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HEAT INACTIVATION KINETICS OF YEAST β-FRUCTOFURANOSIDASE. A POLYDISPERSE SYSTEM

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SUMMARY

1. The kinetics of heat inactivation of yeast β-fructofuranosidase were studied at 65° in the presence of 10 mM acetate buffer (pH 5.0).

2. The orders of reaction with respect to time (n_t) and with respect to concentration (n_c) were determined on a relatively pure enzyme preparation. It was found that $n_t > 1$ and $n_c = 1$.

3. The same enzyme preparation was chromatographed on DEAE-cellulose with a linear gradient. The symmetrical elution pattern displayed almost constant specific activity on a protein basis. This represented only slight improvement on the starting specific activity.

4. Individual chromatographic fractions gave $n_t = 1$. However, the magnitude of their first-order rate constants (k) increased continuously from the "front" to the "rear" of the peak.

5. There was a trend in the polysaccharide content of chromatographic fractions from 54% at the "front" to 39% at the "rear".

6. It was concluded that this preparation of β-fructofuranosidase is polydisperse with respect to polysaccharide content and to heat-inactivation susceptibility.

7. Heat inactivation of unfractionated enzyme is evidently a complex of simultaneous unimolecular processes. This is sufficient to explain the apparent anomaly that $n_t > 1$ but $n_c = 1$.

INTRODUCTION

Purified preparations of yeast β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) (trivial name: β-fructofuranosidase, and formerly called invertase) contain substantial quantities of polysaccharide (mannan)[1]. The application of DEAE-cellulose chromatography[2-4] has lowered the polysaccharide content to about 50%, and this is said to be covalently bonded to the enzyme protein[4]. Another report[5] claims at least two fractions of the enzyme with different polysaccharide contents. Speculations on the function of the polysaccharide moiety include the concept that it

stabilizes an enzymatically active conformation in the polypeptide[6]. Our interest in this hypothesis has prompted a reexamination of the kinetics of heat inactivation of yeast β-fructofuranosidase together with an exploration of a possible correlation between heat stability and the polysaccharide content of enzyme preparations.

VON EULER AND LAURIN[7] found that heat inactivation of yeast β-fructofuranosidase exhibited an order of reaction greater than one. Other investigators have assumed first-order kinetics for convenience, if not rigor. The majority of enzyme inactivation studies have, according to LAIDLER[8], involved monitoring the time course of inactivation at fixed temperature (*e.g.*, VON EULER AND LAURIN[7]) and subsequent curve fitting. This treatment yields what LETORT[9] calls the order with respect to time (n_t). On the other hand, if the initial rates ($-dc/dt$) of inactivation are determined over a range of starting concentrations (c) of enzyme, then a plot of log ($-dc/dt$) *versus* log (c) yields a straight line of slope n where n is the order with respect to concentration (*i.e.*, n_c). The theory has been discussed in detail by LAIDLER[8].

We now report on the determination of n_c for the heat inactivation of yeast β-fructofuranosidase. The discrepancy between n_t and n_c led us to a closer examination of heat inactivation kinetics in individual fractions of chromatographed enzyme. These results, together with chemical analyses on the same fractions, explain this discrepancy and also provide clues to the function of the polysaccharide moiety.

MATERIALS AND METHODS

Enzyme units and assay

One international unit of enzyme is that amount which brings about the hydrolysis of 1 μmole of sucrose per min at 30°. Incubation mixtures contained 100 mM sucrose, 50 mM sodium acetate–acetic acid buffer (pH 5.0), 2 mM ethylenediaminetetraacetic acid, and, typically, 0.5 units of enzyme. Reducing sugar formation was monitored with SUMNER'S[10] 3,5-dinitrosalicylic acid reagent as described previously[11].

Preparation of enzyme

High yields of β-fructofuranosidase were obtained from fresh, commercial baker's yeast by the method of MYRBÄCK AND SCHILLING[3]. Briefly, the procedure involves autolysis under toluene, pH adjustment, and fractional precipitation with ethanol at 4°. Our only modification was a batch-wise application of DEAE-cellulose ion exchange adsorption. The final preparation was dialyzed against distilled water, lyophilized, and stored at 4°. 400 g of fresh yeast yielded 1 g of lyophilized powder, specific activity 2670 units per mg protein. This specific activity is comparable with recent literature values[3,4].

Protein determination

A solution of bovine serum albumin was used as a working standard in conjunction with the Folin–Ciocalteu reagent and procedure described by LOWRY *et al.*[12]. Kjeldahl nitrogen was determined[13] for the working standard and for a purified β-fructofuranosidase preparation. The Folin–Ciocalteu color yield for purified yeast β-fructofuranosidase is 27% higher on a nitrogen basis than that for bovine serum albumin. All protein determinations have been appropriately corrected. The factor 6.25 was used to convert nitrogen assays into protein values.

Polysaccharide determination

A standard solution of mannan (ex-yeast, Pierce Chemical Co.) was prepared from a sample which had been dried *in vacuo* over phosphorous pentoxide. The tryptophan–sulfuric acid reagent of SHETLAR, FOSTER AND EVERETT[14] was adapted to smaller volumes, and quantification was performed at 505 mμ. In addition, a spectrum (650 mμ–450 mμ) was recorded as part of each analysis.

Heat inactivation

All treatments were carried out in a well-stirred, thermostated bath set at 65°. We used 18 mm × 150 mm tubes which were immersed approx. 100 mm. The tubes were preheated at 65°, and then, at zero time, approx. 1 ml of enzyme solution was delivered to the tube from a large-bore pipet. After the prescribed interval the tube was removed from the heating bath and plunged into ice water. An accurate aliquot was subsequently assayed for enzyme activity. The standard medium for heat treatments was 10 mM sodium acetate–acetic acid (pH 5.0).

In plotting heat inactivation data we use the convention ln $(a/(a-x))$ *versus* t where a is the concentration of enzyme at zero time and $a-x$ is the concentration remaining after t min heating. Where linear relationships obtained, the slopes and standard deviations (S.D.) were evaluated by the least squares method.

DEAE-cellulose chromatography

DEAE-cellulose (Calbiochem. 0.9 mequiv/g) was washed and regenerated[15] and finally equilibrated with 5 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.0). It was packed under 4 lb/inch^2 pressure in a jacketed column of internal dimensions 1.44 cm × 30 cm. The dry weight of cellulosic material was later estimated at 5.1 g. The void volume of a similarly packed column was 44 ml, according to the elution volume of the non-adsorbed dye, methyl green. After the column had been packed, 5 mM phosphate buffer (pH 6.0) was pumped through at 1 ml per min for about 2 h; and then the sample of yeast β-fructofuranosidase (22 mg lyophilized powder in 2.2 ml of the same buffer) was applied. The column was washed to waste with about 250 ml of the same buffer, and then a linear gradient[16] 0 to 200 mM NaCl in 5 mM phosphate buffer (pH 6.0) was formed. 150 ml were used in each reservoir. Collection of 5-ml fractions of the eluate was then started. Chromatography and fraction collection were performed at 4°.

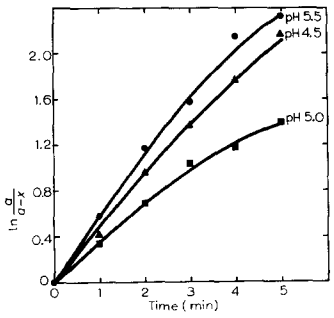
Dialysis

Fractions from DEAE-cellulose chromatography were dialyzed overnight against several liters of distilled water at 4°. Before use, regenerated cellulose dialysis tubing was cleansed by boiling for 3 min in 0.1% sodium carbonate and by subsequently washing in copious quantities of distilled water.

RESULTS

Order of reaction with respect to time, n_t

Solutions of lyophilized β-fructofuranosidase preparation were prepared in 10 mM acetate buffers of pH values 4.5, 5.0, 5.5. Enzyme (5 units per ml) was treated at 65° for each pH for periods of 1 to 5 min. The results are presented in Fig. 1. It is apparent that all three curves depart from the straight line demanded by a first order



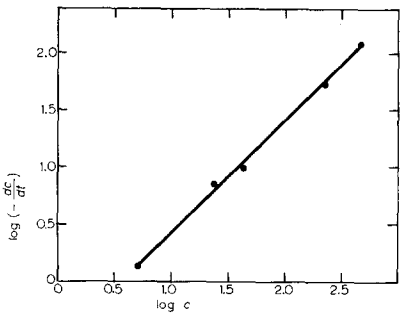


Fig. 1. Heat inactivation curves for β-fructofuranosidase at 65°. Solutions were prepared with lyophilized powder in 10 mM acetate buffers at the pH values indicated.

Fig. 2. Demonstration of order of reaction by the differential method. Slope, $n_c = 1$. Details are given in the text.

reaction and consequently that $n_t > 1$ (*cf.* ref. 7). Also it can be noted that the enzyme exhibits maximum stability in the region of pH 5.0 (*cf.* ref. 7), and this value was used in all subsequent heat-inactivation trials. Preliminary trials recommended 65° as a test temperature because measurable inactivation rates could be obtained in 2 to 5 min. Inactivation was irreversible.

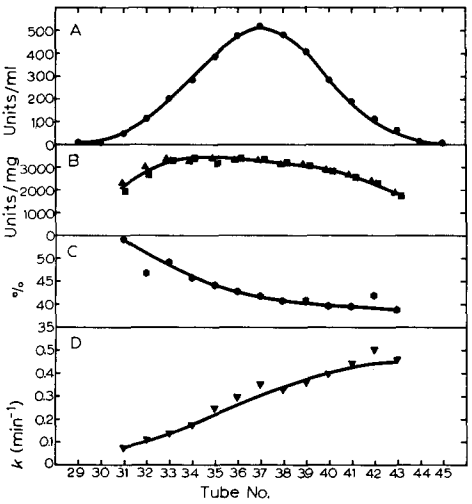
Order of reaction with respect to concentration, n_c

Solutions of lyophilized powder were prepared in the standard buffer to contain 475, 232, 43.2, 24.6, and 5.24 units per ml. These were heat treated, in triplicate, for 0, 1, and 2 min. Suitably diluted aliquots were assayed, and an estimate of the initial rate of inactivation for each concentration was obtained graphically. A Letort plot[8,9] of the data is given in Fig. 2. The line of best fit has slope, 0.979 ± 0.030 (S.D.). Clearly, $n_c = 1$.

Fractionation of lyophilized powder

DEAE-cellulose chromatography was performed with a linear salt gradient as described in METHODS. The elution pattern of enzyme activity is depicted in Fig. 3A. Approx. 95% of the enzyme activity was recovered in tubes 29–45. The specific activity on a protein basis (Fig. 3B) exhibits a high degree of constancy among tubes 33–39 (mean 3330 units per mg, S.D. 89) with some departure on either side (tubes 31–43, mean 2980 units per mg, S.D. 434). These specific activities represent only 25% and 12% improvement over the starting material. After overnight dialysis against distilled water there was no significant change in specific activities (Fig. 3B).

Polysaccharide concentrations were determined on dialyzed Fractions 31–43, and assuming that this is the only component other than protein, the percent polysaccharide has been computed for each fraction (Fig. 3C). There is a gradual change from 54.1–39.0%. The average composition when summed for the number of enzyme units per fraction is 42.8% polysaccharide. No differences were apparent in the spectra of the various fractions following reaction with the H_2SO_4–tryptophan reagent. This reaction does distinguish between glucans and mannans, for instance; but with all tubes the spectra were indistinguishable from that obtained with mannan.



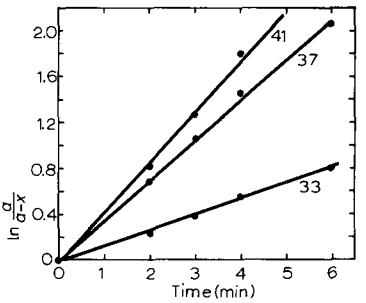


Fig. 3. Fractionation of β-fructofuranosidase by DEAE-cellulose chromatography. Fractions of 5 ml were collected in each tube. A, Elution pattern showing enzyme concentration per tube; B, specific activity on a protein basis, ▲, before dialysis; ■, after dialysis; C, percent polysaccharide; D, first-order rate constants for heat inactivation at 65°.

Fig. 4. Heat inactivation curves for selected fractions of β-fructofuranosidase at 65°. The curves pertain to material from tubes 33, 37, and 41 in Fig. 3A.

Aliquots of each chromatographic fraction were diluted with 10 mM sodium acetate–acetic acid buffer (pH 5.0) so that the final concentration in each case was 5 units per ml. 1-ml aliquots were then treated at 65° for 2 min and finally assayed for residual activity. Apparent first order rate constants (k) were determined from the relationship $k = \frac{1}{2} \ln (a_0/a_2)$, where a_0 and a_2 represent the concentration of enzyme at zero time and after 2-min heat treatment, respectively. The values are plotted in Fig. 3D. Clearly there is a continual increase in heat inactivation susceptibility from the front of the chromatographic peak to the rear. That first order kinetics are followed is evident from a more detailed study of Fractions 33, 37, and 41 (Fig. 4) which yielded constants of 0.137 min^{-1}, S.D. 0.006; 0.350 min^{-1}, S.D. 0.011; and 0.445 min^{-1}, S.D. 0.065, respectively.

DISCUSSION

The true order of reaction for heat denaturation of yeast β-fructofuranosidase was shown to be one, and we confirmed previous reports that $n_t > 1$ for relatively pure preparations. However, by chromatography, an arbitrary number of small subfractions was achieved which individually exhibited first order kinetics ($n_t = 1$), and together displayed a continuum in the magnitude of their first order rate constants.

In unfractionated preparations the observed departure from first-order kinetics (in time-course studies) is due to a relative enrichment in more stable species as the inactivation process proceeds. In other words, we are studying a complex of simultaneous unimolecular processes which in sum give the appearance that $n_t > 1$. On the other hand, the differential method, which employs initial rates of inactivation,

avoids this complication by distinguishing only the most sensitive species (with some apparent weighting due to frequency) and gives $n_c = 1$.

Fractions which had the same specific activity on a protein basis exhibited different heat inactivation susceptibilities. An inverse trend in polysaccharide composition was noted such that increased stability obtained for fractions with higher carbohydrate content. If we assume that these assays are a true reflection of covalently bonded polysaccharide, then some credence is given to the old hypothesis that this moiety has a stabilizing function for the enzyme.

Our preparation of yeast β-fructofuranosidase is clearly polydisperse with regard to polysaccharide content and to heat inactivation susceptibility. Other evidence to this effect will be published elsewhere. It is worth mentioning at this time that we have been able to rechromatograph arbitrary sub-fractions (*i.e.*, the "front", the "middle", and the "rear" of the peaks) from an elution pattern such as that in Fig. 3A. With a linear gradient on DEAE-cellulose columns the subfractions individually rechromatograph with elution peak volumes which reflect their positions in the parent peak. Also, mixtures of "front" and "rear" material exhibit bimodal elution patterns. It might be speculated that the chromatographic behavior of this enzyme is in large part due to the polysaccharide moiety. NEUMANN AND LAMPEN[4] suppose that their preparation of β-fructofuranosidase might contain molecules with slightly varying amounts of polysaccharide. Their evidence appears to rest on the inexact fit of the carbohydrate and the ultraviolet absorption profiles eluted from DEAE-Sephadex.

Modifications in the structure of yeast β-fructofuranosidase might arise during autolysis, and this might result in the polydispersity which we observe in purified preparations. Alternatively, it might be argued that the variable polysaccharide content is a reflection of the mode of release of β-fructofuranosidase from the cell wall and that its normal habitat involves association with the cell wall constituents through the polysaccharide moiety. If this is true, it follows that mechanical means of solubilization might be just as imprecise as the enzymic processes in autolysis are random.

If, as the present work indicates, the polysaccharide moiety stabilizes the polypeptide of the enzyme, then the mechanism remains to be elucidated. It should be mentioned that VON EULER AND LAURIN[7] speculated in 1919 that heat stability might be associated with degree of hydration and that different amounts of bound water might exist in different invertase molecules. They proposed that the most labile are inactivated first and, provided that the species are not quickly interconverted, that there is a sequence of events in which the more stable species gradually dominate the inactivation pattern. If the vicinal water structure is sufficiently dependent on the relative amount of polysaccharide in the enzyme, then our finding of a continuum of polysaccharide content is indirectly in sympathy with their suggestion, and the restriction of slow interconversion of species is removed.

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