THE IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF SOME CARBOHYDRASES PRESENT IN DEBARYOMYCES HANSENII

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ABSTRACT

The soluble carbohydrases α-D-galactosidase, β-D-fructofuranosidase, and trehalase were isolated from *Debaryomyces hansenii*. About 120-fold and 50-fold purifications were obtained with β-D-fructofuranosidase and trehalase, respectively. Broad optimal pH's existed around pH 5 for both of these enzymes. The Michaelis constant for β-D-fructofuranosidase was 0.017 M (0.1 M acetate, pH 5.5, 37°C, with sucrose as the substrate).

INTRODUCTION

Debaryomyces hansenii is a sporogenous yeast capable of reproducing itself in aqueous solutions nearly saturated with sodium chloride at temperatures near the freezing point of water. Occasionally it finds its way from its marine habitat into a variety of food products via the production of solar salt.

Merdinger *et al*. have investigated the nutritional requirements, the lipid composition, and the response to various anesthetics of this organism (Merdinger and Shair, 1962; Merdinger, 1965; Merdinger and Devine, 1965; Merdinger and Frye, 1966; Merdinger, Guthmann and Mangine, 1969). The purpose of this investigation was to isolate and characterize some of the carbohydrases present in uninduced *Debaryomyces hansenii*.

MATERIALS AND METHODS

The yeast was grown under aeration in a fermenter at 30°C in an aqueous solution at pH 5.5. Each 100 ml contained the following: 2.0 gm glucose, 1.5 gm NaCl, 0.5 gm $(NH_4)_2SO_4$, 0.3 gm malt extract, 0.3 gm yeast extract, 0.1 gm asparagine, and a mixture of nutrient salts containing lesser amounts of K, Mg, Ca, Fe, Cu, An, I, and P. After harvesting by centrifugation and checking microscopically for purity, cells were suspended in 0.1 M acetate, pH 5.5, and ruptured at 5°C for 35 minutes with 125 μm diameter glass beads in a Sorvall Omni-mix. Microscopic examination revealed greater than 90% breakage of cells.

This homogenate was centrifuged at 600 x g for 20 minutes. The sediment was then subjected to 25,000 x g for 30 minutes, the supernatant liquids combined, and the sediment discarded. Differential centrifugation was used to sediment different cell organelle types. The final centrifugation (120,000 x g for 2 hours) yielded a supernatant liquid containing only the soluble proteins.

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Precipitation of some of these proteins was achieved by the slow addition of ammonium sulfate to a portion of the 120,000 x g supernatant liquid with stirring at 5°C. When the concentration reached 40% of saturation, the stirring was continued for one hour. The suspension was then centrifuged at 25,000 x g for 20 minutes at 5°C and the supernatant liquid decanted. This procedure was repeated two additional times for 70% and 100% levels of saturation.

Further purification was attempted on a 20 by 240 mm column packed with diethylaminoethyl cellulose (DEAE-cellulose) which had been equilibrated with 0.01 M acetate, pH 7.0 (Lange, Lee, and Merdinger, 1969). Four hundred mg of dialyzed and lyophilized material from the supernatant liquid resulting from the 70% level of saturation with ammonium sulfate was dissolved in 0.1 M acetate, pH 7.0, layered on the column, and eluted in a stepwise fashion with the solutions listed in Table 1. Protein elution was detected by noting the absorbance at 280 mu.

TABLE 1. Eluting solutions used in DEAE-cellulose column chromatography.

pH	Molarity of acetate	Mg lyophilized material recovered* (%)	Enzymes detected
7.0	0.01		
6.5	0.02	171 (43.2)	----
6.0	0.03		
5.5	0.04		
5.0	0.06		
4.5	0.08	9 (2.3)	----
4.0	0.10		
3.5	0.12		
3.0	0.25 (plus 1.0 M NaCl)	13.3 (3.4)	α-D-galactosidase β-D-fructofuranosidase trehalase

*396 mg present initially, total recovery 48.8%

Purification was also attempted using a batch fractionation procedure (4). Three hundred mg of dialyzed and lyophilized material from the 120,000 x g supernatant liquid was dissolved in 20 ml of 0.01 M phosphate, pH 7.8, and added to 1.5 gm wet weight of DEAE-cellulose which had been equilibrated in the same solution. The suspension was stirred for one hour at 5°C, filtered with suction, washed with 5 ml of the solution, and resuspended in

20 ml of the next eluting solution as presented in Table 2. The stirring, filtering, and washing was repeated with each of the succeeding eluants. The absorbance at 280 mμ was used as a measure of protein concentration.

TABLE 2. Eluting solutions used in the DEAE-cellulose batch fractionation procedure.

pH	Molarity of phosphate	Units of protein* (ml x A_{280})	Enzymes detected
7.8	0.01	183	---
7.0	0.02	22.5	---
6.0	0.03	3.5	---
5.0	0.05	2.9	---
4.0	0.5	22.6	{α-D-galactosidase β-D-fructofuranosidase
4.0	0.5 (plus 0.5 M NaCl)	15.5	---

*236 units present initially

Appropriate dialyzed fractions were assayed for carbohydrase activity using the following thirteen carbohydrates: inulin (Fischer Scientific Company, Fair Lawn, N. U.), cellobiose, gentiobiose, maltose, melezitose, methyl-α-D-glucoside, and stachyose (Mann Research Laboratories, N. Y., N. Y.), methyl-β-D-glucoside, sucrose, lactose and trehalose (Difco Laboratories, Detroit, Mich.). Assays were done in 0.10 M acetate, pH 5.5, at 37°C for 30 and 60 minute intervals. The concentration of the nonreducing sugars was 0.58 M, whereas lower concentrations of the reducing sugars were employed. The amount of reducing sugar was determined by the Folin-Wu method (Folin and Wu, 1920). The purification of the two enzymes were followed quantitatively using sucrose and trehalose as substrates. A unit of enzyme activity was defined as the amount of enzyme which hydrolyzed on μmole of carbohydrate per minute. Specific activity was stated as enzyme units per mg of protein. Protein was determined by the method of Lowry et al. (Lowry, Rosebrough, Farr, and Randall, 1951).

Michaelis constants were determined by the method of Lineweaver and Burk (1934). Sucrose and trehalose were the substrates in 0.1 M acetate, pH 5.5, incubated at 27°C for 60 minutes. These sugars were also used to determine pH optima by incubating 0.58 M concentrations in 0.1 M acetate at 25°C for 60 minutes.

RESULTS

Enzyme assay following differential centrifugation detected carbohydrases only in the 120,000 x g supernatant liquid. Those present in this fraction were estimated to be of 30-fold greater purity than the original homogenate. Of the carbohydrates tested, activity was detected with melibiose, raffinose, stachyose, sucrose, and trehalose. No activity was detected with cellobiose, gentiobiose, inulin, lactose, maltose, melezitose, methyl-α-D-glucoside, and methyl-β-D-glucoside.

Treatment with ammonium sulfate failed to precipitate any carbohydrases. The 100% of saturation supernatant liquid was 50% less active with sucrose (11% yield) and 50% more active with trehalose (33% yield) when compared to the 120,000 x g supernatant liquid.

DEAE-cellulose column chromatography gave no resolution of enzyme activities with all carbohydrases being eluted with the final solution (0.25 M acetate, 1.0 M NaCl, pH 3.0). The assays with sucrose and trehalose indicated the chromatography had increased the respective specific activities 50 and 60%.

The batch fractionation procedure with DEAE-cellulose gave some resolution. All detected carbohydrases were eluted with one solution (0.50 M phosphate, pH 4.0). This fraction could catalyze the hydrolysis of sucrose (400% increase in specific activity over the 120,000 x g supernatant liquid, 25% yield) and melibiose, but not trehalose.

The Michaelis constant using sucrose as a substrate was 0.017 M. An accurate determination of this constant with trehalose was not possible due to the limited sensitivity of the Folin-Wu method. It was estimated to be between 0.0002 and 0.0020 M. Both determinations were made in 0.10 M acetate, pH 5.5, at 37°C.

There was a broad pH optimum (90% of maximal velocity) from pH 3.6 to 5.0 with sucrose as the substrate. That for trehalose was between pH 4.6 and 5.9. Both optima were determined in 0.10 M acetate containing 0.58 M substrate concentrations at 25°C (Fig. 1 and 2).

CONCLUSION AND DISCUSSION

There are at least seven different enzymes that could have catalyzed the hydrolysis of the carbohydrates assayed (Table 3). Of these, inulase (International identification no. 3.2.1.7), α-D-glucosidase (3.2.1.20), β-D-glucosidase (3.2.1.21), and β-D-fructofuranosidase (3.2.1.26), and trehalase (3.2.1.28) were detected.

The failure of ammonium sulfate to precipitate carbohydrases was not unexpected since they tend to be glycoproteins.

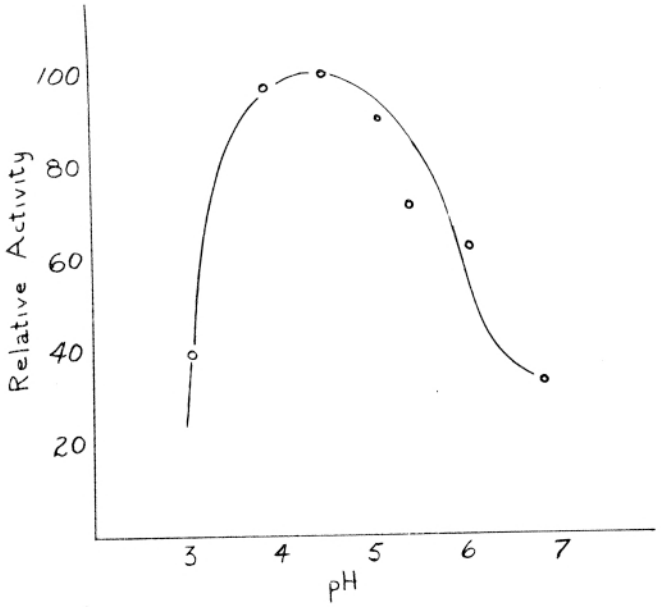

Fig. 1. pH curve with sucrose

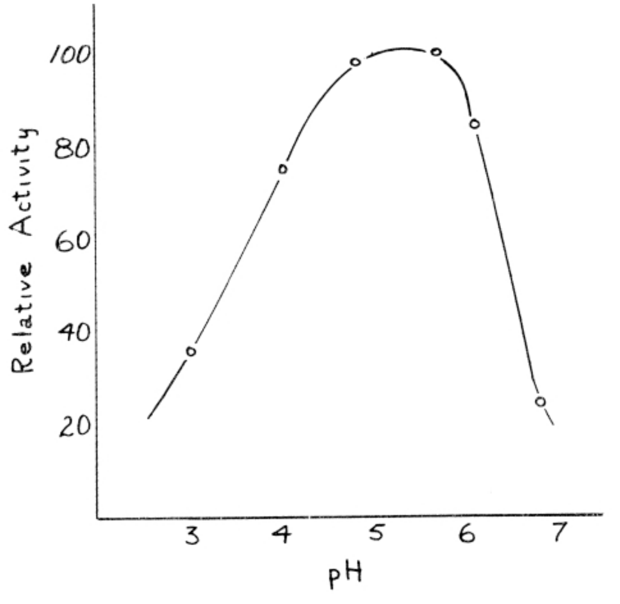

Fig. 2. pH curve with trehalose

TABLE 3. Carbohydrates assayed and their types of glycosidic linkages.

	α-D-glucoside	β-D-glucoside	α-D-galactoside	β-D-galactoside	β-D-fructofuranoside
1. cellobiose		X			
2. gentiobiose		X			
3. inulin	X				X
4. lactose				X	
5. maltose	X				
· 6. melezitose	X				X
*7. melibiose			X		
8. methyl-α-D-glucoside	X				
9. methyl-β-D-glucoside		X			
*10. raffinose	X		X		X
*11. stachyose	X		X		X
*12. sucrose	X				X
*13. trehalose	X				

*Carbohydrates hydrolyzed

he only resolution of enzymes noted was with the batch fractionatio ure in which trehalase was removed from β-D-fructofuranosidase and actosidase. The acidity and/or phosphate may have inactivated the se.

The pH optima (about 5) and the Michaelis constants for the β-D-fructofuranosidase and trehalase were not very different when compared to those of other yeasts and molds (Andersen, 1960; Fischer and Kohtés, 1951;Merdinger, Lange, and Booker, 1971; Metzenberg, 1963; Panek and Souza, 1964).

The earlier study by Merdinger et al. with the yeast Pullularia pullulans achieved much greater purification of trehalase than the present one with Debaryomyces hansenii -- 800-fold versus 50-fold. The earlier purification was achieved using ammonium sulfate fractionation and DEAE-cellulose column chromatography with phosphate eluants (Merdinger, Lange, and Booker, 1971).

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