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Properties of an Extracellular β-Galactosidase Secreted by *Neurospora crassa*

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An extracellular β-galactosidase with an activity optimum at about *p*H 4 was found to occur in the filtrates of *Neurospora crassa* strain 74A when this mold was grown on certain sugars. This activity accounted for a substantial portion of the β-galactosidase activity in the culture. The β-galactosidase in the medium appeared to be a secreted, extracellular enzyme, not a product of cell lysis. The extracellular activity was found to have physical and kinetic properties similar to those of an intracellular β-galactosidase previously found in *Neurospora*. Some conditions for the production and concentration of the enzyme are described.

The occurrence of β-galactosidase activity in *Neurospora crassa* has been determined by several workers (3, 9, 13), and it has been demonstrated that at least two distinct proteins exhibit this type of enzymatic activity. The distinctiveness of these two activities has been established (2, 3, 13) on the basis of several criteria including kinetics of activity, effects of various compounds and treatments as activators and inhibitors, physical separation, and the factors affecting the rates of formation of these enzymes. These β-galactosidases can be distinguished most simply by their *p*H optima, one exhibiting optimal activity at about *p*H 4 and the other at *p*H 7.

Studies of these enzymes have been concerned exclusively with activities obtained from cell extracts or fractions thereof. However, it was recently found in this laboratory that considerable β-galactosidase activity is present in the extracellular fluid of cultures of *N. crassa* grown under certain conditions. The occurrence of extracellular β-galactosidase activity raises questions about its origin and identity which, in addition to their intrinsic interest, may have important bearing on considerations of the function(s) and the regulation of formation of β-galactosidase activities in *N. crassa*. The present report considers the relationship between intracellular and extracellular β-galactosidase activities and the origin of the extracellular enzyme. A method for the concentration of β-galactosidase from culture fluids is described.

MATERIALS AND METHODS

Organism. *N. crassa* strain 74A was the organism used.

Culture. Vogel's medium N (18) was used; chloramphenicol, 5 μg per ml, was added to prevent bacterial contamination. Cultures were grown at 30 C under forced aeration or with shaking, as will be described.

Enzyme assay. β-Galactosidase activity was determined by use of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The volume of the reaction mixture was 2.5 ml, containing 5 μmoles of ONPG and 1.5 ml of McIlvain's standard citrate-phosphate buffer. Incubation was carried out at 37 C, and the reaction was terminated with the addition of 2.5 ml of 0.5 M K_2CO_3. The amount of *o*-nitrophenol (ONP) released was measured with a Klett-Summerson colorimeter equipped with a no. 420 filter. Under these assay conditions, 400 colorimeter units correspond to 1.0 μmole of ONP released. A unit of β-galactosidase activity is designated as the release of 1.0 nmole of ONP per hr under the above conditions. Specific activity is designated as the nanomoles of ONP formed per hour per milligram of protein.

Enzyme extraction. Samples of mycelium were collected by vacuum filtration, washed with cold distilled water, blotted, cut into strips, and lyophilized. Large samples were ground in a Wiley mill equipped with a 60 mesh screen; for small samples, the Fankauser method as described by Carsiotis and Lacy (6) was used. The powdered mycelium was extracted with 0.9% NaCl (50 ml per g of dry mycelium); the suspensions were held at 0 C for 60 min with frequent stirring and then were centrifuged for 40 min at 18,800 × *g*. The resulting supernatant fluid was then assayed in the manner described above. Extracellular activity was determined in untreated culture filtrates.

Preparation of intracellular pH 4 enzyme. Dry, powdered mycelia were extracted as described above, and the extracts were centrifuged at 27,000 × *g* for 15 min to remove cell fragments. The following fractionation was carried out at 2 to 4 C. Saturated ammonium sulfate solution was added slowly to the supernatant fluid with constant stirring until 60% saturation was

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reached. Stirring was continued for 1 hr and then the suspension was centrifuged. The precipitate was discarded, and the supernatant fluid was brought slowly to 80% saturation. The stirring and centrifugation were repeated, and the precipitate was taken up in saline. The above fractionation was then repeated on the saline suspension; the 80% ammonium sulfate precipitate was dissolved in saline (about one-fiftieth the volume of the original extract). The preparation obtained had no appreciable β-galactosidase activity at *p*H 7.

β-Galactosidase induction. The following sugars were examined as inducers: D-arabitol, L-arabitol, L-arabinose, D-cellobiose, L-fucose, D-galactose, D-galactitol, D-lyxose, and D-melibiose (Nutritional Biochemicals Corp., Cleveland, Ohio); D-arabinose, lactose, D-ribose, D-xylose, and L-xylose (Mann Research Laboratories, Inc., New York, N.Y.); D-glucose and sucrose (Mallinckrodt Chemical Works, St. Louis, Mo.); and D-fructose (Matheson, Coleman & Bell, Norwood, Ohio). Germinated conidia (10) were used in the induction studies. A 3.0-liter Fernbach flask containing 1,000 ml of medium N and 1% glucose was inoculated with 2.5 $\times 10^7$ conidia per ml and was incubated at 30 C for 24 hr on a rotary shaker. Germinated conidia were harvested by centrifugation, washed with water, resuspended in 1,000 ml of medium N (without a carbon source), and shaken for 3.5 hr at 30 C. Harvesting and washing were repeated at the end of this time, and the germinated conidia were resuspended in double-strength medium N. A 15-ml amount of this suspension was used to prepare cultures with a final volume of 30 ml in 125-ml Delong flasks, with the sugar concentration 0.1 M. The flasks were incubated at 30 C for 20 hr in a reciprocating water bath shaker. The cultures were harvested by filtration and assayed for β-galactosidase activities as described above.

Sucrose. Sucrose was measured by treatment with invertase (Difco, analytical grade) and determination of the glucose released with Glucostat (Worthington Biochemical Corp.).

Protein and RNA. Protein was determined by the method of Lowry et al. (15), with crystalline bovine serum albumin (Nutritional Biochemicals Corp.) as a reference standard. Ribonucleic acid (RNA) was measured by an orcinol method (5), with yeast RNA (Nutrional Biochemicals Corp.) as a standard.

Gel electrophoresis. Electrophoresis was carried out at *p*H 8.7 with tris(hydroxymethyl)aminomethane (Tris)-glycine buffer (0.005 M in Tris). Polyacrylamide columns were prepared in tubes 5 mm (inner diameter) by 100 mm. All electrophoresis was carried out at 4 C. A Heathkit power supply model IP-32 was used with a current of 1 ma per tube. The location of β-galactosidase activity in the gels was determined by incubation at 37 C in 0.001 M bromonaphthol-β-D-galactoside for 30 to 60 min with subsequent staining of the insoluble bromonaphthol released with diazo blue B to give a brown color. Protein was determined by staining with naphthol blue; gels were destained electrophoretically in 7% acetic acid.

Sucrose gradients. Linear 5 to 20% sucrose gradients were made with a Buchler gradient maker at 4 C. Onto 4.8 ml of sucrose gradient was layered 0.15 ml of β-galactosidase in 0.9% NaCl. Centrifugation was carried out in a Spinco model L ultracentrifuge equipped with a SW-39 rotor at a speed of 35,000 rev/min. The bottoms of the tubes were punctured, and 15-drop fractions were collected. Recrystallized bovine liver catalase (grade A, Calbiochem) with a molecular weight of 248,000 (17) served as a standard for molecular weight estimations. Catalase was identified by its absorbance at 404 nm. None of the *Neurospora* enzyme preparations showed any appreciable absorbance in this region. The catalase preparation had no β-galactosidase activity.

Gel chromatography. The elution and preparation of all columns was carried out in one-tenth strength McIlvain's *p*H 4 buffer. Gels were allowed to equilibrate with the buffer before columns were poured, and samples were dialyzed against the same buffer before being put on the column. Sephadex G-200 and Bio-Gel 300 were used. The void volume of the Sephadex column was determined by use of Blue Dextran 2000 (Pharmacia); the dextran in eluate fractions was measured with a Klett-Summerson colorimeter equipped with a no. 600 filter. All chromatographic procedures were carried out at 4 C.

RESULTS

β-Galactosidase induction. Table 1 shows the occurrence of β-galactosidase activity in cultures grown on various sugars. Extracellular activity accounted for a significant portion of the total *p*H 4 activity of the cultures, even with this rela-

TABLE 1. *β-Galactosidase induction with various sugars*

Sugar (0.1 M)	Dry wt[a]	Enzyme activity[b]			
		Intracellular		Extracellular	
		*p*H 4	*p*H 7	*p*H 4	*p*H 7
Inoculum[c] ..	166	0	4,580	—	—
Control[d]	165	0	3,130	0	0
D-Arabitol ..	210	0	3,410	0	0
L-Arabitol ..	183	4,830	4,360	3,600	0
D-Arabinose .	198	0	3,460	0	0
L-Arabinose .	220	15,650	8,500	7,360	0
D-Cellobiose .	421	0	78,800	0	0
D-Fructose ..	346	0	5,190	0	0
L-Fucose	182	0	4,100	0	0
D-Galactose .	210	1,840	6,050	750	0
D-Galactitol (dulcitol) ..	173	2,390	3,800	2,100	0
D-Glucose ...	330	0	5,350	0	0
Lactose	214	795	7,660	450	0
D-Lyxose ...	207	0	2,340	0	0
D-Melibiose .	166	0	2,830	0	0
D-Ribose	194	0	2,950	0	0
Sucrose	415	0	9,350	0	0
D-Xylose	297	3,500	6,330	900	0
L-Xylose	195	0	2,950	0	0

[a] Milligrams per 30 ml of culture.
[b] Units per 30 ml of culture.
[c] Activity and dry weight before incubation.
[d] Incubated without sugar in medium N only.

tively short incubation period of 20 hr. It may be noted that differential induction of the *p*H 4 and *p*H 7 intracellular enzymes did occur, as in the case of D-cellobiose. Also, the ratio of intra- to extracellular *p*H 4 enzyme in cultures was not constant, but appeared to depend on the sugar used. No extracellular *p*H 7 activity was found, regardless of the level of intracellular enzyme.

Appearance of β-galactosidase activity in culture filtrates. The *p*H 4 β-galactosidase activities in the cells and in the filtered culture medium were examined to determine the relative amounts of these enzymes during cell growth. One liter of Vogel's medium N containing 0.75% L-arabinose and 0.5% sucrose was inoculated with 2.5×10^7 conidia per ml, and the culture was incubated in a rotary water bath at 30 C. Cells and culture fluid were collected periodically and assayed for β-galactosidase activity. As can be seen in Fig. 1, significant β-galactosidase activity appeared in the medium and surpassed the intracellular activity at about 14 hr. The extracellular activity eventually reached a level approximately three times that of the intracellular activity. At no time was *p*H 7 activity found in the medium.

Origin of the β-galactosidase activity in culture filtrates. The question of whether or not the appearance of the activity in the culture filtrate was due to cell lysis was next considered. The observation (Fig. 1) of activity in the medium early in the culture period while the cells are still actively growing tends to contraindicate cell lysis as a source of the activity. The absence of extracellular *p*H 7 β-galactosidase in cultures grown under various conditions (Table 1 and Fig. 1) also suggests that gross cell lysis does not account for the presence of *p*H 4 β-galactosidase in the culture filtrate.

Other intracellular markers were examined to estimate the extent of cell lysis in cultures grown on 0.5% sucrose plus 0.75% L-arabinose. Analyses for RNA in the culture filtrates were made according to the method of Brown (5). The filtrates of 30-hr cultures were found to contain 7 μg of orcinol reacting material per ml. As about 480 μg of RNA may be calculated (11) for the mycelia in the same volume, it may be estimated that less than 1.5% of the cells in the culture underwent lysis. Likewise, it was estimated that the protein in the filtrate was less than 1% of the amount found intracellularly.

A qualitative estimation was also made of the types of proteins in the mycelium and in the medium by gel electrophoresis. Samples of concentrated medium protein and crude extracts of mycelia were compared. (The method of concentration of the medium is described in the next section.) When equivalent amounts of protein were run, 5 distinct bands were discernible in the medium samples but more than 30 were observed in cell extracts (Fig. 2). This also indicates that extensive lysis did not occur.

Concentration of β-galactosidase activity in the culture filtrate. The extracellular β-galactosidase activity was concentrated by means of osmotic ultrafiltration. Polyethylene glycol with a molecular weight of 15,000 to 20,000 (Carbowax 20M, Union Carbide Corp.) was used to reduce the water content of the culture filtrates. The typical concentration of a 15-liter batch of culture filtrate was accomplished as follows. Dialysis tubing was laid in a trough [6 inches (15 cm) wide, 4 inches (10 cm) deep, and 72 inches (183 cm) long] and covered with Carbowax. The medium was circulated through the tubing by means of a centrifugal action pump; 12 ft (3.7 meters)

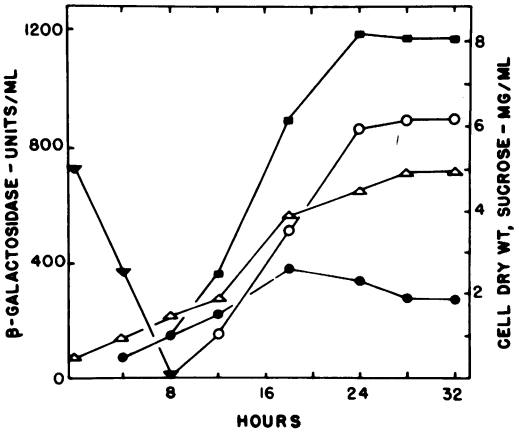

FIG. 1. *Formation of pH 4 β-galactosidase during growth. Dry weight of cells (Δ), sucrose in culture fluid (▼), intracellular β-galactosidase (●), extracellular β-galactosidase (○), total β-galactosidase (■). See text for growth conditions.*

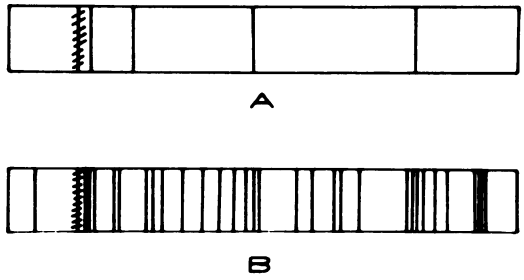

FIG. 2. *Gel electrophoresis patterns in proteins in concentrated culture filtrate (A) and in crude cell extract (B). The diagram represents only the position of discernible bands, not their intensity. The cross-hatched area is the location of pH 4 β-galactosidase. The preparations applied contained about 125 μg of protein. The origin is at the left; the running time was 8 hr.*

of tubing [2.25-inch (5.7-cm) flat width] was used. Water-laden Carbowax was removed at intervals and replaced with fresh material. The concentration was carried out at 4 C; a reduction of the volume from 15 liters to about 150 ml was achieved in about 36 hr. The concentrate was then placed in a smaller section of tubing, and water reduction was continued to a volume of about 50 ml. The concentrate was then centrifuged at 27,000 × *g* for 30 min. The sediment from this centrifugation contained 90% of the original activity of the culture, and it was resuspended in 0.9% NaCl to a final volume of less than 5 ml. Table 2 shows the results of one such concentration procedure. Although some counterdialysis of small polyethylene glycol fragments does take place (8), this material seemed to have no effect on the β-galactosidase activity. The high rate of recovery with this process indicates its possible usefulness in the concentration of other extracellular enzymes.

Comparison of intracellular and extracellular pH 4 activities. The intracellular and extracellular *p*H 4 β-galactosidase activities were examined to determine their relationship. Ultracentrifugation was employed to determine the molecular weights of the two enzymes, with the use of the estimation method of Ames and Martin (1). The enzymes were run separately and in equal activity mixtures. The results of a typical run are illustrated in Fig. 3. When bovine liver catalase was used as a standard, the molecular weight of each of the enzymes was determined to be about 106,000; no peak splitting was observed in the case of the mixture. This value of 106,000 is consonant with the molecular weight of 96,000 reported by Bates and Woodward (3) for the intracellular *p*H 4 enzyme.

The intracellular extract and the concentrated extracellular activity were also run separately and in equal activity mixtures on Sephadex G-200 and on Bio-Gel 300 columns. On both types

TABLE 2. *Concentration of β-galactosidase activity in a culture filtrate*[a]

Vol (ml)	Activity (units/ml)	Total activity (units)	Recovery (%)	Specific activity
13,200	561	7.40×10^6	—	—
138	50,400	6.94×10^6	94	—
45	148,000	6.65×10^6	90	409×10^3
3.4[b]	1,960,000	6.65×10^6	90	538×10^3

[a] The culture filtrate was obtained from a culture grown on 0.5% sucrose plus 0.75% L-arabinose. A 1-liter inoculum of germinated conidia was used; the culture was incubated at 30 C for 24 hr with vigorous aeration.

[b] Resuspended precipitate.

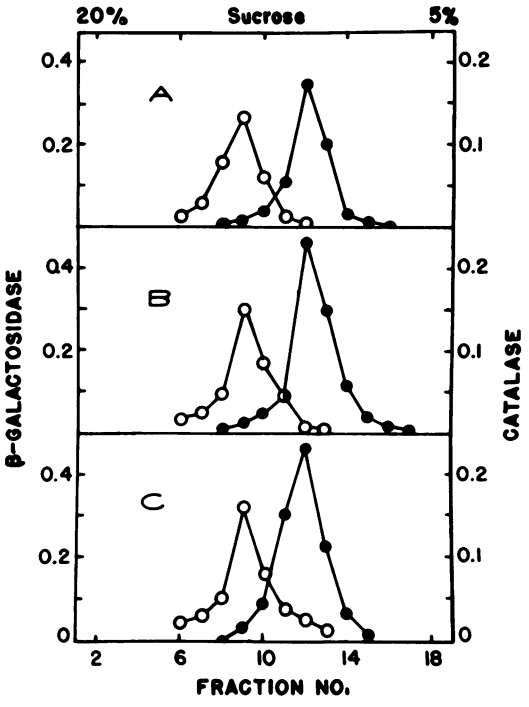

FIG. 3. *Sucrose gradient sedimentation profiles of purified intracellular and concentrated extracellular β-galactosidase (●) and catalase (○); extracellular (A), intracellular (B), and a mixture (C) of the two β-galactosidase preparations. The running time was 8 hr. The recovery of activity was greater than 85% in every case. The ordinates represent absorbance of ONP (420 nm) and catalase (404 nm).*

of columns, the two enzymes showed identical elution patterns and no peak splitting was observed in the case of the mixture. A typical elution profile is shown in Fig. 4. An estimation of molecular weight was made by the method of Determann and Michel (7) with the use of Blue Dextran 2000 to determine the void volume; a molecular weight of 92,000 was calculated from the data.

When the intracellular and extracellular enzymes and equal activity mixtures of the two were examined by acrylamide gel electrophoresis, the two activities were observed to migrate at the same rate, and the mixture showed only a single band of activity.

The Michaelis-Menton constants for the β-galactosidases were determined by Lineweaver-Burk plots (14) with points fitted by the method of least squares. The K_m values obtained for the two preparations and for equal activity mixtures were all within $(5.1 \pm 0.5) \times 10^{-4}$ M. These values are close to those obtained previously for the intracellular *p*H 4 enzyme, 6.2×10^{-4} M (13)

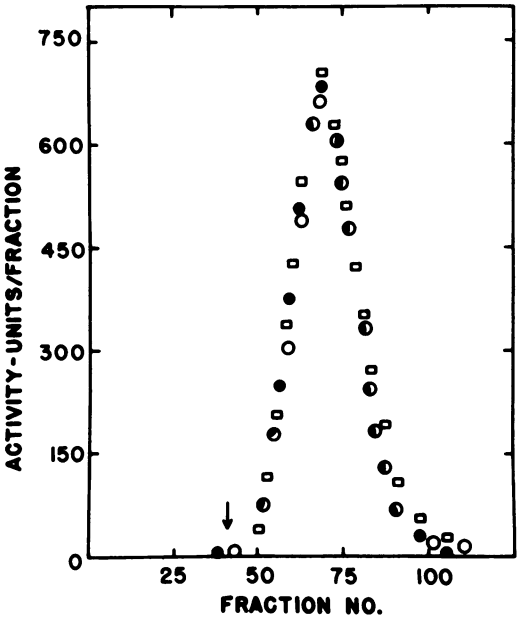

FIG. 4. *Chromatography of purified intracellular β-galactosidase* (○), *concentrated extracellular β-galactosidase* (●), *and a mixture* (□) *of the two on Sephadex G-200. The column was 2.5 cm in diameter, 50 cm long; samples containing about 8,500 units of activity were applied in a volume of 2.0 ml. The flow rate was about 0.3 to 0.5 ml per min; the fraction size was 2.5 ml. Recovery of activity was greater than 85% from each column. The arrow indicates the peak of Blue Dextran 2000.*

and 3.8×10^{-4} M (9). One may conclude that the two β-galactosidase activities have very similar substrate affinities.

DISCUSSION

The *p*H 4 β-galactosidase found in the culture filtrates has the characteristics of a true extracellular enzyme. It appears during the growth phase of the culture and, as shown by intracellular markers, it is not released as a result of cell lysis. This extracellular β-galactosidase activity appears identical to the intracellular *p*H 4 enzyme, by several physical and kinetic criteria. Furthermore, the extracellular activity appears only when intracellular *p*H 4 activity is present. The extracellular *p*H 4 enzyme may account for a large portion of the total β-galactosidase activity of the culture, especially over longer culture periods. It also should be noted that the ratio of intracellular to extracellular enzyme in the same culture does not remain constant but varies with time.

The *p*H 4 enzyme is specifically induced by sugars which, with the exception of D-xylose, are structurally related to galactose. On the other hand, the best inducer of the *p*H 7 enzyme is cellobiose, a β-glucoside, which raises the possibility that the *p*H 7 enzyme may be a β-glucosidase with some cross-specificity for β-galactosidase.

Extracellular enzyme does not begin to appear until sucrose is exhausted from the medium. Similarly, with glucose and fructose as the major metabolizable carbon sources, extracellular enzyme did not appear until these sugars disappeared from the medium (*unpublished data*). These findings, along with the varying ratios of intracellular to extracellular *p*H 4 enzyme which occur with growth on different sugars, suggest that sugars or metabolic activity rates may play a role in determining the rate of secretion of the enzyme from the cells.

Extracellular glycosidases are not uncommon in *Neurospora*. Conidia produce an extracellular β-glucosidase which is distinct from another intracellular β-glucosidase (4). Such extracellular enzymes may play an important role in the metabolism of poorly permeable glycosides. *Neurospora* is unable to transport sucrose across the cell membrane, and invertase has been located in the intermural space between the cell membrane and wall (16). *Neurospora* cannot take up certain β-galactosides such as ONPG and can take up lactose only at a rate that is very slow compared with glucose uptake (12). The presence of an extracellular β-galactosidase could facilitate the metabolism of such galactosides.

In any event, it is important that studies of the β-galactosidase system of *Neurospora* take into account the occurrence of extracellular enzyme.

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