THE INVERTASE ISOZYME FORMED BY NEUROSPORA PROTOPLASTS[1/]

John R. Trevithick and R. L. Metzenberg
Department of Physiological Chemistry
University of Wisconsin
Madison 6, Wisconsin

Received May 19, 1964

Recent studies in our laboratory have shown that the invertase of Neurospora crassa can exist in two active forms, "light" and "heavy" (1). Light invertase, the subunit, is formed from heavy invertase under a variety of conditions which promote dissociation. The heavy form, which predominates in vivo, can in turn be formed by combination of the subunits. Several reports of the existence of inactive protein precursors during the synthesis of enzymes at the ribosome level (2, 3) stimulated our interest in the process of formation of invertase. The subunits of invertase are active, while those of the β-galactosidase of E. coli are not. The possibility occurred to us that the aggregation of subunits could occur elsewhere than at the ribosome. This could take place at the cell membrane, or in the space between the cell wall and the cell membrane, where invertase appears to be localized (4). The high concentration of invertase at the latter site would be expected to favor the aggregation of subunits.

We have investigated this possibility using protoplasts of Neurospora which, like yeast protoplasts (5), secrete invertase. We find that under a wide variety of conditions the enzyme form which predominates inside the protoplasts and the form secreted by them is the heavy form.

1/ This study was supported in part by a U. S. Public Health Service Grant (GM-08995-03).

EXPERIMENTAL

The method used to prepare protoplasts of Neurospora was essentially that of Bachmann and Bonner (6), with the following modifications. Fries' salts medium (7) supplemented with 1.5% sucrose as carbon source was used to grow the conidia and young mycelia. Snail juice (Suc d' Helix Pomatia), obtained from Industries Biologiques Francaises, was treated with 0.002 M. mercaptoethanol to complex the mercurial preservative. The residual mercaptoethanol was removed by precipitating the five fold-diluted snail juice with three parts of ammonium sulfate, and washing the precipitate once with a 3.65 M. solution of ammonium sulfate. The precipitate was taken up in 0.02 M. pH 6.0 potassium phosphate buffer, giving a protein concentration 20% that of the original snail juice. This solution was rendered essentially aseptic by centrifugation for 20 minutes at 20,000 g., followed by careful removal of the supernatant solution.

The digestion and all other incubations were carried out in a basal medium which contained NH_4NO_3 (4 g./l.), $MgSO_4.7H_2O$ (1 g./l.), KH_2PO_4 (.284 g./l.), K_2HPO_4 (.034 g./l.), biotin (10 μg./l.), and trace elements at twice the concentrations used by Beadle and Tatum (7). To maintain the correct tonicity, mannitol was dissolved in the above medium to 0.59 M. All incubation and digestion solutions contained both penicillin and streptomycin at a concentration of 2 mg./ml. The digestion mixture contained 2.5% lysozyme in addition to 10% snail juice, since this seemed to prevent regeneration of cell walls.

Young mycelium, harvested by centrifugation from 100 ml. of medium, was pressed dry between two sheets of sterile filter paper. The pad was suspended in 2 ml. of the digestion medium and treated for 7 hours in a shaker-incubator at 30°C. After 6 hours and at the end, the pulpy mixture was passed up and down in a wide-bore pipette (2.0 ml.) 10 times to disperse any aggregates of protoplasts. Ten volumes of basal

medium containing mannitol were added to dilute the mixture and 10 ml. aliquots were filtered through sterile glass wool filters into centrifuge tubes. The protoplasts were collected by centrifugation for 20 minutes using a clinical centrifuge set at very low speed so as to give about 50 to 100 g. The supernatant fractions were discarded. The protoplasts were all combined and resuspended, using a pipette as described above. The combined residues were washed six times in 5 ml. of basal medium containing mannitol. The buffy layer of debris above the protoplasts was removed by this procedure. The yield of protoplasts was usually 0.3 to 0.5 ml., loosely packed by centrifugation in a calibrated tube. The protoplasts were found to contain some pieces of cell wall when examined under the phase contrast microscope, but none of these pieces contained living protoplasm.

For incubation, the protoplasts were mixed with buffered incubation medium or were taken up in incubation medium and added to an isotonic buffer. When hypotonic solutions were added to the incubation medium of 1.0 ml., their total volume never exceeded an additional 0.05 ml.

The incubations for secretion of invertase were carried out at 30°C. Samples (0.2 ml.) were taken aseptically as the experiment progressed, diluted with cold basal medium containing mannitol (1.0 ml.), and centrifuged. The supernatant was saved for analysis of the secreted protein; the protoplasts were washed again with the above solution (2.0 ml.), then lysed with water (1.0 ml.). All samples were frozen for storage. Samples were analysed by three methods: 1) invertase assay in the standard assay medium of Metzenberg (8), with estimation of the glucose liberated by the glucose oxidase method (8); 2) precipitation of radioactive protein in the presence of 100 μg. of carrier bovine serum albumin with 5 ml. of 10% trichloroacetic acid. After being heated at 90°C. for 15 minutes, the suspensions were cooled and the protein precipitates were collected on Millipore filters. The

filters were washed twice with 5 ml. of cold trichloroacetic acid, and glued to a copper planchet for counting in a Tracerlab thin window gas flow counter; 3) analysis by polyacrylamide gel electrophoresis (9) using the technique of Metzenberg (1) to stain the gels for invertase activity.

RESULTS

When protoplasts were incubated with a large excess of each form of invertase, at pH values at which the forms are stable, there was no interconversion of the two forms of invertase. This indicates that neither the protoplasts nor any materials secreted by them catalyse the interconversion of these forms.

If protoplasts were incubated in the presence of .25 m M. C^{14}-phenylalanine (0.25 μ c./ml.) in 0.04 M. sodium succinate buffer, pH 5.5, they released invertase into the incubation medium at a nearly linear rate, which roughly followed the rate of release of radioactive protein into the medium (see Fig. 1). The amount of invertase contained inside the protoplasts and released on lysis in distilled water appeared to remain relatively constant for at least 12 hours. The slight increase in invertase inside the protoplasts sampled at 18 hours is probably due to retention of the invertase by cell walls which had partially formed at 18 hours.

When actidione, which is a known inhibitor of protein synthesis in yeast (10) and in Neurospora (11), was added to the above incubation medium to a concentration of 10 μg./ml., the incorporation of C^{14}-phenylalanine was reduced to 3% of the value obtained in the absence of the inhibitor. With actidione present the amount of invertase released into solution was just matched by the amount lost by the protoplasts. This loss is probably due to gradual lysis of the inhibited protoplasts. When distilled water was allowed to flow under the cover slip of a preparation which was being observed with a phase

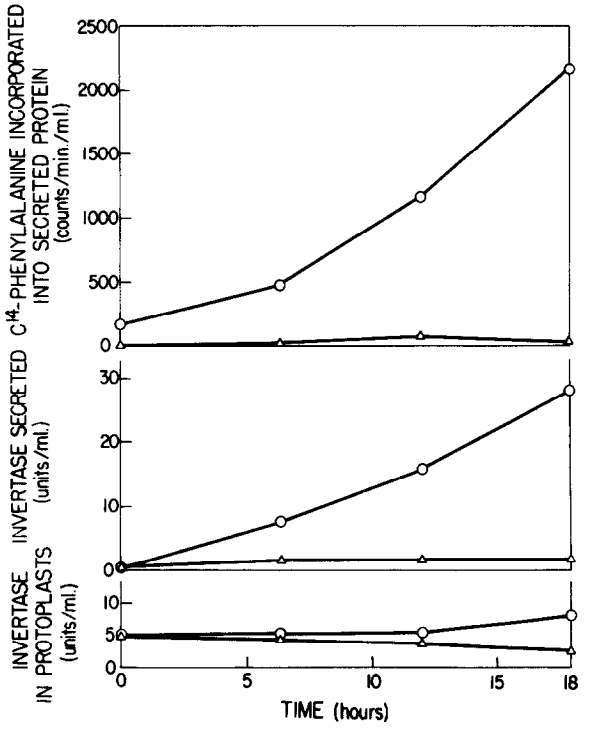

Fig. 1. Invertase activity in protoplasts and in secreted protein compared with incorporation of C^{14}-phenylalanine into secreted protein. The incubations were performed as described in the text. Symbols: -O, without actidione; △, actidione, 10 μg./ml.

---

contrast microscope, the protoplasts were seen to lyse even after long periods of incubation in mannitol medium. If not all protoplasts lysed, this fact was noted, and is reported as an indication of cell wall formation.

An experiment was performed to study the effect of pH on the secretion of invertase into the medium. In the presence of sodium succinate buffers of various pH values (each 0.04 M.), invertase was released into the medium at different rates, as seen in Fig. 2. The invertase secretion was essentially linear with respect to time, and the values plotted are the total increment of secreted invertase obtained over an incubation period of 12 hours. The pH was measured on a duplicate sample made without C^{14}-phenylalanine and protoplasts. The data indicate that both protein synthesis and secretion of in-

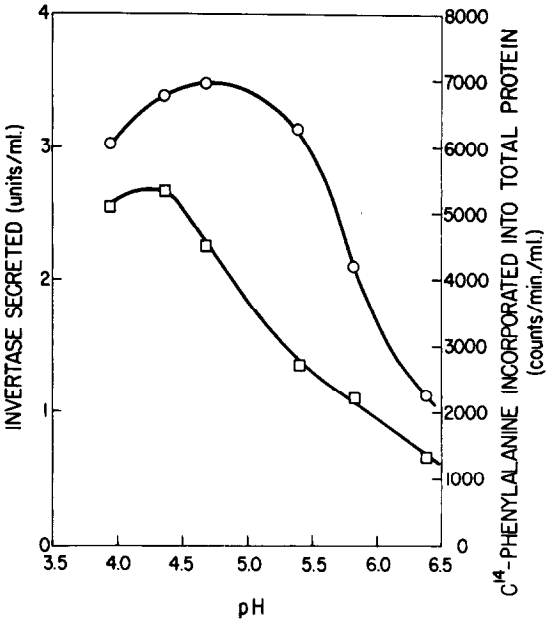

Fig. 2. Secretion of invertase and incorporation of C^{14}-phenylalanine into total protein at different pH values during a 12 hour incubation. Symbols: -O, increment of invertase activity; □ , increment of C^{14}-phenylalanine radioactivity in protein. Incubations were performed as described in the text.

---

vertase are accelerated at low pH values, although their pH dependence is not identical. Some synthesis of cell walls occurred during the experiment, particularly at low pH values. A majority of the protoplasts could not be lysed in distilled water after four hours at pH 3.94, 4.36, and 4.68, and after 12 hours at pH 5.39. Since the rate of invertase secretion did not decrease, these observations indicate that under the conditions noted, the Neurospora existed mainly as spheroplasts.

All experiments reported showed only the heavy form of invertase to be present both in the secreted protein and in the protoplasts, except for the protein secreted at low pH. When analysed by polyacrylamide gel electrophoresis, the invertase present in pH 4.68, 4.36, and 3.94 succinate media existed in the following forms, respectively: mostly heavy, substantial amounts of both, and mostly

light. It has previously been found that low pH favors the dissociation of pure invertase into subunits (1).

These data clearly indicate that the major form of invertase inside the protoplasts is the heavy enzyme. Further, this heavy form is secreted through the protoplast membrane and appears as such in the medium. In intact cells, presumably the heavy invertase is retained between the cell wall and the cell membrane, as has been deduced on other grounds (4). These results would seem to indicate that the aggregation of the subunits must occur at some site interior to the cell membrane, during or soon after their synthesis.

REFERENCES

1. Metzenberg, R. L., Biochim. Biophys. Acta, in press (1964).
2. Zipser, D., J. Molec. Biol., 7, 739 (1963).
3. Hartwell, L.H., and Magasanik, B., J. Molec. Biol., 7, 401 (1963).
4. Metzenberg, R. L., Biochim. Biophys. Acta, 77, 455 (1963).
5. Islam, M. F., and Lampen, J. O., Biochim. Biophys. Acta, 58, 294 (1962).
6. Bachmann, B. J. and Bonner, D. M., J. Bacteriol., 78, 550 (1959).
7. Beadle, G. W. and Tatum, E.L., Amer. J. Botany, 32, 678 (1945).
8. Metzenberg, R. L., Arch. Biochem. Biophys., 96, 468 (1962).
9. Ornstein, L. and Davis, B.J., preprint available from Distillation Products Industires, Rochester, New York.
10. Siegel, M. R. and Sisler, H. D., Nature 200, 675 (1963).
11. Hsu, K. S., J. Gen. Microbiol. 32, 341 (1963).