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Bioconversion of compactin to pravastatin by *Actinomadura* sp. ATCC 55678

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Dedicated to the memory of Professor Saburo Fukui

Abstract

The screening of known and unidentified actinomycetes revealed three strains which could hydroxylate compactin to pravastatin. The best was a new culture identified as *Actinomadura* sp. (ATCC 55678). *Actinomadura* sp. effectively converted compactin to pravastatin. The degree of conversion by cells was 65–78% of compactin added and 65–88% of compactin taken up, depending on the concentration of compactin and duration of the experiment. Increasing the compactin concentration resulted in a higher final pravastatin concentration especially when compactin was added intermittently. The conversion was linear for 16 h. The system required no induction with compactin. The hydroxylase in cell-free extracts of *Actinomadura* sp. converted compactin to pravastatin. It required NADPH as coenzyme and Mg^{2+} as cofactor; Mn^{2+} partially replaced Mg^{2+}. In contrast to the inducible cytochrome *P*450 system of *Streptomyces carbophilus* which catalyzes the same overall reaction, this constitutive hydroxylase was stimulated by ATP and ascorbic acid and was not inactivated by CO. *Actinomadura* sp. required vitamins to support its growth. Addition of folic acid, thiamine and cyanocobalamin allowed growth in chemically defined medium. Cells grown in chemically defined medium were as capable of converting compactin to pravastatin as cells grown in complex medium.

Keywords: Compactin; Pravastatin; Bioconversion; *Actinomadura*

1. Introduction

In 1976, compactin (ML-236B; mevastatin) was discovered independently in Japan and England. Endo et al. [1] isolated it from *Penicillium citrinum* as a cholesterol biosynthesis inhibitor and Brown et al. [2] obtained it from *Penicillium brevicompactum* as an antifungal antibiotic. Three years later, Endo [3] reported that *Monascus ruber* produced monocolin K (lovastatin, mevinolin) and in 1980, Alberts et al. [4] in the USA announced its independent discovery as a product of *Aspergillus terreus*. Lovastatin is a methylated form of compactin. Although both compactin and lovastatin competitively inhibited the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase [5], lovastatin

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was found to be more active than compactin in vitro against the enzyme and in vivo against cholesterol synthesis in the rat. In 1983, Serizawa et al. [6] showed that the fungus, *Mucor hiemalis*, could hydroxylate compactin to produce the more active drug, pravastatin.

High blood cholesterol, especially LDL cholesterol, is a risk factor for coronary heart disease, and the lowering of total and LDL cholesterol levels reduces the incidence of this disease. By 1980, Tsujita et al. [7], Kuroda et al. [8] and Yamamoto et al. [9] had shown that compactin markedly lowered the levels of LDL cholesterol in both experimental animals and humans. By inhibiting HMG-CoA reductase, plasma cholesterol levels in the body were reduced. These findings stimulated the worldwide development of compactin derivatives [10,11]. Lovastatin is more effective than compactin in lowering serum cholesterol in most animal species including humans [11–15] and became a highly successful drug. Pravastatin, a bioconversion product of compactin, is even more active [6,13–23]. It is the 3 β-hydroxy derivative of compactin.

Many fungi, but extremely few actinomycetes, are known to hydroxylate compactin to pravastatin [24]. The first actinomycetes to be used for this purpose was *Streptomyces carbophilus* which contains a cytochrome *P*450 monooxygenase catalyzing the bioconversion [25,26]. We found three actinomycetes to be active, but only a newly isolated strain, *Actinomadura* sp. 2966 (later designated as ATCC 55678), was found to be able to carry out the hydroxylation of compactin to pravastatin in high yield [27]. The bioconversion was found to take place by a mechanism of oxidation different from that of *S. carbophilus* [28,29]. Our findings are summarized in this review.

2. Screening of actinomycetes

Serizawa et al. [30] screened about 500 strains of fungi and 500 strains of bacteria for conversion of compactin to pravastatin. Fifty-three fungi in 26 species representing 13 genera were active. Of the bacteria, they only found a few actinomycetes of interest, i.e. three *Nocardia* strains from soil; *Streptomyces roseochromogenus* showed a trace of activity. Later, the same group [25,26] reported that *S. carbophilus* was active in hydroxylation of compactin. Following their work, we examined 59 actinomycetes strains for conversion of compactin to pravastatin. Among three active strains, strain 2966 showed the highest conversion yield, was identified as *Actinomadura* sp. [27], and later designated as ATCC 55678.

3. Conversion of compactin to pravastatin by *Actinomadura* sp. ATCC 55678

Table 1 shows the production of pravastatin from 500 μg/ml compactin added after 2 days of growth in YM medium (per liter; glucose 10

Table 1
Bioconversion of compactin to pravastatin by *Actinomadura* sp.[a]

Days after compactin addition	Compactin (μg/ml)	Pravastatin (μg/ml)	Bioconversion	
			Of compactin added (%)	Of compactin used (%)
1	32	252	50.4	53.9
2	0	267	52.4	52.4
3	0	290	58.0	58.0
4	0	318	63.6	63.6
5	0	326	65.2	65.2

[a]Compactin was added at 500 μg/ml after 2 days of growth.

Table 2
Bioconversion of a higher compactin (700 μg/ml) charge[a]

Days after compactin addition	Compactin (μg/ml)	Pravastatin (μg/ml)	Bioconversion	
			Of compactin added (%)	Of compactin used (%)
0	678	0	0	0
1	209	271	40.0	57.8
2	149	311	45.9	58.8
3	139	332	49.0	61.6
4	137	349	51.5	64.5
5	142	368	54.3	68.7
6	152	405	60.0	77.0
7	172	446	65.8	88.1

[a]Compactin was added at 700 μg/ml after 2 days of growth.

g, soluble starch 20 g, yeast extract 5 g, NZ amine 5 g) [28]. By the first day, most of the compactin had disappeared from the medium, apparently being taken up into the cells, and the conversion of compactin to pravastatin was over 50%. By the end of the second day, there was no detectable compactin left in the culture fluid, but the pravastatin concentration continued to increase up to 5 days. By the end of the fifth day, the conversion reached 65%. In an experiment using a higher concentration of compactin (700 μg/ml), uptake was incomplete but conversion reached 88% of the compactin used and 66% of the compactin added (Table 2). In an attempt to increase the pravastatin titer, intermittent addition of compactin was tested. An amount of 500 μg/ml was added to the flasks at 2 days, an additional 300 μg/ml of compactin was added on the third day, and then again on the fourth day. Results are shown in Table 3. The highest pravastatin concentration (821 μg/ml) and the highest biotransformation yield (78% of compactin added; 80% of compactin used) were reached at 7 days. Thus, adding compactin in shots does not markedly affect the bioconversion yield but does lead to a higher final pravastatin concentration.

Increased concentrations (25–100 mg/ml final concentration) of glucose, added at time of inoculation, slowed the uptake of compactin

Table 3
Bioconversion with intermittent addition of compactin[a]

Days after first compactin addition	Total compactin added (mg/ml)	Compactin (μg/ml)	Pravastatin (μg/ml)	Bioconversion	
				Of compactin added (%)	Of compactin used (%)
0	500	481	0	0	0
1	500	0	271	57.3	57.3
1[b]	800	304	258	–	–
2	800	37	398	50.7	53.2
2[c]	1100	299	380	–	–
3	1100	146	479	46.0	50.2
4	1100	86	536	51.2	52.9
5	1100	70	585	55.9	56.8
6	1100	65	684	65.3	66.1
7	1100	78	821	78.4	80.3

[a]Compactin was added at 500 μg/ml after 2 days of growth; at 300 μg/ml one day later and at 300 μg/ml one day later.
[b]Immediately after second compactin addition.
[c]Immediately after third compactin addition.

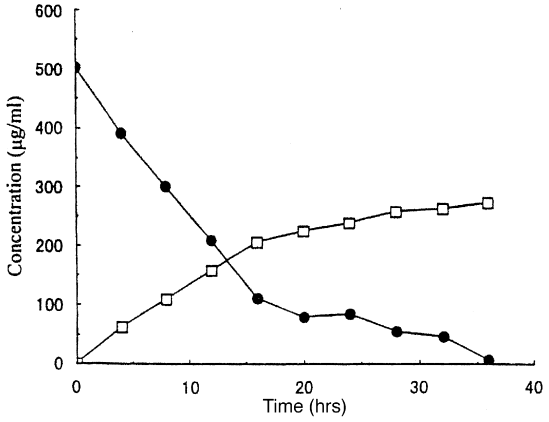

Fig. 1. Bioconversion of compactin (●) to pravastatin (□) by *Actinomadura* sp. over the course of 36 h.

from the medium but did not modify the yield of pravastatin based on compactin taken up.

Pravastatin production was examined at short intervals (3–6 h) during a 36-h bioconversion of 500 μg/ml compactin (Fig. 1). It can be seen that pravastatin production and compactin consumption were linear for the first 16 h, after which the conversion slowed down.

4. The hydroxylase system in cell-free extracts of *Actinomadura* sp. converting compactin to pravastatin

The conversion activity of cell-free extracts was tested using NADH or NADPH [29]. Table 4 shows that cell-free hydroxylation activity was observed but only in the presence of a proton donor, NADPH being more effective than NADH.

Table 4
Activity of NADH and NADPH as H^+ donor in the enzymatic conversion of compactin to pravastatin[a]

Time (h)	Pravastatin (μg/ml) with		
	No H^+ donor	0.26 mM NADH	0.26 mM NADPH
5.5	0	2.8	12.8
21.5	0	2.8	11.9

[a]Compactin at 0.23 mM, 160 μl cell-free extract, 200 μl total volume, 30°C, agitated at 250 rpm.

S. carbophilus contains an inducible cytochrome *P*450 monooxygenase converting compactin to pravastatin [25,26]. To determine whether the enzyme in *Actinomadura* sp. is inducible or constitutive, we tested the effect of growth in the presence of compactin on the subsequent cell-free conversion reaction. We found that prior exposure to compactin had no effect, thus indicating that the *Actinomadura* hydroxylase is constitutive.

A number of factors were tested in the cell-free conversion reaction. As shown in Table 5, ATP plus ascorbic acid markedly enhanced the conversion. $MgCl_2$ showed a mild stimulation, whereas Fe^{2+} and Fe^{3+} had no effect on the conversion. ATP may provide energy for the reaction and ascorbic acid is a reducing agent often required for dioxygenase reactions. Fe^{2+} and Fe^{3+} were tested since they are in the active site of many oxygenases.

To further study the importance of magnesium for enzyme activity, the effect of ethylenediaminetetraacetic acid (EDTA) on enzyme activity was tested. The metal chelator was added to the cell-free extract in different concentrations and incubated for 10 min before the reaction was started. Magnesium was excluded from the reaction mixture. The reaction was inhibited by increasing concentrations of EDTA from 0.1 to 2.0 mM (data not shown) presumably by removal of Mg^{2+} carried over from cells and present in the cell-free extract. The inhibitory effect of EDTA preincubation was reversed by adding $MgCl_2$ to the reaction mixture. A similar

Table 5
Stimulation of hydroxylation by $MgCl_2$ and ATP plus ascorbic acid[a]

Additive	Concentration (mM)	Pravastatin (μg/ml)
None	–	3.2
$MgCl_2$	2.4	4.4
ATP + ascorbate	1.9 + 12.0	11.3
$FeSO_4$	0.09	2.3
$FeCl_3$	0.1	3.3

[a]Compactin at 0.23 mM, NADPH at 0.72 mM, 160 μl cell-free extract, 200 μl total volume, 30°C, agitated at 250 rpm, 30 min.

reversal was observed with $MnSO_4$, showing that Mn^{2+} could replace Mg^{2+} in the conversion reaction.

In order to determine whether or not the hydroxylase is a heme protein, the effect of CO on hydroxylase activity was tested. A heme-containing *P*450 enzyme would be expected to be inhibited by CO. Our cell-free extract was gently flushed with CO for 30 s before testing its activity. The subsequent conversion reaction was not influenced by the CO treatment [29]. Thus, the hydroxylase from *Actinomadura* sp. differs from *P*450 system in *S. carbophilus* in the following ways: (i) it does not need induction; (ii) it is stimulated by ATP and ascorbic acid; and (iii) it is not inactivated by CO.

5. Development of a chemically defined medium

For future work on factors which affect the bioconversion, it is desirable to have a chemically defined medium [31]. When *Actinomadura* sp., previously grown in the complex YM medium, was inoculated into chemically defined medium A (per liter: sucrose 30 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, salt solution 1 ml which contained per liter $FeSO_4 \cdot 7H_2O$ 1.0 g, $MnCl_2 \cdot 4H_2O$ 1.0 g, $ZnSO_4 \cdot 7H_2O$ 1.0 g, $CaCl_2$ 1.0 g), the organism grew well. However, subsequent inoculation from this medium A culture to another medium A flask failed to sustain growth. A vitamin mixture was used to examine the growth factor requirements. Without the addition of vitamins, the culture grew only slightly. The light growth was probably the result of utilization of a small amount of vitamins carried over from the YM medium. The addition of vitamins markedly increased growth, showing that one or more vitamins were needed. Vitamins were removed sequentially from the medium and the resulting media tested for support of growth. We found that removal of pyridoxine, riboflavin, biotin, pantothenic acid, myo-inositol, ascorbic acid, choline and niacin had no effect. However, removal of thiamine decreased growth and removal of thiamine and *para*-aminobenzoic acid (PABA) virtually eliminated growth. Since PABA is a precursor of folic acid, the effect of folic acid, thiamine and B_{12} combinations was tested for support of growth in sequential cultures. The results showed that only the medium supplemented with thiamine + folic acid + B_{12} could support growth for four sequential cultures. Thus, chemically defined medium B was developed which is medium A plus thiamine, PABA and vitamin B_{12}, each at 10 mg/l. Cells grown in medium B were as capable as cells from complex medium in producing pravastatin from compactin.

6. Discussion

Actinomadura sp. ATCC 55678 has a strong ability to convert compactin to pravastatin. The conversion reached a level greater than 50% in 1 day. At later times, up to 78% of the compactin added and up to 88% of the compactin used were converted to pravastatin. Increasing the glucose concentration in the culture interfered with compactin uptake but did not affect the percent bioconversion of compactin taken up, suggesting that the compactin was not used by cells as a carbon source and that the bioconversion of compactin taken up was not repressed by glucose. Increased charges of compactin added at the normal time of compactin addition or intermittently were converted very well, yielding higher pravastatin concentrations. To our knowledge, the extent of bioconversion effected by *S. carbophilus* has not been published. Bioconversion started immediately after compactin was added into the culture, whether or not the culture was previously exposed to compactin, indicating that induction of the hydroxylase was not required. Hydroxylation of compactin to pravastatin in *S. carbophilus* is carried out by a cytochrome *P*450 system which must be induced with compactin [25,26]. Cy-

tochrome *P*450 systems found in actinomycetes active on other substrates also require induction [32].

Cell-free extracts made from *Actinomadura* sp. were able to hydroxylate compactin to pravastatin. The hydroxylase required NADPH and was stimulated by ATP, ascorbate and Mg^{2+} as cofactors; Mn^{2+} could replace Mg^{2+}. The enzyme thus differs from the cytochrome *P*450 hydroxylating system which catalyzes the conversion of compactin to pravastatin in *S. carbophilus* in the following ways: (i) it does not need induction; (ii) it is stimulated by ATP and ascorbic acid. Unlike most heme enzymes including *P*450 monooxygenases [33], it is not inactivated by CO. These properties suggest that the *Actinomadura* enzyme is not only different from that in *S. carbophilus* but is also a unique hydroxylase [32,34].

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