The Pattern of Action of Inulinase from *Saccharomyces fragilis* on Inulin

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Inulin is a naturally occurring plant polyfructoside. It consists of linear chains of approximately 35 D-fructose molecules, united by \( \beta(2 \rightarrow 1) \) linkages, and terminated by a D-glucose molecule, which is linked to fructose by an \( \alpha(1 \rightarrow 2) \) bond as in sucrose (1). In a previous paper (2) it was shown that *Saccharomyces fragilis* excreted an inulin-hydrolyzing enzyme when the yeast was grown in a medium with inulin as the carbon source. After purification, the enzyme was shown to hydrolyze inulin, the fructose portion of raffinose, sucrose, and branched bacterial levans of high molecular weight.

The terminal chains of the bacterial, branched levans consist of linear chains of approximately 35 D-fructose molecules, linked to each other by \( \beta(2 \rightarrow 6) \) bonds (3, 4), the branch connections being attached by \( \beta(2 \rightarrow 1) \) bonds, the same as those found in inulin. In all cases free fructose was the first detectable product of the hydrolysis. Thus, inulinase appears to be a \( \beta \)-fructosidase which hydrolyzes terminal \( \beta \)-fructosyl units from oligosaccharides or from linear or branched fructans irrespective of the linkage of the terminal fructosyl unit to the next hexose molecule. Inulinase was found to differ from invertase (yeast sucrase) by its much higher activity on polymers, especially on those of very high molecular weight. In addition, the pH-activity curves of yeast invertase with sucrose or with inulin as substrates (5) are quite different from those of inulinase (2). The results presented in this paper indicate that inulinase completely degrades 1 polymer molecule before hydrolysing another one (single-chain mode of attack).

**EXPERIMENTAL PROCEDURE**

**Inulinase**—The enzyme solution was the culture fluid of *Saccharomyces fragilis*, strain No. 351, grown for 48 hours at 30°C on a rotary shaker (250 r.p.m.) or in a flask gassed with sterile air. The synthetic medium contained 1% inulin as the carbon source and 1.2% (NH₄)₂HPO₄ as nitrogen and phosphate sources. Additional minerals, trace elements, and vitamins were listed in our previous paper (2). The culture liquid was centrifuged and purified (2) and finally dialyzed against 0.1 M acetate buffer, pH 5.1.

**Paper Chromatography**—Aliquots of enzymic digests of inulin were inactivated briefly in boiling water, concentrated in vacuum, and placed on Whatman filter paper No. 4. Descending chromatography was done with 9-propanol, ethyl acetate, and water (7:1:2) as the developing solvent. Spots were detected by spraying with benzidine trichloroacetic acid (6) or by silver nitrate (7).

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 aliquots of enzymic digests were heavily inoculated with Candida
krusei, a yeast which ferments hexose sugars but not oligosaccha-
rides. After 2 days the cells were removed by centrifugation.

The supernatant liquid was concentrated in vacuum and retested for
the presence of oligofructosides. The absence of hexoses and of
glucose could be indicated by a negligible reducing value of the
samples and by paper chromatography.

Quantitative Measurements of Glucose Liberation from Inulin—
First, a sample of inulin was hydrolyzed for 15 minutes at 120°
in 0.01 N H₂SO₄ to determine the ratio of total hexose glucose
(hereafter designated as the H:G ratio). This ratio was 36
for the inulin used in these experiments. Next, the rate of pro-
duction of free glucose and of total reducing sugars was deter-
dined during enzymic digestion of inulin. Fig. 1 shows an ex-
periment in which a 2% solution of inulin was hydrolyzed 70% by
inulase. Glucose was already detectable during the initial
stages of the reaction. The H:G ratios of the successive samples
decreased until they became comparable to the H:G ratio of
acid-hydrolyzed inulin.

To observe the very early stages of the hydrolysis in more de-
tail, another reaction mixture with a lower enzyme concentra-
tion was followed with short time intervals. This is shown in
Fig. 2. The rate of production of total hexose was constant for
the first 7% of the hydrolysis (corresponding to approximately
1.4 mg of hexose formed per ml). During this period the rate
of glucose liberation gradually increased and it became maximal
after 7% hydrolysis was completed. The initial H:G ratio
dropped from nearly infinity, after 30 seconds reaction time, to
about 450 after 4 minutes and to 175 after 8 minutes. The in-
crease in the rate of glucose production during the initial stages
of the hydrolysis is probably a reflection of the fact that not all
enzyme molecules attack substrate molecules at the same time
and that a certain number of fructosyl units must be hydrolyzed
before a glucose molecule can be liberated. The early appear-
ance of glucose and the general course of the reaction in both
examples given in Figs. 1 and 2 indicated that a substantial part
of the hydrolysis occurred by single-chain action of the enzyme
under our experimental conditions.

It has been shown (9, 10) that for the β-amylase-amylose re-
action the degree of single-chain action was reduced at abnor-
mally high or low temperatures (70° and 0.5°) or when the pH
of the reaction mixture was 2 to 3 pH units removed from the
optimum. A similar approach was made with the inulinas-
inulin system. In one set of experiments the reaction was al-
lowed to proceed at pH 5.1 (the optimum) but at temperatures
of 50° and at 0.5°. In a second series the reaction mixture was
incubated at 30° but at pH 3.0 and at pH 7.0. At 50°, 0.5°, and
at pH 7.0 (30°) the patterns were almost the same as shown in
Fig. 1. However, at pH 3.0 a significant shift toward a greater
degree of multichain action was observed. A comparison of the
pH effect is shown in Figs. 3 and 4. The slower appearance of
hexose at pH 3.0 is reflected by H:G ratios well above 35 during
the first 40% of the hydrolysis (corresponding to approximately
8 mg of hexose per ml). Although the initial rate of the re-
action was faster at pH 7.0 than at pH 3.0, the percentage of
total hydrolysis and the amount of glucose liberated after 90
minutes were greater at pH 3.0. It is possible that the enzyme
was more stable at pH 3.0 than at pH 7.0, but the higher rate
of the reaction at pH 3.0 during the later stages can also be ac-
counted for by the shift toward multichain action. The result
of such a shift is that the substrate concentration remains higher
for a longer time than would be the case with mainly single-
chain action.

Kinetics of Inulin Hydrolysis—A study of the kinetics of inulin
hydrolysis by inulase could give additional information on the
nature of the reaction. A first order reaction should occur when
terminal hydrolysis of a polymer occurs by a single-chain mecha-
nism. With a multichain mechanism little, if any, decrease in
substrate concentration occurs during a considerable part of the
hydrolysis and a zero order reaction would result. As shown
previously (2) the reaction rate does not decrease when oligo-
saccharides form the substrate.
The course of hydrolysis of a 0.25% solution and of a 0.5% solution of inulin was plotted as a function of time. The first order rate constants, determined graphically for 0.25% substrate and for 0.5% substrate, were 0.0502 min⁻¹ and 0.0495 min⁻¹, respectively (Fig. 5, A and B). The fact that the first order rate constants were valid for at least 80% of the hydrolysis (Fig. 5, A and B) supports the view that the decrease in rate of hydrolysis was due to substrate depletion. Thus, the first order reaction kinetics forms an independent element of proof for the single-chain mechanism of inulin hydrolysis.

**DISCUSSION**

Enzymes which attack polysaccharides by an endwise action may hydrolyze the substrate by a single-chain mechanism in which the enzyme completely hydrolyzes 1 substrate molecule at a time and by a multichain mechanism in which the enzyme attacks the ends of the polymer chains in a completely random manner. The multi-versus the single-chain mode of action has been studied most extensively for the amylose-β-amylase system. Some investigators (11-13) have provided evidence in favor of the single-chain mechanism of action, but others (14-16) have criticized their evidence and favor multichain action. More recently, Bailey and Whelan (10) have shown that under their conditions the action pattern was always intermediate between single- and multichain, but that the proportion of the two reaction mechanisms varied considerably with the degree of poly-
merization of the substrate and with pH and temperature conditions. The amyloglucosidase of \textit{Aspergillus niger} appears to attack amylose by a single-chain mechanism (17, 18) although oligosaccharides up to maltopentaose are attacked almost exclusively by a multichain mechanism (19). Information on the two mechanisms of action in the case of starch was based on the chromatographic detection of intermediates, rate of disappearance of the starch iodine color, fractionation of the products of partial degradation, and on labeling nonreducing chain ends with radioactive carbon.

Inulin is an ideal substrate by which to study the mechanism of action of the terminally acting inulinasins, since the substrate ends with a glucose molecule and inulinasin hydrolyzes the substrate from the opposite end of the chain. Thus, the rate of appearance of free glucose in relation to the total increase in reducing value can furnish information on the degree of single- or multichain action. The hexose to glucose ratios (H:G) of the reaction products were determined after various times and under various experimental conditions, after it had been established that the H:G ratio of acid-hydrolyzed inulin was approximately 35. Under most conditions the hydrolysis occurred largely by the single-chain mechanism as the H:G ratios of the reaction products soon approached the level of 35. At pH 3.0 (2.2 pH units below the optimal pH) there was a significant shift toward the multichain mechanism, as evidenced by significantly higher H:G ratios.

At least during the initial stages of the hydrolysis, the H:G ratios reported with the various curves may be used to estimate the percentage of the reaction products formed by the single-chain mechanism. This percentage can be expressed at a certain time, \( t \), as:

\[
\text{Amount of hexose formed as result of complete hydrolysis of inulin} \times 100
\]

or

\[
\text{Total hexose produced during same time} \times 100
\]

The value of the numerator can be obtained by multiplying the glucose values by 35, as the ratio of total hexose to glucose in our batch of inulin was shown to be 35. The denominator values (total hexose) were determined separately and are recorded in Figs. 1, 2, and 4. Thus,

\[
\% \text{Single-chain reaction} = \frac{35G}{H} \times 100 \text{ or } \frac{35}{H:G} \times 100
\]

When this relationship is applied to Figs. 3 and 4, it can be seen that after 20% hydrolysis (or 4 mg of free hexose per ml) was reached at pH 7.0, the reaction at that point, as well as during later stages of the hydrolysis, was almost completely of the single-chain type. On the other hand, when 20% hydrolysis was reached at pH 3.0, only 35/59 \( \approx 60 \% \) of the hexose sugars had been formed by the single-chain mechanism. During the later stages of the hydrolysis the relationship becomes less and less applicable since, because of multichain action, the substrate molecules become gradually shorter. Thus, the ratio of fructose to glucose hydrolyzed as a result of single-chain action, becomes smaller and as a result the H:G ratio decreases with time (cf. Fig. 4). Nevertheless, oligofructosides could not be detected at any time on paper chromatograms, indicating that the shorter molecules were hydrolyzed essentially by the single chain mechanism. Additional support for this mechanism under optimal reaction conditions was derived from the first order rate curves.

**SUMMARY**

Inulinasin is produced extracellularly by \textit{Saccharomyces fragilis}. The purified enzyme hydrolyzes inulin by an endwise action. The action commences at the \( \alpha \)-fructose end of the polymer and yields fructose until the last linkage is broken, which yields 1 \( \alpha \)-glucose molecule per molecule of inulin. By following the rate of glucose liberation in relation to the over-all rate of hydrolysis at pH 5.1 and 30\(^\circ\), it was shown that the degradation of inulin occurs largely by the single-chain mechanism. At 50\(^\circ\) and at 0.5° (pH 5.1) and at pH 7.0 (30°), essentially the same mechanism of action was observed. Only at pH 3.0 (30°) was a significant shift toward multichain action demonstrated. At pH 5.1 (30°) the reaction was first order, which supports the single chain mechanism of action.

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