Rapid screening of *Aspergillus terreus* mutants for overproduction of lovastatin

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Summary

A novel rapid screening method is demonstrated for isolating lovastatin-overproducing strains of *Aspergillus terreus*. The screening methodology, based on the activity of lovastatin against the yeast *Candida albicans*, is nearly three times as fast as the selection methods used earlier. The new 6-h assay shows a linear correlation between the quantity of lovastatin generated by *A. terreus* isolates and the inhibition zones obtained on plates of *C. albicans*. The new technique is less expensive and requires less labour.

Introduction

Lovastatin is a potent cholesterol-lowering drug. Lovastatin acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) which catalyses the rate-limiting step of cholesterol biosynthesis. Lovastatin is produced as a secondary metabolite by a variety of filamentous fungi, including *Penicillium* sp. (Endo *et al.* 1976a), *Monascus ruber* (Endo *et al.* 1976b; Juzlova *et al.* 1996), and *Aspergillus terreus* (Alberts *et al.* 1980). Commercial production of lovastatin uses *A. terreus* batch fermentation which has been investigated extensively (Novak *et al.* 1997; Manzoni *et al.* 1998, 1999; Szakács *et al.* 1998; Kumar *et al.* 2000a).

Random mutation and selection strategies have been reported for obtaining overproducing isolates of *A. terreus* (Vinci *et al.* 1991). A particularly useful rapid screening method for identifying lovastatin overproducers was described by Kumar *et al.* (2000b). The method was based on the anti-fungal properties of the β -hydroxyacid form of lovastatin and its inhibitory effect on the mycelial fungus *Neurospora crassa*. This bioassay required 16–18 h of plate incubation to measure zones of inhibition.

This paper describes a new method for screening for overproduction of lovastatin. Activity of lovastatin against the yeast *Candida albicans* is used as the basis for this method. Zones of inhibition proportional to lovastatin concentration were generated on agar plates that had been previously inoculated with *C. albicans*. This new 6-h assay allowed relatively rapid identification of lovastatin-overproducing strains of *A. terreus*. A mutation-selection program was carried out with

A. terreus ATCC 20542. After two mutation runs, a 4-fold enhancement in production of lovastatin was achieved compared to the original strain.

Materials and methods

Microorganism and inoculation

The fungus used was obtained from the American Type Culture Collection, as *Aspergillus terreus* ATCC 20542. A suspension of spores was prepared by washing petri dish (potato dextrose agar, PDA) cultures with a sterile aqueous solution of 2% Tween[®] 20. The spore concentration was determined spectrophotometrically at 360 nm by reference to a standard curve that had been made by direct counts (Coulter EPICS XL-MCL flow cytometer) of spores in suspension.

Mutation with EMS

Aspergillus terreus spores were exposed to methanesulphonic acid ethyl ester (EMS) at three concentrations (0.15, 0.3 and 0.5 M) for four durations of exposure (30, 60, 90, and 120 min). The spores were grown for 4 days on PDA slants. Colonies were collected in agar plugs (3 mm diameter $\times$ 7 mm height) when more than 90% mortality had been attained in a dish. Each agar plug was transferred to an Eppendorf tube containing 500 μ l of the lactose-yeast-extract medium and incubated at 28 °C for 5 days. This medium consisted of (per litre): 120 g lactose, 1.85 g yeast extract, 2 g KH₂PO₄, 0.52 g MgSO₄·7H₂O, 0.40 g NaCl, 2 mg Fe(NO₃)₃·9H₂O, 1 mg ZnSO₄·H₂O, 0.04 mg biotin and 1 ml of a trace

element solution. The trace element solution contained (per l of solution): $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 100 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 50 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 250 mg. The pH of the medium was adjusted to 5.5 before sterilization. Spores were harvested and plated at a concentration of 10^6 spores per ml on duplicate fresh petri dishes with the same medium that had been solidified with 15 g of agar/l. The plates were incubated for 7 days at 28 °C.

Bioassay with *Candida albicans*

After incubation, *A. terreus* colonies from one of the duplicate dishes (A) were isolated in agar plugs punched using a sterile 10-mm stainless steel cork borer and transferred to a screw-capped test tube. One millilitre of ethyl acetate was added for lovastatin extraction at 50 °C (15 min; with vortex agitation at 2 min intervals). The lovastatin extract was recovered by centrifugation ($2800 \times g$, 5 min). The other slant (B) was stored at 4–6 °C for further culture.

Candida albicans was grown for 12 h on PDA dishes at 28 °C, then harvested and transferred at a concentration of $5\text{--}7 \times 10^2$ cells per ml on fresh PDA slants. Fifty microliters of lovastatin extract were transferred onto a 6 mm diameter paper disk and placed on the surface of a 90 mm diameter *C. albicans* plate. The spacing between lovastatin impregnated disks on a plate was at least 15 mm. Negative standard disks were prepared with ethyl acetate alone. Positive standard disks were made by impregnating the paper with 50 μl of a solution of known concentration of lovastatin in ethyl acetate. The plates were incubated for 6 h and zones of inhibited growth were recorded. A large diameter of the inhibition zone indicated a high titre of lovastatin.

Lovastatin analysis

Lovastatin was quantified as its β -hydroxyacid form, by HPLC. Because the fungus secretes lovastatin in the β -hydroxyacid form, the assay eliminated the conversion step to the active lactone form of the drug. Using the β -hydroxyacid permitted rapid analysis because this form elutes earlier from a chromatography column than does the lactone form of lovastatin. Also, the β -hydroxyacid is quite stable in solution. HPLC was performed on a Beckman Ultrasphere ODS (250×4.6 mm I.D., 5 μm support diameter) column. A diode array detector was used. The eluent was acetonitrile/0.1% phosphoric acid (60:40, by vol). The eluent flow rate was 1.5 ml/min. The detection wavelength was 238 nm. The sample injection volume was 20 μl .

Shake flask cultures

All fermentations were carried out at 28 °C in 100 ml shake flasks filled with 20 ml of the medium and held on a rotary platform shaker (150 rev/min, 2.6 cm stroke). The culture medium used was the lactose-yeast-extract previ-

ously described. The culture lasted up to 7 days. The flasks were inoculated with 1 ml of a spore suspension which had been standardized to contain 10^6 spores/ml.

Results and discussion

In submerged cultures of *C. albicans*, addition of the β -hydroxyacid form of lovastatin caused growth inhibition at lovastatin concentrations greater than 0.06 g/l. On solid medium, when a given amount of lovastatin was placed on the agar surface using a paper disk, zones of no growth were obtained on plates of *C. albicans*. The diameter of the inhibition zones correlated linearly (Figure. 1) with the quantity of lovastatin impregnated in the paper disc, as follows:

$$\begin{aligned} \text{Inhibition zone diameter (mm)} = \\ 0.195 \times \text{lovastatin dose } (\mu\text{g}) \end{aligned}$$

The value of the slope in the above equation will depend on the nature of the agar gel. The slope will be lower for gels containing a high concentration of agar because the agar content is well known to affect the rate of diffusion in the gel. Preliminary experiments showed a 10–20% variation in the inhibition zone diameters for different batches of agar gel. Considering this, a positive standard with a given amount of β -hydroxyacid lovastatin was placed on each plate and the measured diameters of inhibition zones were normalized relative to this standard for comparing with the calibration curve shown in Figure 1.

After establishing the bioassay protocol, two two-stage mutation runs were carried out starting from the parent strain (*A. terreus*, ATCC 20542). Although in routine work only the high-yielding isolates (plate B) would be cultured in a liquid medium to assess the lovastatin production in submerged fermentation, in this study all the isolates were grown in shake flasks to illustrate the feasibility of the proposed methodology.

Figure 1. Standard curve for dose of the β -hydroxyacid form of lovastatin vs. inhibition zone diameter on *C. albicans* plates.

Figure 2. Inhibition zone diameter on plates of *C. albicans* vs. lovastatin concentration (analysed by HPLC) from submerged fermentation of *A. terreus* isolates (duplicate cultures): (a) first stage mutation run; (b) second stage mutation run.

The first run of mutagenesis gave rise to mutants with a wide range of lovastatin production titres (Figure 2a). The parent culture displayed a lovastatin production level that was midway compared to the range for the isolates. There was a positive correlation (correlation coefficient >0.98) between the measured diameter of inhibition zone on agar (plate A) and the lovastatin titre obtained in liquid cultures of the isolate (plate B). The isolate E2B4 was selected for a second mutagenesis run (Figure 2b).

As expected, most of isolates from this second mutagenesis stage gave lovastatin titres higher than that of the parent culture (ATCC 20542). The highest titre (60.3 mg/l), obtained with the isolate E354, was four times the lovastatin production level of the original culture.

Conclusions

The screening methodology demonstrated in this study permitted isolation of lovastatin overproducer mutants

of *A. terreus* in a significantly shorter period compared to the best existing methods. Also, the new technique was less expensive and required less labour. Currently, we have a substantial mutation/selection program underway for isolating better overproducer strains than those obtained already.

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