BBA 12276

THE LOCALIZATION OF β-FRUCTOFURANOSIDASE IN NEUROSPORA

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(Received March 25th, 1963)

SUMMARY

1. The β-fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) of *Neurospora conidia* is fully accessible to substrate, to aniline (an inhibitor), and to H^+, as judged by the similarity of the kinetic properties of the conidial enzyme to those of highly purified preparations. The conidial and mycelial forms of β-fructofuranosidase are rapidly and completely inactivated under conditions which do not impair the viability of conidia, and fail to destroy alkaline phosphatase, which has been inferred on other grounds to be intracellular.

2. A limited fraction of the conidial β-fructofuranosidase is easily removed by washing.

3. The bound conidial β-fructofuranosidase, but not the crude or purified soluble enzyme, is resistant to pepsin inactivation.

4. The results favor the view that this enzyme is located between the cell wall and the cell membrane.

INTRODUCTION

The question of the localization of the enzyme β-fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) (invertase) in yeast has been the subject of considerable attention, but of rather less agreement. MYRBÄCK[1] treated yeast cells with ethyl acetate and observed that the enzyme, though fully active, could be liberated in soluble form only by autolysis or papain treatment. ISLAM AND LAMPEN[2] found that removal of the cell wall of yeast with snail gut juice caused the resulting protoplasts to secrete newly formed β-fructofuranosidase into the medium. Partial disruption of the cell wall by limited treatment with the snail preparation allowed some secretion of the enzyme, but a large proportion was retained by the remaining cell wall. The authors opined that the cell wall contained binding sites for the β-fructofuranosidase, and that secretion of this enzyme might result from the removal of the requisite binding sites.

Studies on the purified yeast enzyme by FISCHER AND KOHTÉS[3] have shown that mannan, a known constituent of the cell wall of yeast, is present in very large amounts (70%), suggesting an association *in vivo* of the enzyme with the cell wall.

In addition, a number of studies[1,2,4,5] have established that the β-fructofuranosidase of Saccharomyces is located virtually exclusively in a position external to the cell membrane. (See however the results of FRIIS AND OTTOLENGHI[6], who studied a hybrid yeast.)

In contrast to these studies, which either support, or are consistent with the notion that β-fructofuranosidase is firmly attached to the cell wall, BURGER *et al.*[7] have found that disruption of either intact or ethyl acetate-treated yeast in the Hughes press yielded most of the enzyme in a soluble form, and that the enzyme was almost quantitatively solubilized by vibrating with glass beads. Osmotic-shock treatment was also effective. On the basis of these and other observations, the authors postulated that β-fructofuranosidase is in a soluble form *in vivo*, being located in a compartment outside the cell membrane.

It has recently been reported by the present author that highly purified preparations of β-fructofuranosidase from *Neurospora crassa* contain hexosamine[8]. As it is known that the cell wall of Neurospora contains hexosamine, it was proposed that β-fructofuranosidase is in some way associated with the cell wall in this organism. EBERHART[9] has reported that this enzyme can be detected in washings of conidia, which strongly suggests that at least some of it is externally located in Neurospora. The evidence to be presented below suggests that virtually all of this enzyme is outside the cell membrane, but that most of it is interior to another permeability barrier, presumably the cell wall.

METHODS

Wild-type *Neurospora conidia* were obtained from petri plates containing the minimal salts medium of Fries as described by BEADLE AND TATUM[10], supplemented with sucrose (1.5%) and solidified with agar (1.5%). The inoculated plates were incubated at 24°, and were harvested with a vacuum line, employing a constricted glass tube to conduct the conidia to a Millipore filter. The conidia were then suspended in water at 0°, filtered through glass wool to remove bits of mycelium and clumps of conidia, and the concentration of conidia was estimated optically as previously described[11]. The conidia were harvested between 1 and 2 weeks after inoculation of the plates. In general, a suspension which had an absorbancy of 1.00 at 420 mμ in a 19-mm diameter Coleman cuvette was prepared. Such preparations contained 2.0 μmoles of protein nitrogen per ml, and had about 2 units of β-fructofuranosidase per ml in the case of 2-week-old conidia. 1-week-old conidia were somewhat less active. Suspension of this concentration provided a suitable enzyme level for the experiments reported below.

β-Fructofuranosidase which was homogeneous in the ultracentrifuge and showed a single band in gel electrophoresis was prepared as previously described[8].

Reducing sugar was estimated by the method of SOMOGYI[12]. The assay of β-fructofuranosidase was conducted as previously described[11], with the modification that the reaction was stopped by adding an equal volume of the SOMOGYI alkaline copper reagent to the reaction mixture. When conidia or mycelia were used as such the tubes were incubated in a gyrorotatory shaker, and the formed elements were removed by centrifugation after addition of the copper reagent.

Alkaline phosphatase (EC 3.1.3.1) was measured essentially as described by TORRIANI[13]. Units were as previously defined[11].

Counts of viable conidia were performed either by plating a suitable aliquot on Fries' agar with 1.5% glucose and counting growth centers microscopically, or by plating on sucrose–sorbose medium to induce colonial morphology as described by TATUM *et al.*[14]. DESERRES *et al.*[15] have observed that the efficiency of plating in the presence of substantial concentration of sorbose is conditioned by such factors as the autoclaving time, the commercial source of the sorbose, *etc.* Therefore, the following precaution was observed. The conidia were initially plated on 0.01 M sucrose plus 12.5 mM sorbose Fries medium. This ratio of sorbose to sucrose only mildly inhibits growth, and provides a good start for the growing conidia. After 24 h at room temperature, the plates were overlaid with warm agar (0.75%) containing 0.01 M sucrose plus 0.05 M sorbose. After an additional 2 days at room temperature, the colonies were readily observed macroscopically.

RESULTS AND INTERPRETATIONS

pH curve

The activities of whole conidia and of highly purified β-fructofuranosidase as a function of pH, employing sucrose as a substrate, are shown in Fig. 1. It will be seen

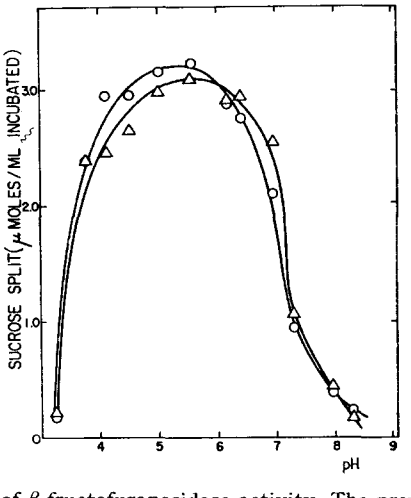

Fig. 1. The pH dependence of β-fructofuranosidase activity. The previously reported[8] pH curve of the purified enzyme is included for purposes of comparison. The buffers used at each pH value were as described[8]. △, conidia; ○, purified enzyme.

that the conidial invertase is completely responsive to the pH of the external milieu. A similar comparison has been made by WILKES AND PALMER[4], using yeast autolysate and intact yeast.

Michaelis constant for sucrose

Experiments in which whole conidia were incubated with various concentrations of sucrose yielded a linear Lineweaver–Burk plot, from which the value $K_m = 6.6$ mM

was obtained. The purified enzyme gave $K_m = 6.1$ mM. These values are regarded as being the same within experimental error. Thus is it clear that the access of sucrose to the enzyme *in vivo* is not greatly modified by a permeability barrier.

Relative activity upon sucrose and raffinose

Whole conidia and highly purified enzyme were incubated under standard conditions[11] with either sucrose or raffinose (0.05 M). The relative rate of hydrolysis (sucrose split:raffinose split) for conidia was 4.50; for purified enzyme, 4.55.

If the substrate for the conidial enzyme were obliged to diffuse through an effective barrier prior to cleavage, it would be anticipated that the trisaccharide would diffuse more slowly than the disaccharide, and thus the ratio sucrose split: raffinose split would be larger for the conidial enzyme than for the soluble system.

Inhibition by aniline

Fig. 2 shows the hydrolysis of sucrose by conidia in the uninhibited system and in the presence of aniline (12.5 mM). The relative slopes of the curves correspond to 86% inhibition by this concentration of aniline. A similar experiment with purified β-fructofuranosidase showed 84% inhibition. An analogous line of evidence has been reported for the corresponding enzyme from yeast[1].

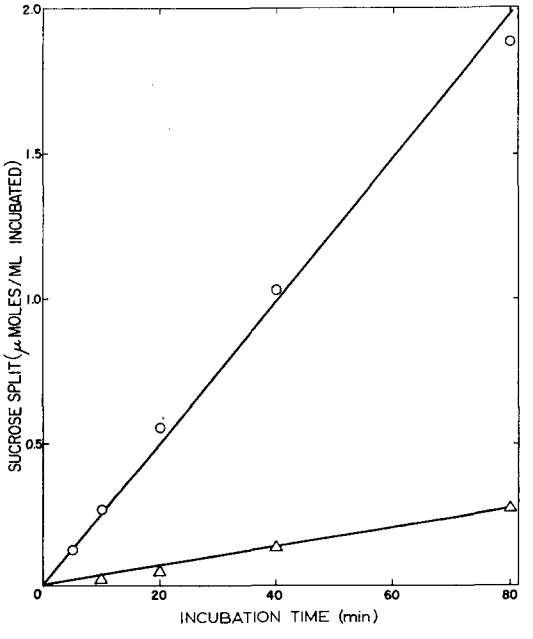

Fig. 2. Inhibition of β-fructofuranosidase of conidia by aniline. Aliquots of the usual incubation mixture, with or without aniline, were removed at intervals and analyzed for reducing sugar. ○, without aniline; △, 12.5 mM aniline.

Partial removal of β-fructofuranosidase by washing with water

Conidia were suspended in cold water, filtered, and gently stirred at 0°. Samples were removed at intervals ,quickly centrifuged at about 700 × *g* for 5 min, and the supernatant solutions assayed for β-fructofuranosidase. An aliquot which was not subjected to centrifugation was saved for determination of the total activity.

Table I shows that an appreciable amount of the enzyme (13.6%) was soluble at the earliest time measured (15 min), and that the soluble fraction increased very

TABLE I

EXTRACTION OF β-FRUCTOFURANOSIDASE WITH WATER

Extraction time (min)	*Activity extracted (% of total)*
15	13.6
30	14.1
45	14.5
60	15.1
120	16.4

slowly thereafter. It has been found that the soluble fraction is quite variable from one preparation of conidia to another, ranging from extremes of 9% to 19% of the total activity.

Successive and continuous extraction of conidia with water

It seemed possible that the enzyme in question might dissociate reversibly from the cell surface, and that the data presented in Table I might reflect the approach of such a dissociation to equilibrium. To test this hypothesis, a suspension of conidia was divided into two portions. The first, which served as a control, was treated as above. The second was likewise stirred, but the entire preparation was centrifuged at intervals. Following each centrifugation the supernatant solution was saved for assay, and the conidia were resuspended in water and the extraction continued.

TABLE II

UNINTERRUPTED AND SUCCESSIVE EXTRACTION OF β-FRUCTOFURANOSIDASE WITH WATER

Type of extraction	*Time interval (min)*	*Activity extracted (% of total)*
uninterrupted	0– 15	11.8
	0– 30	11.7
	0– 60	12.0
	0–120	12.7
successive	0– 10	11.2
	10– 25	0.3
	25– 55	0.2
	55–115	0.4

Table II shows the per cent of the total activity found in the supernatant fraction in the case of uninterrupted as opposed to successive extration. It will be seen that very little β-fructofuranosidase is solubilized in successive extractions.

In an additional test of the hypothesis, conidia were continuously extracted in the cold on a Millipore filter for a period of 2 h with distilled water, and the activity of the preparation was compared with that of a control suspension of once-washed conidia. The former had 89% of the activity of the latter. Thus it is clear that the soluble fraction represents a discrete proportion of the total enzyme rather than a random dissociation from a chemically and topographically homogeneous pool of enzyme.

Extraction of the once-washed conidia with 0.05 M sodium acetate buffer (pH 5.0) or 0.05 M Tris–HCl buffer (pH 8.25) or with either of these plus 2.0 M NaCl gave results essentially identical with those of successive extraction with water; *i.e.*, only negligible additional β-fructofuranosidase was extracted. On these grounds, it seems unlikely that the bound enzyme is held to the cell surface by salt bonds.

Inactivation of the β-fructofuranosidase by acid

A suspension of conidia was warmed in a shaker-incubator to 38° and an equal volume of 0.2 N HCl was added. After 1 min, the inactivation was terminated by adding the calculated amount of 1.0 M sodium acetate to bring the pH to 5.0. As a control, the acid and acetate were mixed prior to addition to the suspension. Fig. 3 shows that the enzyme was completely inactivated by brief acid treatment. Similar results were obtained by 5-min acid treatment at 0°.

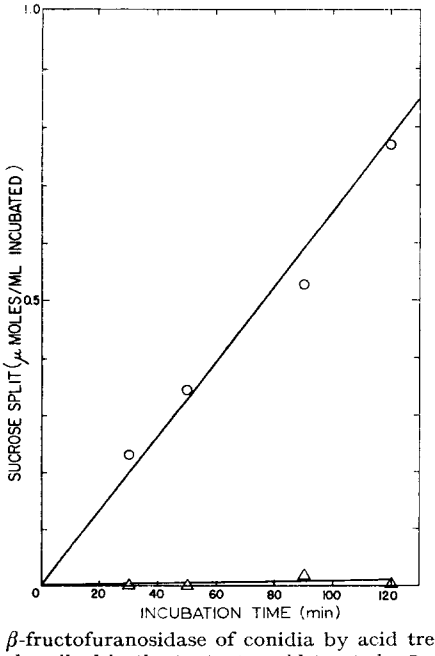

Fig. 3. Inactivation of the β-fructofuranosidase of conidia by acid treatment. Conditions of the experiment are described in the text. △, acid-treated; ○, buffer control.

Conidia treated aseptically for 1.5 min at 38° in 0.1 N HCl as above were diluted and plated on Fries' agar containing sucrose and sorbose in the manner described under METHODS. Comparison with control plates showed that no appreciable killing of the conidia by acid had occured. Treatment for 15 min with acid as above reduced the viable count by about one-third as measured either on glucose or sucrose medium, and, of course, totally destroyed the β-fructofuranosidase. It is apparent that the enzyme is inactivated under conditions that do not appreciably impair the subsequent growth of the spores, even on sucrose. The growth of the acid-treated spores on sucrose could be due either to resynthesis of the β-fructofuranosidase of the cell surface prior to growth, to utilization of traces of glucose and fructose present in the medium, or to the persistance of a small, but sufficient intracellular pool of the enzyme.

Sonic disruption of conidia and release of enzymes

A suspension of conidia was washed three times with cold water by centrifugation and resuspension. The conidia were subjected to sonication at 20 000 cycles/sec with a Branson Model LS-75 sonic oscillator at an output of 4.5 A. The sample was immersed in an ice-bath, and the treatment was interrupted at intervals to remove aliquots and to maintain the temperature below 20° at all times. The samples were centrifuged at 700 × *g* for 5 min and the clear supernatant solutions as well as samples which were not centrifuged were assayed for β-fructofuranosidase and alkaline phosphatase. EBERHART[9] has reported that substantial amounts of the latter are solubilized by acetone treatment of conidia, which provides evidence of its intracellular localization. Fig. 4 shows the release of these two enzymes into the soluble phase. It will be seen that total detectable β-fructofuranosidase remains essentially

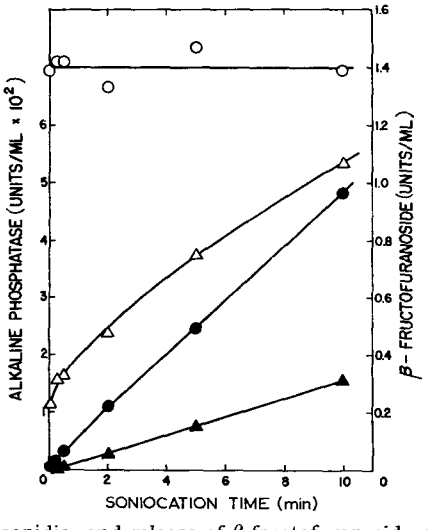

Fig. 4. Sonic disruption of conidia, and release of β-fructofuranosidase and alkaline phosphatase into the soluble phase. Conditions are described in the text. ○, total detectable β-fructofuranosidase; ●, soluble β-fructofuranosidase; △, total detectable alkaline phosphatase; ▲, soluble alkaline phosphatase.

constant during the sonication, but that the proportion in the soluble fraction rises approximately linearly with time. The constancy of the total enzymic activity suggests that the bound enzyme and the soluble form are quantitatively as well as qualitatively simililar in sucrose hydrolysis. The disruption of the conidia does not appear to follow one-hit kinetics, although more rigorous control of the conditions during sonication would be needed to establish this point, as well as separate analyses of the residues from more exhaustively sonicated preparations. In contrast to the β-fructofuranosidase, the total detectable alkaline phosphatase rises sharply during the sonic disruption, which is consistent with the intracellular location of at least a large proportion of the latter enzyme. The increment in total detectable alkaline phosphatase is much greater than is the amount of activity which is actually brought into solution. It seems probable that sonic disruption as observed here is not an all-or-none situation, but that the cell becomes progressively more leaky with increasing sonication. Thus the permeability of the membrane to the *p*-nitrophenyl phosphate molecule could be greatly increased by sonication, whereas relatively few of the much larger phosphatase molecules would escape into the soluble phase. This model would also be in harmony with the apparently linear release of both enzymes with time.

Sonic disruption of acid-treated conidia

A suspension of thrice-washed conidia was divided into two portions. One portion was treated for 5 min at 0° with 0.1 N HCl and sodium acetate was added to give pH 5.0. The second portion was treated with similarly prepared acetate buffer. Both preparations were sonicated as before for 10 min, following which a portion of each was centrifuged for 5 min at 700 × *g*. The supernatant solutions were saved for assay, and the residues were resuspended to the original volume in water. The preparations were again centrifuged and the first washings saved for assay. The residues were again washed as above to give second washings and twice-washed residues. Table III shows the activities of β-fructofuranosidase and alkaline phosphatase in these fractions. No measurable activity of the former was seen in the supernatant from disrupted conidia, suggesting that there was no appreciable β-fructofuranosidase which was not accessible to acid. Qualitatively similar results

TABLE III

β-FRUCTOFURANOSIDASE AND ALKALINE PHOSPHATASE IN SONICALLY DISRUPTED CONIDIA

Type of preparation	*β-Fructofuranosidase units**		*Alkaline phosphatase units* × 10²**	
	Buffer-treated	*Acid-treated*	*Buffer-treated*	*Acid-treated*
Washed conidia	1.30	0.00	1.38	1.23
Whole sonicate	1.47	0.00	1.87	1.40
Sonicate, supernate and washings	0.58	0.010	0.67	0.55
Sonicate, washed residues	0.86	0.016	1.12	0.79

* All units are referred back to 1 ml of the original washed conidia.

have been obtained by grinding conidia with fine carborundum powder and by disruption with five freeze–thaw cycles. The latter two procedures were, however, less definitive, as they gave considerably less disruption of controls which were not treated with acid.

In contrast, alkaline phosphatase was readily observed in sonicated acid-treated conidia, albeit at somewhat lower levels than untreated controls. A control showed that solublized alkaline phosphatase was completely denatured by acid under the conditions to which the conidia were subjected.

Inactivation of mycelial β-fructofuranosidase with acid

Conidia were harvested aseptically and were suspended in Fries' salts supplemented with glycerol (2%). The suspension of conidia was filtered through glass wool, and the suspension, which was equivalent to 1.25 mg of conidial protein nitrogen, was centrifuged and resuspended in 40 ml of the above medium. The preparation was shaken for 17 h at 24°. The wispy young mycelia, which were sufficiently disperse to handle reasonably accurately with a large-orifice pipet, were washed twice with water. The final suspension was divided into two 20-ml portions, which were respectively treated with acid or buffer as in the preceding experiment. One-fourth of each portion was then assayed directly for β-fructofuranosidase as described for conidia, aliquots being removed at intervals for the estimation of reducing sugar. The results will not be separately presented, as they were essentially identical with those obtained with conidia (Fig. 3).

The remainder of each preparation of treated mycelia was recovered on a Millipore filter, washed, and lyophilized. The lyophilized pads were ground in a mortar with dry ice. 1 ml of 0.05 M sodium acetate buffer (pH 5.0) was added to each frozen sample, and the preparations were ground for 5 min with additional dry ice. The powders were thawed, centrifuged, and the supernatant solutions were assayed for β-fructofuranosidase, alkaline phosphatase, and protein in the manner previously described for mycelia[11]. The specific activities of these two enzymes, respectively, were 33.0 and 0.127 in buffer-treated mycelia, and 0.00 and 0.125 in acid-treated mycelia. Thus the localization of these enzymes is probably the same in mycelia as in conidia.

The action of proteolytic enzymes on soluble and "bound" β-fructofuranosidase

In view of the fact that the latter enzyme is readily solubilized by disruption of either mycelia or conidia, it seemed likely that the enzyme was located between the cell membrane and the cell wall. If this were the case, it might be expected that the porosity of the cell wall, which allows free penetration of sugars, aniline, and H^+, but prevents the loss of β-fructofuranosidase, might also prevent the ingress of such macromolecules as proteolytic enzymes. A preliminary study with the highly purified β-fructofuranosidase showed no loss of activity with trypsin (EC 3.4.4.4), even after prolonged incubation with high concentrations of the latter; chymotrypsin was barely more effective. However, pepsin (EC 3.4.4.1) in 0.05 N acetic acid readily attacked the enzyme. While there was invariably some inactivation of the enzyme by the acid alone, pepsin greatly increased the rate of inactivation. Attempts to

circumvent the moderate acid inactivation by use of formate or citrate buffer (pH 3) during the pepsin digestion were without success. Surprisingly, β-fructofuranosidase is much more labile to these buffer *per se* than to acetic acid.

Table IV shows that, as predicted, the activity of highly purified enzyme and of conidial washings is very labile to pepsin, whereas the β-fructofuranosidase of washed conidia is completely insensitive to a concentration of pepsin 50-fold greater than that which sharply reduces the activity of the soluble forms of the enzyme.

TABLE IV

THE ACTION OF PEPSIN ON VARIOUS PREPARATIONS OF β-FRUCTOFURANOSIDASE

Purified enzyme, conidial washings, and washed conidia, 13.0, 3.0, and 3.0 units/ml, respectively, were added to an equal volume of pepsin in 0.1 M acetic acid and preincubated for 5 min at 38°. The action of pepsin was terminated and the action of β-fructofuranosidase was initiated by adding an equal volume of 0.1 M sucrose in 0.1 M sodium acetate. After 30-min incubation, the reaction was stopped and reducing sugar determined in the usual way. All results are referred to control tubes which were preincubated with acetate buffer (pH 5.0) rather than acetic acid.

Pepsin (μg/ml) in preincubation	*β-Fructofuranosidase activity: % surviving treatment in 0.05 M acetic acid + pepsin*		
	Purified enzyme	*Conidial washings*	*Washed conidia*
0	61	43	79
2.5	30	—	—
5	2.3	29	76
25	2.5	3.7	81
50	2.2	3.6	81
250	—	—	80

It is also to be noted that the three enzyme preparations vary appreciably in their lability to acetic acid alone, the bound form being the most stable. The meaning of the latter phenomenon is unclear, although a number of obvious *ad hoc* explanations could be offered.

It must be emphasized that the insensitivity of the bound enzyme to pepsin does not unequivocally establish that the former is protected from the latter by a permeability barrier. It is possible that β-fructofuranosidase is bound to the outer surface of the cell wall, and that the binding changes the configuration in such a way as to prevent the attack of pepsin. There are numerous examples of stabilization or labilization of enzymes to thermal or enzymic degradation by their substrates or coenzymes. However, in the present case, it seems likely that such a drastic change in stability to proteolytic attack would be accompanied by changes in at least some of the other properties of the enzyme (K_m, V_{max}, pH curve, etc.) if the stabilization were primarily due to a change in configuration of the enzyme.

ACKNOWLEDGEMENT

This work was supported in part by a U.S. Public Health Service grant (RG-8995).

The author is grateful for the excellent technical assistance of Miss J. WATSON.

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