AFFINITY CHROMATOGRAPHY PURIFICATION OF PENICILLINASE OF BACILLUS LICHENIFORMIS 749/C AND ITS USE TO MEASURE TURNOVER OF THE CELL BOUND ENZYME

L. J. CRANE[1], G. E. BETTINGER And J. O. LAMPEN

Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903

Received November 16,1972

SUMMARY: Affinity chromatography purification of small amounts of penicillinase using cephalosporin C covalently linked to Sepharose 4B has been used in examining the turnover of cell-bound penicillinase by B. licheniformis 749/C. Under conditions in which most of the nascent penicillinase is retained by the cells, turnover could be demonstrated (15% of the cell-bound enzyme in 2.5 hours), although most of the secreted enzyme is derived from newly-formed molecules.

INTRODUCTION: During our studies on the secretion of penicillinase by B. licheniformis 749/C (1 - 4) it became necessary to devise a rapid and gentle means of purifying microgram amounts of the enzyme. Affinity chromatography (5) using the substrate analog cephalosporin C covalently bound to agarose has been successful in isolating penicillinase from crude preparations.

Penicillinase from B. licheniformis 749 and 749/C is found in three locations: in the medium, in periplasmic vesicles released upon protoplasting, and in the plasma membrane (3). We have examined the supernatant penicillinase from ^{14}C labelled intact cells and protoplasts using the affinity column to see if any of the cell-bound enzyme is released into the medium as part of the normal secretion pattern; and if so, which of the two cell-bound fractions it derives from.

---

[1] Formerly published as L. J. Sharkey.

MATERIALS AND METHODS: Affinity chromatography — Cephalosporin C (gift from Eli Lilly & Co.) was covalently bound to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey) activated by CNBr (Eastman Chemicals) as described by Cuatrecasas (6). 50 ml of packed, washed Sepharose and 15 gm of CNBr were combined as described; 500 mg of cephalosporin C were added at a coupling pH of 10.4. At the completion of the reaction the suspension was washed with pH 4.0, 0.1 M CH_3CO_2Na until no more material absorbing at 260 nm was removed.[1] 32 - 40 μmoles of cephalosporin C was bound to the Sepharose.

Turnover experiments — Supernatant penicillinase was applied to the column as a culture supernatant after overnight dialysis against distilled water and lyophilization. Penicillinase was assayed by the method of Sargent (8), and protein estimated by the method of Lowry (9). Cells of B. licheniformis 749/C were grown in pH 6.5 CH/S medium as previously described (10). The turnover of cell-bound penicillinase from intact cells or protoplasts was investigated using cells pre-labelled for four generations in a ^{14}C amino acid mixture (New England Nuclear Corp., Boston, Mass.), 1 μCi/ml in pH 6.5 CH/S medium, or protoplasts prepared from them as previously reported (11). Cells were washed twice with 0.85% NaCl and resuspended in fresh pH 6.5 CH/S medium without ^{14}C amino acids supplement at 0.35 mg/ml dry weight of cells; the protoplasts were washed twice with the pH 6.5 succinate protoplast growth medium of Sargent (2) and resuspended in the same medium at 1 mg/ml equivalent dry weight of cells at pH 6.5 or 7.5. Resuspended cells or protoplasts were divided into

---

1 $E^{260\ nm}_{1\ cm\ 1\ M}$ = 8900 of cephalosporin C (7).

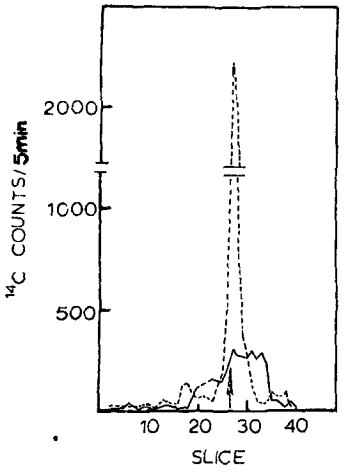

Fig. 1.

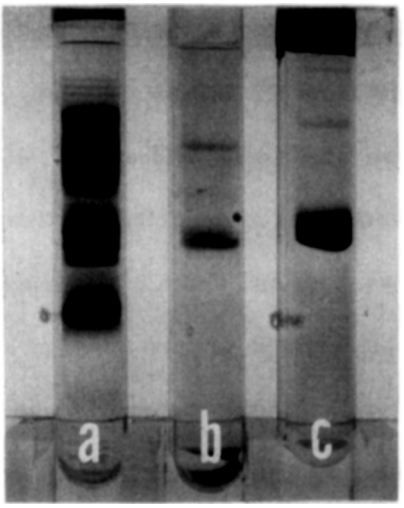

Fig. 2.

Fig. 1. SDS electrophoretogram of ^{14}C-exo-penicillinase purified by affinity chromatography. The column was equilibrated with pH 4.0, 0.1 M CH_3CO_2 Na buffer. Crude exo-enzyme was applied in the same buffer, and the column washed until no more O.D. 280 nm eluted. Removal was effected by elution with pH 6.2, 0.1 M NaH_2PO_4 buffer. (———), profile of proteins not bound at pH 4.0; (----), profile of protein eluted at pH 6.2. Arrow indicates mobility of pure exo-penicillinase.

Fig. 2. SDS acrylamide gel electrophoresis of: a) plasma membrane penicillinase, partially purified by the method of Sargent & Lampen (3); b) the same enzyme, after purification by affinity chromatography. The same conditions are used as for the exo-enzyme (Fig. 1), except that the enzyme was eluted with pH 6.5, 0.05 M Tris-maleate buffer, and 0.1% TDC [1] was added to all buffers. c) exo-penicillinase purified by Pollock's method (15).

[1] TDC - sodium taurodeoxycholate, synthesized by the method of Norman (16). Purity was checked by thin layer chromatography (17) and I.R. (18).

two aliquots, with 40 μg/ml chloramphenicol (CAP) added to one. Both aliquots were incubated 2.5 hours at 30° C in a gyratory water bath (200 rev/min). The penicillinase from equal volumes of both supernatants was isolated using the affinity column. Penicillinase activity was monitored in a similar experiment.

Sodium dodecyl sulfate (SDS) electrophoresis was performed by the method of Maizel (12). Radioactive gels were sliced into 1.8 mm lengths, solubilized (13) and counted using the scintillation cocktail of Turner (14).

RESULTS: ^{14}C exo-penicillinase purified by affinity chromatography shows the presence of one minor impurity (Fig. 1), amounting to less than 5% of the total radioactivity in the two peaks. Enzyme purified by Pollock's method (15) shows the same impurity upon SDS gel electrophoresis (Fig. 2 C).

Use of the affinity column for purification of plasma membrane penicillinase resulted in substantial, but not complete purification of plasma membrane penicillinase. Enzyme partially purified by the method of Sargent and Lampen (3) is complexed with at least 18 other proteins by SDS electrophoresis, as shown in Fig. 2A. When these preparations are subjected to affinity chromatography,[1] one other protein band consistently remains with the enzyme (Fig. 2 B). Minor amounts of other protein bands sometimes also occur. This protein impurity also persists with a number of other treatments (unpublished results), and seems to be tightly complexed to the enzyme, rather than non-specifically bound to the column.

Turnover experiments --- The experimental results from labelling of intact cells under synthetic and non-synthetic conditions are shown in Figs. 3 and 4. As can be seen from Fig. 3B, a significant increase in both cell-bound and exopenicillinase occurred under synthetic conditions during the course of the experiment (approximately 2400 units and 800 units respectively), enough to permit synthesis-linked turnover of cell-bound enzyme if such a process occurs. Fig. 4 shows that synthesis-linked turnover does, indeed, occur, for the electrophoretograms of isolated penicillinase show that there is approximately 2.5 times as much ^{14}C

[1] The plasma membrane enzyme must be purified at least 10-fold from fresh membrane before it is applied to the affinity column to prevent precipitation at the low pH of adsorption.

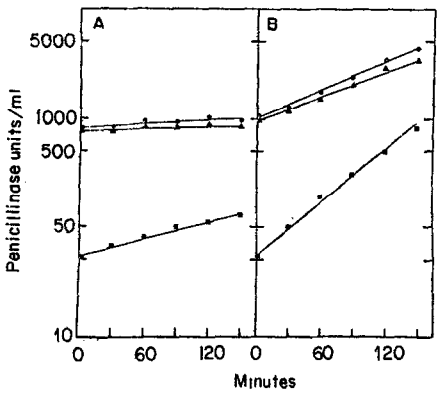

Fig. 3.

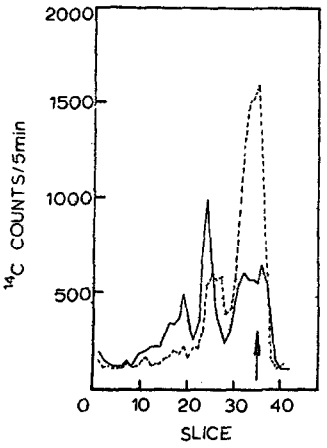

Fig. 4.

Fig. 3. Penicillinase synthesis in intact cells. Experimental conditions as described for turnover experiments. Cells incubated under (A) non-synthetic (CAP, 40 μg/ml) or B) synthetic conditions. (O), total penicillinase; (▲), cell-bound penicillinase; (■), exo-penicillinase.

Fig. 4. Penicillinase turnover in intact cells. SDS acrylamide gel electrophoretogram of purified penicillinase from the supernatant of intact cells. The electrophoretic mobility of ^{14}C-radioactivity was compared to the stained position of unlabelled, pure exo-penicillinase standard (arrow). (----), cells incubated under protein synthetic conditions; (_____), cells incubated in the presence of 40 μgm/ml CAP.

---

radioactivity migrating as exopenicillinase in the supernatant from growing cells as in CAP-treated cells. This radioactivity reflects the amount of penicillinase that was initially cell bound and then secreted as exo-enzyme. Fig. 3A shows that CAP-treated cells release as exo-enzyme 10% of their cell-bound penicillinase (presumably from the vesicle fraction (1)) during the course of the experiment; therefore, the synthesis-linked turnover of bound enzyme by growing cells was 15%. The total turnover from the initial cell-bound fraction (25%) yielded approximately 250 units of exo-enzyme. This represents only about 30% of the total exo-enzyme formed; thus, turnover is not a major path for secretion of exo-enzyme.

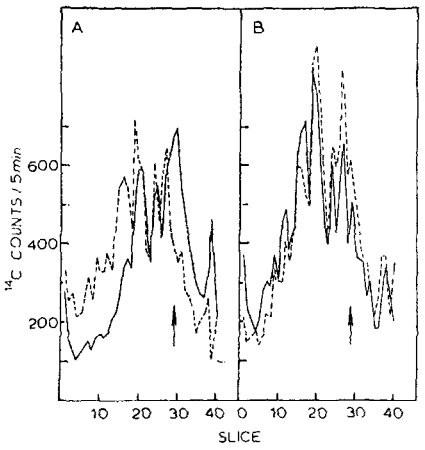

Fig. 5. Turnover in protoplasts. Protoplasts incubated at pH 6.5 (A) or pH 7.5 (B). Experimental conditions as in Fig. 3 (_____), protoplasts incubated under synthetic conditions; (-----), protoplasts incubated in the presence of 40 μgm/ml CAP. Arrows indicate mobility of pure exo-penicillinase.

A similar experiment was carried out with protoplasts in an attempt to determine whether the turnover occurred from the plasma membranes or from periplasmic vesicles (Fig. 5). However, the large number of proteins eluting from the affinity column along with penicillinase precludes definite demonstration of turnover and hence analysis of the specific site of turnover by this method. It is improbable that the other proteins are the result of non-specific adsorption to the column since a variety of membrane and soluble proteins have been tested and failed to adsorb (unpublished results).

DISCUSSION: Cephalosporin C is an excellent ligand for the affinity column for several reasons. It has a high affinity for B. licheniformis penicillinase (K_M = 10 - 20 μM (this lab)), but is hydrolyzed at a very low rate (15). Hence the binding of enzyme is non-destructive to the column. The amino adipoyl side chain offers a non-essential and distant amino group for coupling. Finally, it is stable to non-enzymatic hydrolysis, unlike

penicillin analogs; the same column preparation has been used for two years with no noticeable loss in binding ability.

Turnover studies of penicillinase secretion from intact cells indicate that under synthetic conditions in which most of the nascent enzyme is retained (2), by far the major portion of the secreted enzyme derives from newly-synthesized molecules. Only approximately 15% of the enzyme bound to the cells is released in a synthesis-linked process over a period of 2.5 hours. The specific source of this enzyme, whether from the plasma membrane or from periplasmic vesicles remains undetermined because of the inconclusive nature of the protoplast experiments. The large number of proteins isolated with penicillinase in the protoplast experiments may indicate that penicillinase released from protoplasts is not initially like the exo-enzyme. Experiments with pH-released enzyme from protoplasts (unpublished results) and with the isolated plasma membrane enzyme itself show that it has a marked tendency to aggregate with other proteins (3).

ACKNOWLEDGMENTS: This investigation was supported by Public Health Service grant AI-04572 from the National Institute of Allergy and Infectious Diseases. G.E.B. was a predoctoral trainee under PHS Training Grant GM-507 from the National Institute of General Medical Sciences. L.J.C. is an NDEA Title IV Fellow.

REFERENCES:

1. Sargent, M.G., Ghosh, B. K. & Lampen, J. O., J. Bacteriol. 96: 1231 (1968).
2. Sargent, M.G., Ghosh, B. K., & Lampen, J. O. J. Bacteriol. 97: 820 (1969).
3. Sargent, M.G., & Lampen, J. O., Arch. Biochem. Biophys. 136: 167 (1970).
4. Sargent, M.G., & Lampen, J. O., Proc. Natl. Acad. Sci. (U.S.A.) 65: 962 (1970).
5. Cuatrecases, P., Wilchek, M., & Anfinsen, C.B., Proc. Natl. Acad. Sci. (U.S.A.) 61: 636 (1968).
6. Cuatrecasas, P., J. Biol. Chem. 245: 3059 (1970).
7. Abraham, E. P., Quart. Rev. 21: 231 (1967).
8. Sargent, M. G., J. Bacteriol. 95: 1493 (1968).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J., J. Biol. Chem. 193: 265 (1951).

10. Ghosh, B. K., Sargent, M. G., & Lampen, J. O. J. Bacteriol. 96: 1314 (1968).
11. Bettinger, G. E., & Lampen, J. O., Biochem. Biophys. Res. Commun. 43: 200 (1971).
12. Shapiro, A. L., Vinuela, E., & Maizel, J. V., Biochem. Biophys. Res. Commun. 28: 815 (1967).
13. Goodman, D., & Matzura, H., Analyt. Biochem. 42: 481 (1971).
14. Turner, J. C., Int. J. Appl. Radiation and Isotopes 20: 499 (1969).
15. Pollock, M. R., Biochem. J. 94: 666 (1965).
16. Norman, A., Arkiv. Kemi. 8: 331 (1955).
17. Hofmann, A. F., J. Lipid Research 3: 127 (1962).
18. Fischmeister, I., Arkiv. Kemi 16: 166 (1960).