AMMONIUM ION SUPPRESSES THE BIOSYNTHESIS OF TYLOSIN AGLYCONE BY INTERFERENCE WITH VALINE CATABOLISM IN *STREPTOMYCES FRADIAE*

Sir:

We have reported that tylosin production by *Streptomyces fradiae* is inhibited by ammonium ion. The inhibition appears to occur at a step before the formation of the aglycone moiety, protylonolide. Protylonolide biosynthesis by a mutant of *S. fradiae* was enhanced under ammonium ion-depressed conditions produced in the presence of an ammonium ion-trapping agent such as natural zeolite[1]. A recent paper from this laboratory revealed that protylonolide biosynthesis involved valine metabolism[2]. The amino acid is deaminated and decarboxylated to give *iso*-butyrate, which is then isomerized to *n*-butyrate, a direct precursor for protylonolide biosynthesis. This finding prompted us to examine if there is a correlation between the inhibitory effect of ammonium ion on tylosin production and valine catabolism, since amino acid degradation is under the general control by ammonium ion in enteric bacteria[3]. This communication presents evidence that ammonium ion suppresses protylonolide production by inhibition and repression of valine dehydrogenase in *S. fradiae*.

A protylonolide-producing mutant, strain KA-427-261, of tylosin-producing *S. fradiae* was grown at 27°C for 7 days in Sakaguchi flasks containing 100 ml of defined medium (2% starch, 0.5% glucose, 0.17% ammonium sulfate, 0.55% (v/v) of 50% sodium lactate, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$, 3 mg each of trace metals (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}) per liter, pH 7.0 before sterilization). When an increase of ammonium ion concentration was required, ammonium sulfate was further supplemented. Two flasks were withdrawn each day and were subjected to various analyses. The amounts of protylonolide and of ammonium ion were determined as described previously[1]. Cell-free extracts were prepared as follows. The mycelia obtained by centrifugation (5,000 rpm, 10 minutes at 5°C) were suspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol, and disrupted by sonication (100 W, 5 minutes at 2~5°C), then centrifuged (21,000×*g*, 30 minutes at 2°C). The supernatant fluid (2~4 ml) was dialyzed at 7°C for 5 to 16 hours against two changes of one liter of the same buffer. A pale brown solution thus obtained was used as enzyme source. The amount of protein was estimated by the LOWRY's method[4]. Valine dehydrogenase activity was assayed essentially according to the method of OHSHIMA *et al.* originally described for leucine dehydrogenase[5]. The reaction mixture in a total volume of 1.5 ml contained: 3-cyclohexylaminopropanesulfonic acid (CAPS, Dotite Co.), 107 mM; L-valine, 20 mM; NAD, 0.4 mM; and enzyme protein, 0.5~1.5 mg. The final pH was 10.6. The reaction was allowed to proceed at 25°C. The increase of NADH was monitored at 340 nm. One unit of activity is defined as the amount of enzyme which results in an increase in A_{340} of 0.001 per minute.

Fig. 1-A shows that when the ammonium ion concentration was elevated, mycelial growth increased. Ammonium ion was consumed by the second day in the control medium (Fig. 1-B). It disappeared later or remained throughout the fermentation at higher ammonium sulfate concentrations. Protylonolide production began at the second day, and increased up to the seventh day (Fig. 1-C). In media with higher levels of ammonium sulfate, specific production of protylonolide *i.e.* amount of protylonolide per mg of dry mycelial weight, declined markedly.

In the cell-free extracts of the cultures, NAD(P)-dependent valine dehydrogenase activity was detected. This enzyme is most probably responsible for the following reaction:

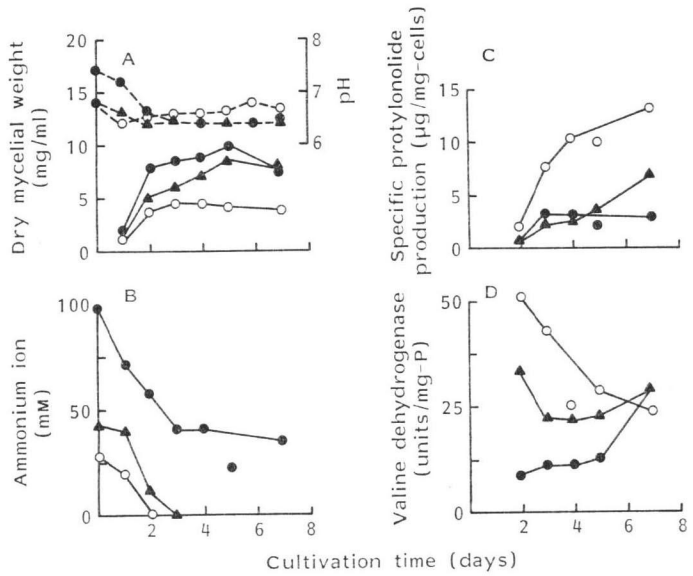
L-Valine + $NAD(P)^+$ + H_2O →
2-keto-*iso*-valerate + NH_3 + NAD(P)H

NAD and NADP were nearly equally effective as cofactors. L-Valine was the best substrate for NAD reduction, although L-leucine and L-isoleucine served as substrates to one third or lesser extents. The optimal pH for activity was approximately 10.6 with L-valine as substrate. These properties largely resemble those of leucine dehydrogenase from *Bacillus* which converts L-leucine to 2-keto-*iso*-caproate[5]. Fig. 1-D shows that at high ammonium ion concentration, intracellular valine dehydrogenase activity

Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XXVIII. Part XXVII see reference 1.

Fig. 1. Effects of ammonium ion on protylonolide production and on valine dehydrogenase formation by *S. fradiae* KA-427-261.

A protylonolide-producing *S. fradiae* mutant, strain KA-427-261, was grown in defined medium containing 25 mM (○), 50 mM (▲) or 100 mM (●) of ammonium ion. An aliquot was taken from each flask and assayed for (A), cell growth (——) and pH (------); (B), residual ammonium ion concentration; (C), specific protylonolide production and (D), intracellular valine dehydrogenase activity. Mean values of duplicate flasks are shown.



declined as did protylonolide production in the early idiophase. The enzyme activity increased later in the fermentation. The mechanism of this later increase is not known. While valine dehydrogenase activity was lowest at 100 mM ammonium ion (Fig. 1-D), specific protylonolide production was almost the same at 50 mM and 100 mM ammonium ion early in the idiophase (Fig. 1-C). Although the reason for this is unclear, the final specific protylonolide production was lower under the higher ammonium condition. The *in vitro* activity of valine dehydrogenase was inhibited by about 50% and nearly 100% at 25 and 100 mM of ammonium ion, respectively. These results suggest that valine dehydrogenase is one of the targets of the inhibitory action of ammonium ion in protylonolide biosynthesis. With cells of the parent strain, KA-427, valine dehydrogenase activity was lowered when tylosin production was inhibited by high levels of ammonium ion.

Supporting evidence of the above view was obtained with blocked mutants. *S. fradiae* KA-427-261 was irradiated with UV light. Three colonies out of 10^4 were selected which could grow on ammonium ion but could not on L-valine as sole nitrogen source. They were defective in valine dehydrogenase activity and produced little protylonolide (Table 1). One mutant, No. 210, grew on valine better than the parent. It exhibited higher valine dehydrogenase

Table 1. Protylonolide production by mutants of *S. fradiae* KA-427-261 altered in intracellular valine dehydrogenase activity.

Strain (No.)	Packed cell volume (ml/10 ml)	pH	Valine dehydrogenase activity (units/mg-P)	Protylonolide produced (μg/ml)
119	1.2	6.4	2.7	<1
257	1.4	6.4	0.0	<1
261	1.0	7.2	2.9	<1
210	2.1	6.6	13.6	77
Parent	1.8	6.4	9.5	44

The fermentation was carried out at 27°C for 6 days in Sakaguchi flasks containing 100 ml of a defined medium.

Cell growth levels measured by packed cell volume and by protein content (mg/ml) were substantially parallel.

activity, and produced a larger amount of protylonolide. This is strongly suggestive that the route of valine catabolism *via* valine dehydrogenase-catalyzed oxidative deamination is the major pathway in supplying a key precursor for protylonolide biosynthesis.

Valine is metabolized through an alternative route in which transaminase is concerned. Other amino acids, organic acids or fatty acids possibly participate in protylonolide biosynthesis. The role of these substances in tylosin production and in its regulation will be described in a forthcoming paper.

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