in 1 M HCl at 100°C, the same procedure as that for free glucose was followed for bound glucose. Standard solution: glucose. Spectrophotometric readings: 460 nm.

Reducing sugar. The method described by Somogyi [15] and Nelson [16] was used. Spectrophotometric readings were made at 500 nm.

RESULTS

Table 1 gives the results from the analysis of the reaction mixture.

Monosaccharides. The concentrations of glucose and fructose as the reaction proceeds are seen in Table 1, sections b and c.

Reducing disaccharides and trisaccharides. The quantitative proportions appear in Table 1, sections f and g. Qualitative examination of the reducing disaccharides and the trisaccharides confirms the observations described in the literature. The reducing disaccharides are hydrolysed exclusively to glucose and fructose as regards to 6-β-fructofuranosylglucose and the two difructosides on hydrolysis yield fructose only. The trisaccharides are hydrolysed to glucose and fructose in the proportion 1:2.

Tetrasaccharides. Table 1, section h, shows the formation of tetrasaccharides. Hydrolysis followed by paper-chromatography established that only glucose and fructose are present. Analysis of the amounts of glucose and fructose gave the proportion 1 part glucose to 3 parts fructose. Since they have not before been described as intermediates in this reaction, an amount of 10 mg was isolated and re-chromatographed on the 4.5 m column to ensure purity. This tetrasaccharide fraction has been separated by paper-chromatography into three parts.

DISCUSSION

If the reaction was a simple hydrolysis of sucrose to glucose and fructose having no consecutive reactions, equal amounts of these products would be formed at any time and the degree of reaction could be defined from the concentration of either of them or from the consumption of sucrose.

Now if the mechanism is a two step transfer reaction with an active enzyme-fructose complex as the first step, it could be described as:

$$\text{I.} \quad EH + GOF \rightleftharpoons EF + GOH$$
$$\text{II.} \quad EF + HOH \rightleftharpoons EH + FOH$$
$$\text{III.} \quad EF + XOH \rightleftharpoons EH + XOF$$

where GOF stands for sucrose, GOH and FOH for glucose and fructose and XOH for any receptor sugar.

Reactions I and II are responsible for the ultimate hydrolysis whereas reactions I and III give the intermediate oligosaccharide formation. From this, a definition of the degree of reaction from the amount of free sucrose leads to serious errors since appreciable amounts of sucrose are used as the acceptor for the fructosyl transfer forming the trisaccharides and the tetrasaccharides. In the same way a definition based on the amount of free fructose is inadequate since significant amounts of fructose are used in all oligosaccharide formation. On the other hand the amount of glucose liberated in reaction I is only slightly reduced by the tiny amount which is used as XOH in reaction III to form one of the oligosaccharides, namely, 6-β-fructofuranosylglucose. Therefore a calculation of the degree of reaction based on the amount of free glucose gives nearly the true value. In Fig. 3 the graph of concentra-

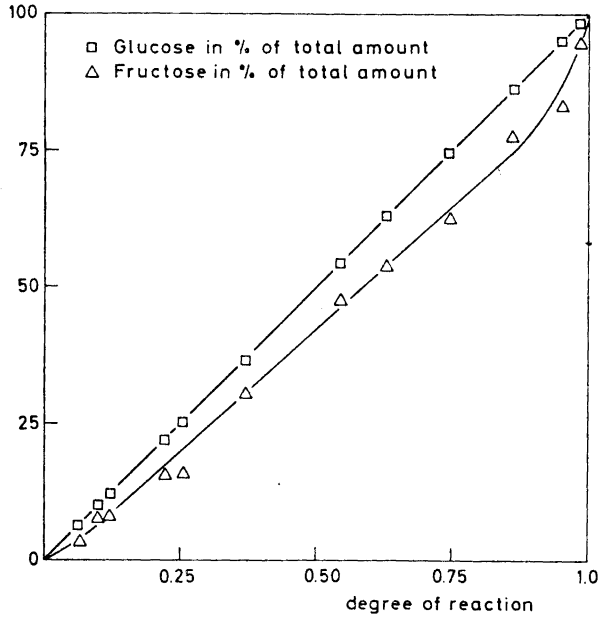

Fig. 3. The difference between the concentration of free glucose and free fructose during the reaction.

tion plotted against degree of reaction is a straight line for glucose (per definition) whereas that for fructose is depressed from the straight line for the whole reaction by an amount corresponding to the transferred part of the fructose.

A survey of the numerous papers treating yeast invertase and its action on sucrose shows that the results of polarimetric measurements have been preferred for calculating the degree of reaction. Since these calculations assume equal amounts of glucose and fructose and neglect oligosaccharides, serious errors have inevitably occurred. A demonstration of this is seen in Fig. 4.

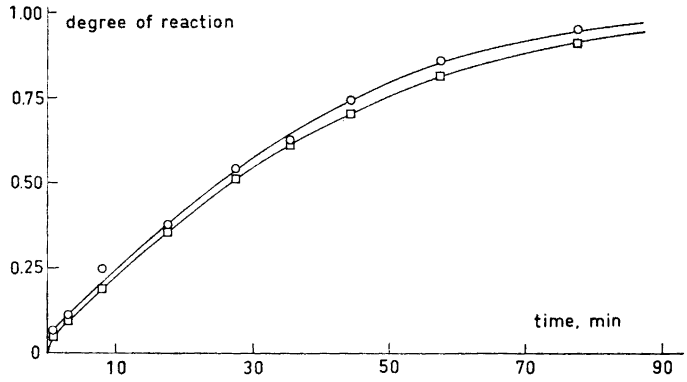

Fig. 4. The figure illustrates the deviation between the reaction degree as calculated from polarimetric measurements and from the amount of free glucose. ○ Measurements of free glucose; □ polarimetric measurements.

Fig. 5 shows that the maximum concentration of the trisaccharides are reached much earlier in the course of the reaction than is the maximum concentration of the reducing disaccharides. This would be expected from the proposed mechanism since the trisaccharides are formed from sucrose, whereas the disaccharides are formed from the reaction products glucose and fructose.

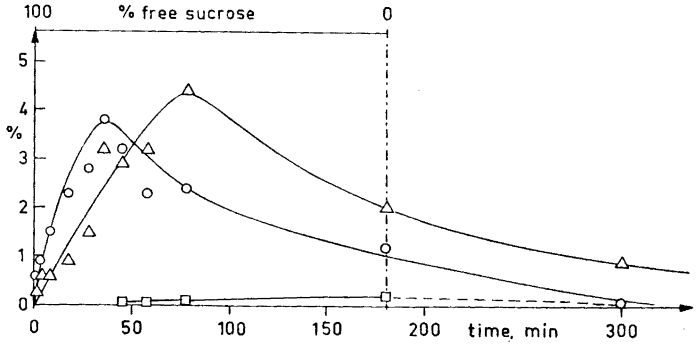

Fig. 5. A graphical representation of the concentrations of the intermediate oligosaccharides as functions of time. △ Reducing disaccharides; ○ Total trisaccharides; □ Total tetrasaccharides.

Another noteworthy feature is that, at the point where all free sucrose is hydrolyzed (Table 1: d=f) significant amounts of oligosaccharides remain to be hydrolyzed by means of the reversible reaction III.

Only moderate amounts of tetrasaccharides occur and they are formed late in the reaction, as expected if they are formed from the trisaccharides. As mentioned the analysis gave 1 part glucose and 3 parts fructose, indicating that they are formed from the trisaccharides. By fructosyl transfer to primary alcohol groups in the 3 trisaccharides 9 different tetrasaccharides are possible, but each of the 3 parts we obtained by the paper chromatographic separation can of course consist of more than one tetrasaccharide.

The results here described taken together with the earlier observation that the enzyme can form an active complex with fructose [10] confirms the proposed mechanism.

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Received January 23, 1969.