USE OF RESINS FOR TRICHOTHECENE PRODUCTION IN LIQUID CULTURES

BRUCE B. JARVIS, CATHERINE A. ARMSTRONG and MING ZENG

Department of Chemistry and Biochemistry,
University of Maryland,
College Park, MD 20742, U.S.A.

(Received for publication May 2, 1990)

During the course of studies on the production and isolation of macrocyclic trichothecenes from fungal isolates,[1] we have developed procedures which may be of general use to the community concerned with antibiotic production. Herein, we report on the use of a resin to significantly improve the production of trichothecenes from *Myrothecium*, by a method which may be useful for the production of antibiotics in submerged cultures.

Resins, such as the XAD's, are used extensively in the isolation of microbial metabolites from fermentation broths. A major use of these hydrophobic materials is to adsorb the organic matter from the aqueous media, obviating the necessity for organic solvent extraction procedures. This methodology proves particularly useful in large scale liquid fermentations. It occurred to us that the presence of such a resin during the fermentation could have a significant effect on the course of secondary metabolism. However, that effect is by no means predictable. For example, if early metabolites were released into the aqueous medium, the resin might trap them and prevent further metabolism, thus leading to their accumulation. Contrarily, if the final metabolites released into the medium build up to a level where they tended to inhibit further production, removal of them from the medium with a resin, should lead to enhanced production.

We have evaluated the efficacy of added resins in the shake culture fermentations of *Mycothecium verrucaria* for affecting the course of trichothecene production. The isolate studied, *M. verrucaria* CL-72 has proven to be a good producer of macrocyclic trichothecenes, particularly verrucarin A. The resins evaluated were the Amberlite resins XAD-4, XAD-7, XAD-8, and Amberchrom CG-71 and CG-161 chromatographic media. The latter two are resins which are of a finer mesh size than the Amberlite resins. The resins were first evaluated for their ability to adsorb the trichothecenes from the aqueous fermentation broth. Both XAD-4 and CG-71 were relatively inefficient at adsorbing the more polar trichothecenes (*e.g.* $<60\%$ of roridin A and baccharinoid B4 were adsorbed by these resins), but performed satisfactorily with the less polar trichothecenes such as verrucarin J ($>80\%$ absorption from solution). The other resins, XAD-7, XAD-8, and CG-161 adsorbed $>80\%$ of the trichothecenes from the fermentation broths, though XAD-8 tended to be somewhat less efficient. Therefore, XAD-7 and CG-161 (chromatographic grade of XAD-7) were chosen for further study.

The fermentations were conducted in shake culture using a two stage procedure where a seed medium was first inoculated with the fungus and after 2 days growth, this seed medium was used to inoculate a production medium. After 5 days in production, the cultures were analyzed by HPLC for the levels of trichothecenes. The levels of the two major toxins, verrucarins A and J, in the controls were *ca.* $600\sim800$ and $300\sim400$ μg/ml, respectively. When XAD-7 resin was added to the production medium at the time of seed medium inoculation (day 1 of production), the production levels of these toxins increased by $75\sim100\%$. When the resin was added at day 3 of production, the yields were only modestly increased ($10\sim20\%$). The levels were increased further, by *ca.* 10% over those observed with the XAD-7 resin, when CG-161 resin was employed. However, this latter resin is significantly more expensive. Furthermore, XAD-7 resin is easily recovered at the end of these fermentations by suspending the resin-mycelium mixture in water and decanting the lighter resin from the heavier mycelium. We were unable to separate the more finely divided CG-161 particles which became imbeded in the mycelium during the fermentation.

We have employed this resin technique for the production of ^{14}C-labeled verrucarin A. From a 1-liter culture of *M. verrucaria* CL-72 to which 2.0 mCi of sodium acetate had been added, a total of 650 mg of labeled verrucarin A was isolated with a specific activity of 0.56 μCi/mg (280 μCi/mmol); the details of this preparation are given in the Experimental section.

Others also have employed the use of a resin in the preparation of radiolabeled antibiotics,[2] though this technique has not been generally employed. However, it is very important to evaluate this technique for each isolate. For example, *M. verrucaria* ATCC 36872 produces verrucarin A (*ca.* 800 mg/liter) and roridin A (*ca.* 1,500 mg/liter) under

normal fermentation conditions; however, yields of these toxins drop significantly (by 50~100%) when the resins are added to the production medium at day 1. Interestingly, for this culture, the levels of trichoverrin B, a trichoverroid precursor to the macrocyclics,[3] increase dramatically (from <100 mg/liter to 700~1,000 mg/liter) when the resin is added to the production medium on day 1. The origin of the difference in the responses of these two *M. verrucaria* isolates to the presence of resins in their liquid cultures is obscure but may be related to the differences in the transport properties of the antibiotics in the two isolates.

Experimental

General

The resins were obtained from ROHM and HAAS, Philadelphia, PA. General procedures for the fermentations (without added resin) have been previously described.[4] HPLC was carried out on a Gilson Model 302 chromatograph equipped with a Knauer D-1000 variable wavelength detector set at 264 nm. Integration of peaks was performed with a Shimadzu C-R3A Chromatopac. The analysis conditions were: 55~92% methanol in water gradient over a 20-minute period at a flow rate of 1.0 ml/minute on a 5-μm Supelco C8 column (250 mm × 4.6 mm). Standard curves for verrucarins A and J,[3] roridin A,[3] and baccharinoid B4[5] were generated by injecting known amounts of standards. At the end of the fermentations, the cultures (10 ml of seed medium +5 ml of resin in 50 ml of production medium in 250-ml Erlenmeyer flasks, in triplicate)[4] were treated with 0.5 g of sodium azide. After 1 hour, 0.5 mg of baccharinoid B4 (as internal standard) in 500 μl of DMSO was added to each flask. The mixture was shaken for 1 hour, filtered and the collected mixture of mycelium and resin washed with 25 ml of distilled water. Filtrate extracts (ethyl acetate) were shown to contain only traces of the trichothecenes. The mycelium-resin mixture was soaked with 2 × 25 ml of acetone (*ca.* 8 hours each), the extracts combined and concentrated to dryness. The crude extracts were dissolved in 10.0 ml of dichloromethane, and HPLC analyses were made, in triplicate, on 10 μl injections. Controls without added resin were worked up by extraction of the mycelium with acetone and extraction of the filtrate with ethyl acetate. These extracts were combined and the total analyzed by HPLC as described above.

Quantitative data were collected for verrucarins A and J, but not for roridin A since there was an interfering peak in the chromatogram. However, qualitatively, the levels of production of roridin A were similar to those of verrucarin J. The following levels of verrucarins A (VA) and J (VJ) in μg/ml were found in the three runs: VA for control = 610, 700, and 790 μg/ml; with added CG-161 resin VA = 1,500, 1,610, and 1,660 μg/ml; with added XAD-7 resin VA = 1,400, 1,480, and 1,550 μg/ml. VJ for control = 300, 380, and 390 μg/ml; with added CG-161 resin VJ = 580, 620, and 630 μg/ml; with added XAD-7 resin VJ = 500, 580, and 530 μg/ml.

Production of ^{14}C-Labeled Verrucarin A

The seed medium consisting of corn steep (1 ml) and glucose (1.8 g) in 100 ml distilled water was prepared in a 500-ml Erlenmeyer flask. The seed medium[4] was autoclaved (20 minutes, 121°C) and allowed to cool to room temperature, inoculated with the spores of *M. verrucaria* CL-72 grown on potato-glucose agar, and the flask was shaken for 2 days (120 rpm, 25°C). One liter of production medium[4] was prepared and divided into ten 500-ml Erlenmeyer flasks. To each flask, 10 ml of XAD-7 resin (Amberchrom AD-7 Grade) was added, and the production media were autoclaved (20 minutes, 121°C) and allowed to cool to room temperature. Each flask was inoculated with 10 ml of seed medium and put on the shaker. After 1 day of growth (24 hours) in production medium, 65 μl of a solution of [1-^{14}C]sodium acetate (1 mCi in 1 ml of ethanol, NEC-084A) was added to each flask. This addition was repeated at 36 and 48 hours production times to give a total addition of 2.0 mCi of [1-^{14}C]sodium acetate. At the end of 60 hours of production growth, the media were filtered and the residues combined and rinsed with 100 ml of water. The residue was extracted with 3 × 100 ml of acetone. The acetone extracts were combined, dried (Na_2SO_4) and concentrated to give 2.8 g of gum. This material was taken up in a small portion of CH_2Cl_2 and chromatographed over 180 g of 40~60 μm silica gel (20~100% EtOAc-hexane). Two fractions contained verrucarin A. The more crystalline fraction was triturated with ether, and the precipitate was recrystallized from acetone/ether to give 280 mg of ^{14}C-verrucarin A. The mother liquor and filtrate from this fraction were combined with the other verrucarin A-containing fraction (total weight of 0.7 g) and subjected to preparative TLC on the Chromatotron (Harrison Research Laboratories, Model 7942) (3 × 4 mm plates, developed with 1.5% MeOH-CH_2Cl_2). From this was obtained 330 mg of ^{14}C-verrucarin A (total weight = 0.61 g). Re-

crystallization of a portion of this sample from acetone-ether to constant radioactivity gave a specific activity of 0.56 μCi/mg.

Acknowledgments

We thank Dr. LARRY STEFFIER of ROHM and HAAS for furnishing the resins used in this study. *Myrothecium verrucaria* CL-72 was kindly provided by Dr. JAMES FRENCH of Warner-Lambert/Parke-Davis Company, Ann Arbor, MI. Support from the National Institutes of Health (Grant No. GM 43724) is gratefully acknowledged.

References

1) JARVIS, B. B.; G. PAVANASASIVAM & G. A. BEAN: Mycotoxin production from *Myrothecium* species. *In* Trichothecenes and Other Mycotoxins. *Ed.*, J. LACEY, pp. 221~229, John Wiley & Sons, 1985

2) HSI, R. S. P.; D. F. WITZ, J. VISSER, W. T. STOLLER & C. L. DITTO: Synthesis of carbon-13 and carbon-14 labeled paldimycin trisodium salt. J. Label. Radiopharmaceu. 27: 147~165, 1989

3) JARVIS, B. B.; G. P. STAHLY, G. PAVANASASIVAM, J. O. MIDIWO, T. DESILVA, C. E. HOLMLUND, E. P. MAZZOLA & R. F. GEOGHEGAN, Jr.: Isolation and characterization of the trichoverroids and new roridins and verrucarins. J. Org. Chem. 47: 1117~1124, 1982

4) JARVIS, B. B.; F. T. COMEZOGLU, Y.-W. Lee, J. L. FLIPPEN-ANDERSON, R. D. GILARDI & C. F. GEORGE: Novel macrocyclic trichothecenes from *Myrothecium roridium*. Bull. Soc. Chim. Belg. 95: 681~697, 1986

5) KUPCHAN, S. M.; D. R. STREELMAN, B. B. JARVIS, R. G. DAILEY, Jr. & A. T. SNEDEN: The isolation of potent new antileukemic trichothecenes from *Baccharis megapotamica*. J. Org. Chem. 42: 4221~4225, 1977