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Expression of Cryptic β-Fructofuranosidase in *Saccharomyces rouxii*

WILFRED N. ARNOLD

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66103

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Raffinose hydrolysis was studied in *Saccharomyces rouxii*. The responsible enzyme was identified as a β-fructofuranosidase (EC 3.2.1.26), which has a pH optimum of 5.5 and a K_m of 83 mM for raffinose. This enzyme was cryptic in cells from a 3-day culture. A 2-min treatment with 0.1 volume of ethyl acetate in sodium acetate buffer (pH 6) gave complete expression of the enzyme, which was still retained by the cell. Ghosts were prepared by modifying membrane structure with small basic proteins in distilled water, and after washing they showed the full complement of enzymatic activity. The enzyme remained cryptic in osmotically protected spheroplasts; however, after lysis (by dilution) release, as well as expression, was effected. Mechanical disruption of fresh cells revealed and released all of the enzyme. Cells in early stationary phase had all of their β-fructofuranosidase in a cryptic state. Aging yielded fractional expression of activity; initially this was proportional to cell death, but later the degree of expression exceeded the death rate. Media from aged cultures or cell-free extracts of aged cells were not effective in revealing the cryptic enzyme of younger cells. *S. rouxii* β-fructofuranosidase has a different autolytic-release pattern from its counterpart in *S. cerevisiae*. Also, high concentrations of glucose do not repress the *S. rouxii* enzyme. The β-fructofuranosidase in young cells of *S. rouxii* must be enclosed by the protoplasmic membrane or a special vesicular structure. This system was compared with other *Saccharomyces* species in connection with the translocation of enzymes across the protoplasmic membrane.

β-Fructofuranosidase (EC 3.2.1.26, trivial name invertase) has been the subject of considerable investigation in *Saccharomyces* species. The enzyme in bakers' yeast (*Saccharomyces cerevisiae* Hansen) is located external to the protoplasmic membrane, where it is accessible to substrate and to hydrogen ions but is restrained from diffusion into the medium by the cell wall (11). Evidence has been presented (2, 6) to indicate that the periplasmic space is the precise locale for that enzyme, and it is of current interest to investigate the spatial and temporal events which culminate in this disposition within the cell.

Differences are encountered in the behavior of the cell envelope of related species of yeast. With respect to β-fructofuranosidase and in comparison with *S. cerevisiae*, variations in the retentivity of the cell wall (e.g., *S. uvarum* [20]), in susceptibility of envelopes to sulfhydryl reagents (e.g., *S. fragilis* [9]), and in vulnerability to osmotic shock (e.g., *S. chevalieri* [17]), are probably due to chemical differences in key structural components.

If periplasmic enzymes such as β-fructofuranosidase and acid phosphatase are synthesized in the cytoplasm, a movement of whole or partially synthesized molecules must take place and a general relationship to the process of enzyme secretion is apparent. A useful working hypothesis includes transport across the protoplasmic membrane as an integral step in the cell's relocation of these enzymes. Here again, some variation among species is probable, and species with extraordinary physiology may present certain investigative advantages in the elucidation of mechanism.

Saccharomyces rouxii Boutroux (19) exhibits a pronounced delay in the fermentation of sucrose as opposed to glucose or fructose. This is due to the eventual appearance of enzymatic sucrose hydrolysis in aged cultures. Pappagianis and Phaff (12) demonstrated that neither mutational selection nor enzyme induction explain this phenomenon. It was shown (12) instead that *S. rouxii* cells express the ability to hydrolyze sucrose upon aging, or after suffering autolysis, drying, or freezing. We have under-

taken a study of the *S. rouxii* system for its inherent interest and also with a view to a more general description of β-fructofuranosidase synthesis and translocation in *Saccharomyces* species.

MATERIALS AND METHODS

Yeast. A culture of *S. rouxii* was kindly provided by H. J. Phaff. The strain bears the Davis collection number 48-28 and was used by Pappagianis and Phaff (12). The yeast was grown on a gyratory shaker at 30 C in a medium (YM Broth, Difco Laboratories, Detroit, Mich.) containing yeast extract (0.3%) malt extract (0.3%), peptone (0.5%), and glucose (1%). The strain was maintained on YM agar slants.

For some experiments, the yeast was grown in flasks with colorimeter tubes attached as side arms (Bellco Glass Inc., Vineland, N.J.). Growth was monitored turbidimetrically in a Klett-Summerson colorimeter with filter CS 2-59 (Klett Mfg. Co., New York, N.Y.). A standard curve was constructed for the Klett reading versus the yeast concentration (mg dry weight per ml). In selected experiments, the percentage of dead cells was ascertained with the methylene blue test in conjunction with a hemacytometer as described previously (3).

Cultures were harvested by centrifugation and washed twice in 0.25 M sodium acetate buffer (pH 6) by sequential resuspension and centrifugation. Finally, the cells were resuspended in the same buffer to approximately 20% (wet weight/vol).

Cells were disrupted in a French pressure cell (American Instrument Co., Silver Spring, Md.) operated at 20,000 lb/in^2. The supernate from centrifugation at 12,800 × *g* for 15 min was employed and is referred to hereafter as cell-free extract.

Enzyme assay. A unit of β-fructofuranosidase in these experiments is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of raffinose to 2 μmol of reducing sugar (i.e., fructose plus melibiose) per min at 30 C and pH 5.5. Incubations were initiated by adding 1 ml of 100 mM raffinose in 200 mM sodium acetate buffer (pH 5.5) to 1 ml of yeast suspension (typically 4% wet weight/vol). After 1 h at 30 C, the reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent, and reducing sugars were determined spectrophotometrically as previously described (3) except that the samples were cleared by centrifugation after color development. For each enzyme assay, matching controls were performed in which the substrate solution was added at the termination of the 1-h incubation, i.e., an incubated control in the absence of raffinose. The subtraction of the corresponding control value corrected for endogenous reducing groups in the sample as well as for possible reducing groups generated (from sources other than raffinose) during the 1-h incubation. Enzyme concentrations were based on the dry weights of washed yeast samples at time of harvest and are expressed as units per gram. If a series of treatments was performed, then the initial dry weight of the yeast was the point of reference.

Analysis of reaction products. *S. rouxii* cells or solvent-treated cells were incubated at 30 C in the presence of 80 mM raffinose and 10 mM sodium acetate (pH 5.0) At zero time and intervals up to 3 h, samples were withdrawn, expressed through a membrane filter (0.22-μm pore size), and plunged into a boiling water bath for 1 min. After cooling, 20 μliters of each sample was applied to Whatman No. 1 paper and subjected to descending development with 1-butanol-ethanol-water (80:22:38) or ethyl acetate-acetic acid-formic acid-water (80:15:5:20) for 89 and 44 h, respectively (5). Sugars were found with alkaline silver nitrate (18).

Spheroplasts. The cells from a 3-day culture were preincubated for 1 h at 30 C in a medium (14) containing 10 mM dithiothreitol, 1.0 M mannitol, and 100 mM sodium phosphate buffer (pH 6.8). The wall-degrading preparation was a crude solution of snail enzymes (Sigma Chemical Co., St. Louis, Mo.) which had been diluted 10-fold in the above medium. A 2-ml sample of preincubated cells was mixed with 2 ml of diluted snail enzymes and allowed to stand, with occasional manual stirring, for 2 h at 30 C. Spheroplast preparations were harvested by centrifugation, washed in a solution containing 1.0 M mannitol and 100 mM sodium phosphate buffer (pH 6.8), and resuspended to 4% (wet weight/vol) based on the starting yeast concentration.

Yeast cell ghosts. The procedures of Schlenk and colleagues (1, 15, 16) which were developed with *Saccharomyces cerevisiae* and *Candida utilis* were followed in detail. In principle, a relatively small, basic protein is used to disrupt membranes which become sensitized by the removal of protective electrolytes. *S. rouxii* cells from a 3-day culture were thoroughly washed in distilled water and subjected to various treatments with ribonuclease and cytochrome *c* according to the optimal proportions established for *S. cerevisiae* (16).

Autolysis. Organic solvents and toluenethiol were used as initiators of autolysis in 0.25 M sodium acetate buffer (pH 6) as described for *S. cerevisiae* (3, 4). The criterion for enzyme release during autolysis was resistance to centrifugation at 12,800 × *g* for 15 min. The exclusion of cells or debris was assured by forcing the supernate through a membrane filter (0.22-μm pore size).

Standardized ethyl acetate treatment. A 1-ml suspension of cells (20% wet weight/vol) in 0.25 M sodium acetate buffer (pH 6) was mixed with 0.1 ml of ethyl acetate and incubated at 30 C for 15 min. Then 10 ml of the same buffer was added with stirring, and the suspension was centrifuged. The pellet was resuspended by adding 4.8 ml of the same buffer. The final concentrations of these treated suspensions (approximately 4% wet weight/vol) were monitored turbidimetrically on samples diluted 1:5.

RESULTS

β-Fructofuranosidase. Aged cells and cell-free extracts of *S. rouxii* generated reducing sugars from sucrose and raffinose. At 50 mM, sucrose was hydrolyzed at 3.2 times the rate of raffinose. However, because of the relatively low

activity of this species and the potential ambiguity of an attack on sucrose by an α-glucosidase (EC 3.2.1.20, [11]) as well as a β-fructofuranosidase, we elected to use raffinose as substrate for these studies.

The products of raffinose [*O*-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl β-D-fructofuranoside] hydrolysis were established by chromatographic analyses of various incubations. The progressive formation of melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) and fructose is shown in Fig. 1. This indicated the participation of a β-fructofuranosidase. Similar results were obtained with fresh cells from a 5-day culture, and cells pretreated with toluene or ethyl acetate. The two chromatographic solvent mixtures described above yielded consistent analyses.

α-Glucosidase is not indicated because, for example, the purified α-glucosidase of *S. cerevisiae* catalyzes the hydrolysis of sucrose but not raffinose, and the substrate spectrum of that enzyme demonstrates a critical requirement for a terminal, unsubstituted α-glucosyl residue (13). In addition, we showed in separate trials that neither melezitose [*O*-α-D-glucopyranosyl-(1 → 3)-β-D-fructofuranosyl α-D-glucopyranoside] nor methyl α-D-glycopyranoside was

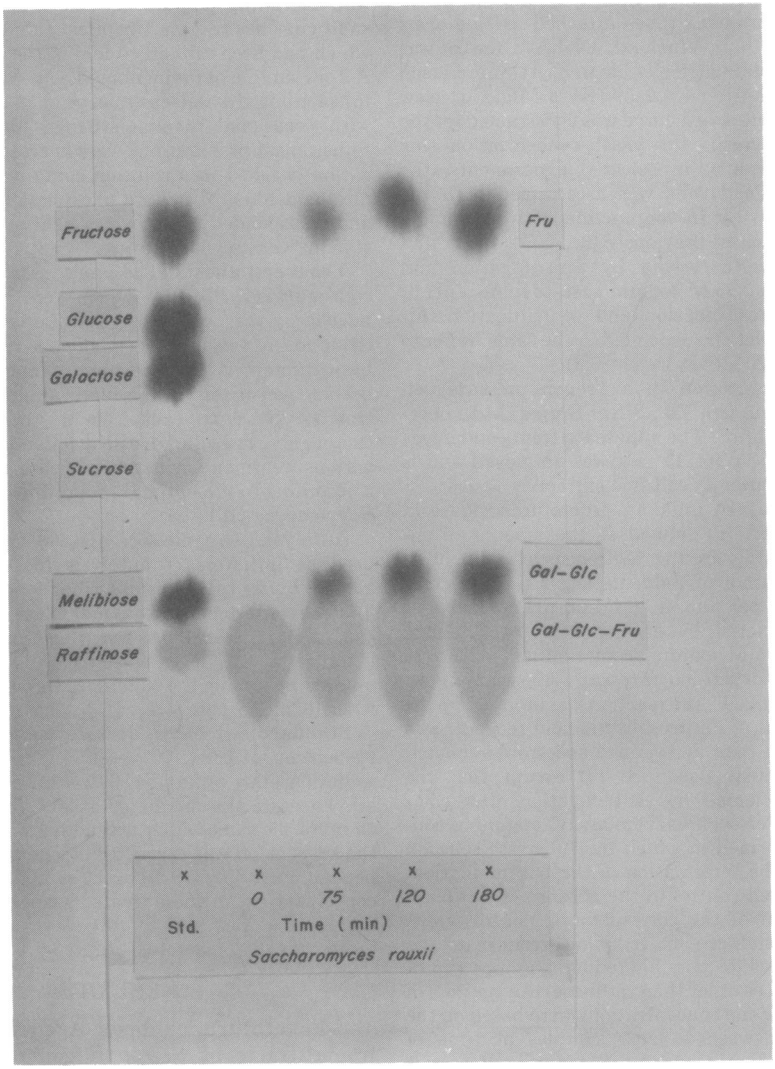

FIG. 1. *Paper chromatogram showing the progressive formation of melibiose and fructose in an incubation of toluenized S. rouxii cells with raffinose. The solvent system was 1-butanol-ethanol-water (80:22:38); descending development took 89 h.*

hydrolyzed to a detectable extent by *S. rouxii* cells during a comparable incubation period.

If an α-galactosidase (EC 3.2.1.22) were present, then raffinose would yield galactose and sucrose, the disaccharide being further hydrolyzed by the indicated β-fructofuranosidase to glucose and fructose. The absence of galactose and glucose in the reaction products (Fig. 1) rules out the participation of α-galactosidase in our assay.

In our enzyme assay, reducing sugar formation from raffinose was linear with time and with amount of *S. rouxii* cells over the employed ranges. Hydrolytic activity was destroyed by 1-min pretreatment in a boiling water bath.

An incubated control (i.e., in the absence of raffinose) was always employed, and this corrected by subtraction for all endogenous compounds reacting with the dinitrosalicylic acid reagent as well as for any reducing groups generated from nonraffinose sources. One product of raffinose hydrolysis is the reducing disaccharide melibiose which is not fermented by *S. rouxii* (Fig. 1, [19]). Further, we were able to quantitatively recover added melibiose from cells of all ages employed in this study (Table 1). The other product, fructose, was of course subject to metabolism; half of the total reducing sugar products would thus be subject to underestimation. Recovery experiments (Table 1) with added fructose demonstrated that this was not a significant problem with older cells or with ethyl acetate-treated cells. For example (Table 1), 4 μmol of fructose after 1 h of incubation with 9-day cells gave an analysis of 4.9 μmol of reducing sugar (i.e., a 3.9-μmol increment over the control). With younger cells (e.g., from a 3-day culture), some fructose was lost to metabolism. However, cells of this age exhibited very low β-fructofuranosidase activity (see raffinose incubation in Table 1, and below), and the values were not significantly increased by the inclusion of 0.03 M sodium fluoride, a known inhibitor of glycolysis. The substrate raffinose was added in large excess and was not significantly depleted as evidenced by semiquantitative paper chromatography. For the above reasons, we have not attempted to correct for the potential underestimation (although small) of enzyme concentration in fresh, younger cells.

Ethyl acetate-treated cells have been assayed against raffinose over a concentration range from 30 to 216 mM. The Michaelis constant (K_m) for this β-fructofuranosidase, acting on raffinose at 30 C in the presence of 100 mM acetate buffer (pH 5.5), was 82.7 ± 4.1 mM. This value and the attendant standard error were computed from the data by a least squares method (21).

TABLE 1. *Control incubations testing the validity of the β-fructofuranosidase assay*

Additions[a]	Reducing sugar (μmol per tube)		
	9-Day cells	3-Day cells	3-Day cells after ethyl-acetate treatment
None	1.0	0.7	1.2
Raffinose (100 μmol)	2.5	0.8	6.0
Raffinose (100 μmol) plus sodium fluoride[b]	2.4	1.1	5.7
Fructose (4 μmol)	4.9	3.2	5.2
Melibiose (4 μmol)	5.1	4.7	5.3

[a] All incubations contained washed *S. rouxii* cells in acetate buffer as described previously. Incubations were for 1 h at 30 C.

[b] Final concentration 0.03 M.

Aging of cells. The β-fructofuranosidase content of 3-day (or younger) cells was always low and typically 0.1 unit/g. Cells expressed increasing activity with further aging. In one trial, for example, the yeast exhibited 1.3 and 2.7 units/g after 5 and 9 days, respectively, on the gyratory shaker. The amount of activity expressed by the amount of aging varied among batches. Our observations suggested that vigorous shaking and a small medium to flask-capacity ratio minimized the degree of enzyme expression at a given age. In no case was significant activity found in the medium of cultures which were examined up to 12 days.

Autolysis. Trials were conducted with the washed cells from a 3-day culture. The cells exhibited 0.1 unit/g at the commencement of the experiment, and, in the absence of additives, this value was constant for 2 days. After 1 day with 0.1 volume of toluene, the cell suspension exhibited 4.4 units/g, which remained unchanged the following day. A few crystals of *p*-toluenethiol were added to another tube and resulted in 2.0 units/g after 1 day and 4.3 units/g after 2 days.

One batch of cells from a 6-day culture had 2.5 units/g at the start of the experiment and 2.8 units/g after sitting in the water bath for 2 days at 30 C. Toluene or *p*-toluenethiol led to the expression of 4.0 to 5.5 units/g after 1 to 2 days, the largest value attained with *p*-toluenethiol after 1 day.

Pappagianis and Phaff (12) allowed cells to autolyze for 3 days in the presence of toluene. They noted the expression of sucrose-hydrolyz-

ing activity at that time and reported no detectable activity in the supernate after centrifugation. We have confirmed these observations, but in addition noted that about 15% of the enzyme was released from the cells after 1 day and that this activity disappears during the next 24 h. With *p*-toluenethiol, about 26% is released after day 1, and this increases to 35% at day 2.

Ethyl acetate and chloroform elicited an unusual response. With cells from a 3-day culture and after 1 day with 0.1 volume of organic solvent, the enzymatic activity was 0.3 units/g, which was not much greater than untreated controls. Repeat experiments, however, showed in a matter of minutes a peak of enzymatic activity, which was followed by a decrease to the low value, at 24 h, already mentioned. Cells from a 6-day culture showed a peak (about 5 units/g) after 30 min, a decline to control values (about 2.5 units/g) at 2.5 h, and a further decrease to less than 0.5 unit/g at 25 h. Insignificant amounts of enzyme were released from the cells by treatment with either chloroform or ethyl acetate. Ethyl acetate was selected for further detailed study.

Characterization of the ethyl acetate treatment. Cells from a 3-day culture were treated with ethyl acetate under a variety of conditions. The results are summarized in Fig. 2; in each panel the values were normalized about the maximum. The standard treatment (0.1 volume ethyl acetate, for 15 min, at 30 C, and pH 6) as described above, was arrived at by varying one parameter at a time (Fig. 2, panels A–D). In this mixture, a 0.086 volume of ethyl acetate correspond to a saturated solution. A presentation time of 15 min was a convenient compromise for handling large numbers of samples. A temperature range from 25 to 30 C was optimal, there was a marked decrease at ice bath temperature, and some enzyme inactivation at 40 C. A pH range of 5.5 to 6.0 was optimal. No activity was expressed at pH 4.5 or lower.

In further experiments, it was found that after enzyme expression at pH 5.5, the ethyl acetate could be washed from the system, and the cells could be resuspended at pH 4.5 and incubated at 30 C for 30 min without change in activity. On the other hand, if ethyl acetate was included during incubation at pH 4.5, then the previously expressed enzyme activity disappeared. Fresh cells which had been preincubated at pH 4.5 in the absence of ethyl acetate were adjusted to pH 5.5, and the full complement of enzyme was then expressed by ethyl acetate treatment at this pH. However, cells that were preincubated with ethyl acetate at pH 4.5 remained inactive when shifted to pH 5.5. Clearly, β-fructofuranosidase was irreversibly inactivated at pH 4.5 in the presence of ethyl acetate. On the other hand, we conclude that enzyme expression, as mediated by ethylacetate, is insensitive to pH.

Equivalent effects of ethyl acetate and mechanical disruption. The standard ethyl acetate treatment and mechanical disruption were compared as methods for β-fructofuranosidase expression (Table 2). The two treatments are clearly equivalent in their ability to expose, or to lead to the expression of cryptic enzyme activity. Mechanical disruption also led to release of the enzyme. Examination under the light microscope showed about 96% broken cells in the case of fresh yeast and 47% with solvent treated cells, which correlated well with the degree of enzyme release. It is worth mentioning that solvent-treated cells of *S. cerevisiae* are also less suceptible to cracking (2).

pH profiles. The effect of varying the pH was tested on aged cells, ethyl acetate-treated cells and a cell-free extract (Fig. 3). The similarity among the three curves is consistent with the expressed enzyme having complete accessibility to the medium.

Yeast cell ghosts. The generation of yeast ghosts by the method of Schlenk is demonstrated by the results in Table 3. The release of ultraviolet-absorbing compounds indicates membrane modification (15). This was not an enzymatic effect (compare denatured ribonu-

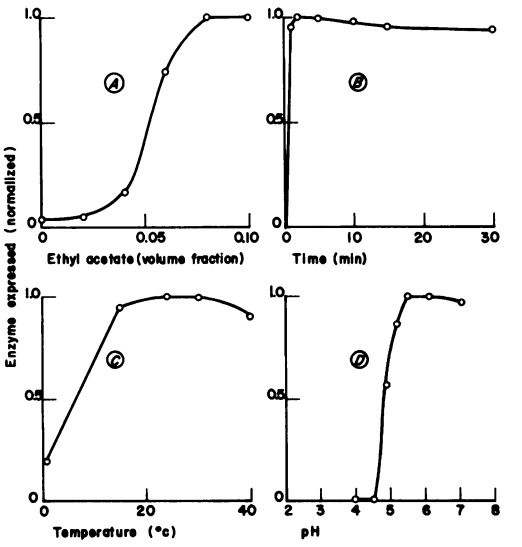

FIG. 2. *Effect of the amount (A); presentation time (B); temperature (C); and pH (D) of the ethyl acetate treatment on the expression of β-fructofuranosidase activity in 3-day S. rouxii cells.*

clease), but was proportional to the amount of ribonuclease, and was negated by the presence of salt (Table 3). We used the standard ethyl acetate treatment to show the full complement of enzyme in each case. However, the inclusion of mannitol (osmoticum) decreased the ribonuclease effect, and washing these cells twice in distilled water (lysis?) still resulted in a suboptimal response (Table 3).

We have observed similar effects with cytochrome *c* (Mann Research Lab., New York). A concentration of 75 μg/ml elicited about half the yield of expressed enzyme as did 100 μg of ribonuclease per ml. We found a binding capacity of about 20 mg of cytochrome *c* per g of moist cells (compare 50 mg/g of cells for *S. cerevisiae* or *C. utilis;* (15)).

Spheroplasts. *S. rouxii* cells were incubated with snail enzymes as described above. Under the light microscope, treated cells exhibited a more spherical form. Lysis occurred in the majority of cells upon dilution in distilled water. This is presumptive evidence for partial conversion to spheroplasts. The expression of β-fructofuranosidase is summarized in Table 4. The results support the conclusion that the enzyme is still cryptic in spheroplasts. The best evidence is the appearance of activity in the supernate after water lysis (Table 4).

It is worth noting that the cells used in this experiment were from the same batch as those used for ghost formation. They had been stored 2 days at 4 C in distilled water which led to the lowering of total enzyme activity to 3.0 units/g. Further storage for 5 days lowered this value still further to 1.7 units/g. The lability of the released enzyme in the presence of ethyl acetate (Table 4) was in agreement with the results of autolysis.

Raffinose was hydrolyzed by the crude snail enzyme preparation. The activity was sufficiently high to suggest that searching for increases from the solubilized cell material would not be meaningful. This aspect awaits a more purified lytic system.

TABLE 2. *Effects of ethyl acetate and mechanical disruption on the expression of cryptic β-fructofuranosidase*

Treatment[a]	β-Fructofuranosidase (units per g dry wt)
Fresh cells (3-day culture)	0.10
Ethyl acetate	4.13
Mechanical disruption	4.24
High-speed supernatant	3.94
Pellet	0.20
Ethylacetate + mechanical disruption	4.12
High-speed supernatant	1.93
Pellet	2.18

[a] Details are given in the text.

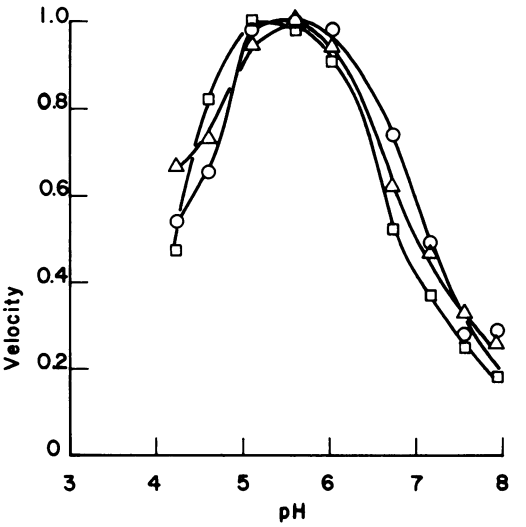

FIG. 3. *Effect of pH on S. rouxii β-fructofuranosidase. Symbols:* ○, *cells from a 6-day culture;* △, *ethyl-acetate-treated cells; and* □, *cell-free extract.*

Effect of glucose concentration in the growth medium. The formation of β-fructofuranosidase in growing cultures of *S. fragilis* (7) or *S. cerevisiae* (8) is repressed by high concentrations of glucose in the medium. YM broth contains 1% glucose together with other carbohydrates derived from the malt extract. We tested glucose concentrations up to 10% (Table 5) in a defined medium (10). There was no evidence of enzyme repression. All of the enzyme was cryptic in 3-day cultures. The 9-day culture in 1% glucose exhibited 90% enzyme expression (compare ethyl acetate treatment).

The cultures which started with 5% and 10% glucose had generally lower activities, which we attribute to inactivation of expressed enzyme in the more acid conditions attained in these media. Confirmatory evidence was obtained with a YM broth culture (14 days old), in which the activities were 4.2 and 5.3 units/g before and after ethyl acetate, respectively. Two-hour incubations in media ranging from pH 6.0 to 3.5 indicated that most of the expressed enzyme was destroyed by pH values of 4.2 and lower, whereas a fairly constant increment of about 1 unit/g was expressed upon ethyl acetate treatment in each case.

Enzyme complement and degree of expres-

TABLE 3. *Effect of ribonuclease on the expression of cryptic β-fructofuranosidase*

Treatment[a]	β-fructofuranosidase (units per g)[b]		Absorbance[c]	
	Initially	After ethyl acetate treatment	260 nm	280 nm
Fresh cells (3-day culture)	0.1	4.9	0.043	0.029
Ribonuclease[d] (50 μg/ml)	1.5	5.8	0.339	0.131
Ribonuclease (100 μg/ml)	3.9	5.8	0.859	0.354
Ribonuclease (200 μg/ml)	5.1	5.7	1.093	0.442
Denatured[e] ribonuclease (100 μg/ml)	4.0	5.8	0.861	0.357
Ribonuclease (100 μg/ml) plus 50 mM Na_2HPO_4 (pH6)	0.1	5.4	0.026	0.001
Ribonuclease (100 μg/ml) plus 1 M mannitol	1.6	4.9	0.639	0.261
The above, after lysis by dilution	2.5	5.9	0.123	0.066

[a] Each treatment contained 2 ml of cells (20% wet wt/vol) which had been thoroughly washed in distilled water and then resuspended to 100 ml with additives as indicated.
[b] Treated cells were concentrated 10-fold prior to assay.
[c] The absorbance of the high-speed supernate was measured in a 1-cm cuvette. The absorbance of a ribonuclease control was subtracted where appropriate.
[d] Crystalized from ethanol (Worthington Biochemicals Corp., Freehold, N.J.).
[e] Boiling water bath for 5 min.

TABLE 4. *Expression of β-fructofuranosidase in spheroplasts*

Treatment	β-Fructofuranosidase (units per g)	
	Initially	After ethyl acetate treatment
Cells[a] washed in:		
1.0 M mannitol-phosphate buffer	0.1	3.0
distilled water:	0.4	
pellet supernatant	0	
Spheroplasts[b] washed in:		
1.0 M mannitol-phosphate buffer	0.1	
distilled water:	0.1	
pellet supernatant	2.6	2.0

[a] Cells were incubated in dithiothreitol-mannitol-phosphate as described earlier.
[b] Spheroplasts were prepared with snail enzymes as described earlier.

TABLE 5. *Effect of glucose concentration on growth of S. rouxii and the expression of β-fructofuranosidase activity*

Age of cells	Percent of glucose in medium[a]	Yield (mg [dry wt] per flask)[b]	pH of medium at harvest	β-fructofuranosidase (units per g [dry wt])	
				Initially	After ethyl acetate treatment
3 days	0	0	5.48		
	1	109	4.81	<0.1	4.86
	5	257	3.90	<0.1	4.94
	10	338	3.40	<0.1	5.28
9 days	0	0	5.46		
	1	106	4.87	2.72	3.04
	5	261	4.01	0.25	1.57
	10	332	3.69	0.12	0.61

[a] The synthetic medium of McMurrough and Rose (10) was used with 1% (wt/vol) ammonium citrate and sodium hydroxide was added to pH 5.5.
[b] Each flask contained 40 ml of medium.

sion. Many observations that bear on this point were made throughout the course of these studies. The experimental results in Fig. 4 are representative of the main features. Stationary phase was attained after about 3 days. The total enzyme content is 4 to 5 units/g as revealed by ethyl acetate and changed little between 3 and 12 days.

The degree of expression increased gradually with aging (Fig. 4) but there was considerable variation in magnitude, as previously mentioned.

Mechanism of expression. A series of experiments was performed with cells from cultures of 3-, 6-, and 9-days duration. The degree of enzyme expression in these ranged from 4% to 22%. Cell-free extracts were prepared from each.

Cells from the 3-day culture were incubated for 2 h at 30 C and for a further 16 h at 4 C with

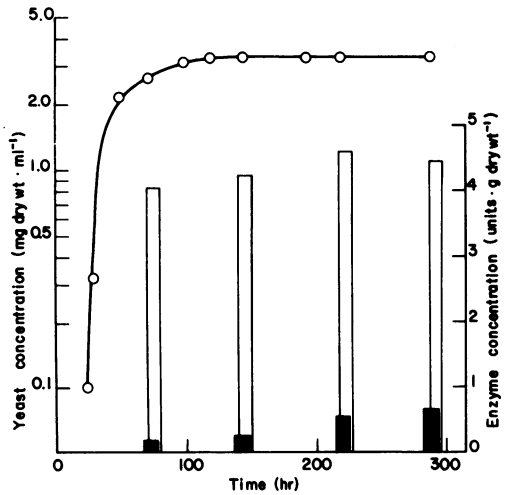

FIG. 4. *Growth, enzyme content, and degree of β-fructofuranosidase expression in S. rouxii. Yeast was grown in flasks containing 50 ml YM broth. Growth (O) was monitored turbidimetrically. The filled bars represent initial enzyme activity. The open bars represent the activity after the standard ethyl acetate treatment.*

media from each of the cultures. There was no activity in the media nor did they lead to the expression of activity in the 3-day cells. The cell-free extracts, which contained most of the activity of the cells from which they were derived, were without effect on the expression of 3-day cells.

In two series of cultures the percentage of dead cells was measured by the methylene blue test. Five other samples, which had been stored at 4 C for various times, were included in an attempt to correlate the percentage of dead cells in a given batch with the degree of enzyme expression (assuming ethyl acetate treatment yielded 100% in each case, Fig. 5). A direct proportionality was indicated in samples with a low dead-cell count (i.e., cells from younger cultures); however, upon further aging the degree of expression seemed to be greater than the death rate.

DISCUSSION

We identified a β-fructofuranosidase in *S. rouxii* by its substrate specificity. There was a very low concentration of this enzyme in this species in comparison with *S. cerevisiae*, which can have 3 orders of magnitude more activity on a dry weight basis (3). However, the use of raffinose as assay substrate was shown to preclude potential interference by other enzymes.

β-Fructofuranosidase was cryptic in cells from a 3-day culture of *S. rouxii* but becomes expressed upon aging. At first, this was approximately proportional to the number of dead cells, but later the degree of expression exceeded the death rate. After the enzyme was expressed, it was fully accessible to the medium as judged from pH activity profiles and increased susceptibility to inactivation at pH values below 4.5.

Cryptic enzyme was revealed by short treatments with ethyl acetate or chloroform, although the enzyme was not released from the cell. Enzyme expression by ethyl acetate was shown to be insensitive to pH (5.5 to 7.0), complete in 2 min at 30 C, and relatively insensitive to temperature in the range of 15 to 40 C (although at 0.5 C the rate was about fivefold slower). The dose response curve for ethyl acetate was sigmoidal and reached a maximum at a concentration approximating a saturated solution. The combined evidence indicated that the action of ethyl acetate was to break a barrier (supposedly including a lipid component) which separates enzyme from substrate in the untreated cell. The intermediation of enzymatic steps such as activation of a zymogen form of β-fructofuranosidase or partial autolysis of the cell wall was not suggested by our data.

This work also confirmed and extended sev-

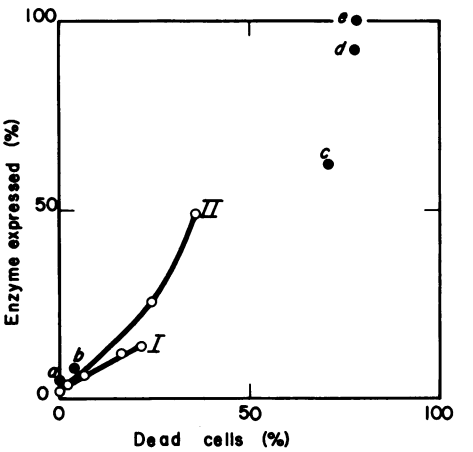

FIG. 5. *Relationship between degree of enzyme expression and death rate. Curve I: cells were grown in 50 ml of medium per flask, stirred vigorously, and assayed on the day of harvest. Analyses were performed on samples on day 3, 6, 9, and 12. Curve II: cells were grown in 500 ml medium per flask, stirred moderately, and assayed on the day of harvest. Analyses were performed on samples on day 3, 5, and 9. Points a–e refer to cells which were stored at 4 C after harvest. At point a: 2-day culture with 24-days storage; b: 3 with 46; c: 9 with 18; d: 14 with 35; and e: 9 with 40.*

eral results from an earlier study by Pappagianis and Phaff (12) on the delayed fermentation of sucrose by *S. rouxii*. They suggested that the cell wall undergoes a rather abrupt change in permeability upon aging. Our characterization of the β-fructofuranosidase system and its mode of expression now shows that a membrane system is modified rather than the cell wall. In particular, the enzyme is still cryptic in spheroplasts in which the cell wall is for the most part removed. Moreover, in the generation of ghosts by the Schlenk technique (15), the cell wall is obviously penetrated by small proteins (which are much larger than the substrate raffinose), and the disruption of some membrane system leads to the expression of β-fructofuranosidase activity. In ghosts, the enzyme is still restrained from diffusion into the medium. The French pressure device clearly breaks both membrane and cell wall, leading to expression plus release of the enzyme.

Our working hypothesis is that the cryptic β-fructofuranosidase is either surrounded by the protoplasmic membrane (i.e., located in the cytoplasm) or that it has some special vesicular location in the vicinity of the protoplasmic membrane. By analogy with a periplasmic location in *S. cerevisiae*, we speculate that the enzyme is in a penultimate transport stage in 3-day *S. rouxii* cells. The process is brought to fruition by aging or artificially by ethyl acetate treatment. Supportive evidence awaits a thorough study of ultrastructure in cells of different ages. An electron microscopy study is now in progress.

S. rouxii is an osmophilic yeast; the present strain was isolated by Phaff from dried prunes. The function of the cryptic β-fructofuranosidase in these cells is at first sight paradoxical when we consider that the natural environment typically has a high concentration of sucrose (plant sources). Perhaps in this ecological niche the species finds some survival value in initially metabolizing other components in the medium. After maturation or death of a fraction of the population, the sucrose would then be hydrolyzed and the osmotic pressure gradually lowered, thus delaying the competition.

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