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L-Tyrosine oxidative metabolism by *Streptomyces aureofaciens*

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Summary. L-Tyrosine metabolism found in normal mammalian system via homogentisic acid was investigated in *Streptomyces aureofaciens* S-834 as L-tyrosine was shown to possess the stimulatory role in the production of chlortetracycline antibiotic. L-Tyrosine as well as the substrates of the intermediary oxidative enzymes-p-hydroxyphenylpyruvate (p-HPPA) hydroxylase and homogentisate (HGA) oxygenase were incubated with the washed, resting cells of the culture and by adding sodium diethyldithiocarbamate and α,α'-dipyridyl, the specific inhibitors of these enzymes to the culture, the oxidative intermediates-p-HPPA and HGA were blocked from further utilisation and were accumulated and isolated by extraction with the appropriate solvent system. The intermediates consisted of keto acids and aromatic phenolic acids were separated by adsorption and elution on silica gel and sephadex gel columns. p-HPPA and acetoacetate (AA) were identified as keto acids by obtaining their 2,4-dinitrophenylhydrazone derivatives. HGA as well as p-HPPA were identified as phenolic acids along with HGA lactone using diazotized reagents. These investigations helped to establish L-tyrosine oxidative pathway in *Streptomyces aureofaciens* via p-HPPA, HGA and AA leading to the biosynthesis of chlortetracycline antibiotic.

Keywords: Amino acids – L-Tyrosine metabolism – p-Hydroxyphenylpyruvate – Homogentisate – Chlortetracycline biosynthesis

Introduction

The stimulation of chlortetracycline antibiotic by L-tyrosine in *Streptomyces aureofaciens* has been shown in this laboratory by Lawate and Maladkar (1960) and by other workers (Zelinka et al., 1962). However, this stimulation was found in complete fermentations comprising of synthetic medium and complex medium respectively. It was not known whether the stimulation was due to increase in growth or the role of L-tyrosine as precursor participating in the building up

of tetracycline molecule. Later during the investigations on the stimulatory effects of aromatic amino acids on chlortetracycline production by the washed, resting cells of *S. aureofaciens* S-834, Maladkar and Deshpande (1978) showed that L-tyrosine produced maximum stimulation of the antibiotic (68%) followed by L-phenylalanine and L-tryptophan which were 52% and 28% respectively. The stimulation was more than the one produced by glucose (26%) added in equimolar quantities and the increase in stimulation was linear with the increase in the concentration of L-tyrosine added externally from 2.5 to 25 mM. It was also observed that in all the incubations, glutamic acid was significantly present at the end of growth phase. The stimulation of antibiotic by L-tyrosine shown in washed, resting cells of the producing culture had marked significance because in the procedure of washed, resting cells technique, growth phase of the organism is separated from the production phase and all the metabolic transformations taking place during production phase contribute to the production of the antibiotic alone which is a secondary metabolite.

Out of major established pathways by which L-tyrosine is metabolished in mammals, bacteria and fungi forming lignins, epinephrine, melanin, thyroxine and acetoacetate, an oxidative pathway in normal human system operating via p-hydroxyphenylpyruvate (p-HPPA), homogentisate (HGA) and acetoacetate (AA) deserved attention for the present studies. In this pathway wellknown for human genetic disease "alcaptonuria", L-tyrosine is transaminated to form p-HPPA and glutamic acid (La Du et al., 1958) indicating that such metabolism might be operating in *S. aureofaciens*. Hence, this pathway was studied by applying the Warburg Oxygen uptake system *in vivo* (Knox, 1955) which led to the presence of the intermediary enzymes of L-tyrosine oxidative pathway-namely p-HPPA hydroxylase (EC 1.14.2.2), HGA oxygenase (EC 1.13.1.5), maleylacetoacetate isomerase (EC 5.2.1.2) and fumarylacetoacetate hydrolase (EC 3.7.1.2) as reported earlier by Maladkar (1984). A technique was further developed to block the oxidative intermediates serving as the substrates of these enzymes by adding sodium diethyldithiocarbamate (DEDTC) (Hager et al., 1957) and α, α'- dipyridyl (Suda et al., 1951) which are the specific inhibitors of the enzymes catalysing their oxidation. These intermediates were then isolated and characterised by chromatographic and spectrophotometric methods.

The present communication describes these isolation and characterization studies in order to establish L-tyrosine oxidative pathway via p-HPPA and HGA and which would unravel the mechanism of stimulation of chlortetracycline antibiotic produced by *S. aureofaciens*.

Material and methods

Chemicals

Silica gel G was from E. Merck, Darmstadt. p-Nitroaniline and sulphanilic acid were from Eastman Organic Chemicals and 2,4-dinitrophenylhydrazine was from Nutritional Biochemical Corporation. All other chemicals and solvents were of analytical grade.

Incubation

The washed, resting cells – suspensions of *S. aureofaciens* were prepared according to the procedure of Maladkar and Deshpande (1978). Na-DEDTC (1×10^{-6} M) and α, α'-dipyridyl

(1×10^{-3} M) were added to the separate incubations containing L-tyrosine (1×10^{-2} M) to block p-HPPA and HGA from further utilisation and to accumulate them in the broth. The incubations were terminated at 10, 20 and 30 h by acidifying to pH 2.0 with 10 N H_2SO_4. After centrifugation for 30 min, the clear supernatant was separated from the cells.

Isolation of phenolic acids

The phenolic acids of L-tyrosine metabolism were isolated by successive extraction of acidified incubation samples with ethyl acetate (Armstrong et al., 1956). They were later adsorbed on silica gel and eluted by two bed volumes of each of benzene, ether, ethanol and ammonia solution successively. The eluted fractions were evaporated to dryness by flash evaporation and the residues were dissolved in 95% ethanol for identification.

The ethyl acetate extract and eluates of silica gel column were analysed by ascending paper chromatographic method (Smith et al., 1969a). After spraying with diazotized sulphanilic acid and p-nitroaniline reagents, the phenolic acids were located as bright and characteristic coloured spots due to the formation of azo-dyes with clear separation.

Isolation of keto acids

Keto acids of L-tyrosine metabolic pathway which were blocked from being further utilised by using the inhibitors were separated and identified as 2, 4-dinitrophenylhydrazones by observing their specific colour reactions and R_F values (Smith et al., 1969b).

Results and discussion

Phenolic acids of the pathway

The isolated compounds were characterised by two methods, paperchromatography and spectrophotometry. R_F values of phenolic acids – p – HPPA and HGA and the characteristic colour reaction responses after diazotization are shown in Table 1. HGA was distinctly marked with a typical pink colour with diazotized sulphanilic acid reagent. The incubation sample also showed the presence of HGA lactone as ethyl acetate extraction of HGA was followed by extraction with 10% $NaHCO_3$ solution which induced lactone formation (Fig. 1).

For the separation of HGA from p-HPPA, an advantage of the difference in their solubilities was taken. HGA is insoluble in benzene while p-HPPA is sparingly soluble. p-HPPA was obtained from benzene eluate of silica gel

Table 1. Characterization of oxidative intermediates as diazotised compounds by paper chromatographic method

Sl. No.	Name of Compound	R_F		Colour reaction	
		Extraction system	Diazotised system	a	b
1	p-HPPA	0.84, 0.90	0.73	Orange, violet	Orange, brown
2	HGA	0.72	—	Pink	Light brown
3	HGA Lactone	0.53	—	Pink	Light brown
4	Acetoacetate	—	0.71, 0.75	—	Yellow

The solvent system contained butanol – pyridine – dioxane – water (70 : 20 : 5 : 5). Diazotized reagents for spraying were (*a*) sulphanilic acid and (*b*) p-nitroaniline.

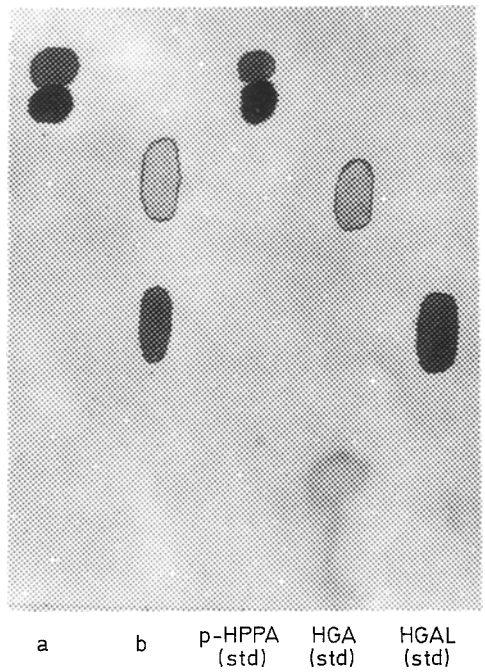

Fig. 1. Identification of phenolic acids, p-HPPA and HGA obtained from the eluates of silica gel column, sample *a* is benzene eluate of silica gel column and sample *b* is water soluble fraction obtained after excluding ether eluate through sephadex G-25. Solvent system for ascending chromatography: Butanol-pyridine-dioxane-water (70 : 20 : 5 : 5). Reagents: Diazotized sulphanilic acid and p-nitroaniline

Table 2. Characterization of oxidative intermediates by spectrophotometric method

Sl. No.	Name of compound	Incubation period (h)	Absorption (nm)
1	p-HPPA	10	300
2	HGA	20	295
3	Acetoacetate (Diazotized)	30	440

column showing maximum absorption peak at 300 nm. HGA was isolated from ether eluate of silica gel column, purified by gel filtration on sephadex G-25 and eluted by water. It showed a characteristic elution pattern at 295 nm. Table 2 shows the absorption maximum values of all these isolated compounds. The presence of HGA in L-tyrosine metabolism was shown earlier in the urine of guinea pig treated with α,α'-dipyridyl after the administration of L-tyrosine (Suda et al., 1951).

Keto acids of the pathway

p-HPPA was located at R_F value, 0.73. Acetoacetate, an end product of L-tyrosine oxidative metabolism was located at two R_F values, 0.71 and 0.75. The

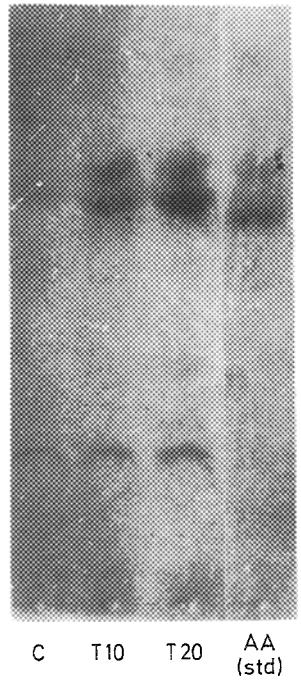

Fig. 2. Identification of acetoacetate from acidified broth samples as 2,4-DNPhydrazone. Control sample represents an endogenous content of acetoacetate. Samples *T10* and *T20* were drawn from the resting cells incubations containing L-tyrosine at the incubation period of 10 and 20 h

control experiment which did not contain L-tyrosine also showed an endogenous presence of acetoacetate. In incubations with L-tyrosine alone, the presence of acetoacetate was distinctly greater than the control (Fig. 2).

Thus, by using DEDTC and α, α'- dipyridyl, the specific inhibitors of p-HPPA hydroxylase and HGA oxygenase respectively in the incubations of the washed, resting cells of *S. aureofaciens* with L-tyrosine as the substrate, it is observed that p-HPPA and HGA were blocked from further utilisation and they were accumulated. These were identified as phenolic acids along with HGA lactone and p-HPPA as keto acid in addition. Acetoacetate in greater amounts was identified as a keto acid in L-tyrosine incubations in contrast to the trace presence of it in the control experiment. L-Tyrosine oxidative pathway operating via p-HPPA, HGA and acetoacetate, has been established in *S. aureofaciens* for the first time.

The further sequence of reactions involved in acetoacetate metabolism by *S. aureofaciens* has been also elucidated by Maladkar (1985). On the basis of the experimental evidence, the scheme is presented in Fig. 3, starting from the activation of acetoacetate and ending up in the building of chlortetracycline molecule. In this scheme, acetoacetate obtained as end product of L-tyrosine oxidative metabolism in turn is activated and cleaved into two moles of acetyl-CoA. Finally, acetyl-CoA is carboxylated to give malonyl-CoA which is the condensing principle in the biosynthesis of the carbon skeleton of tetracycline structure via polyketide (Gatenbeck, 1962) and 6-methylpretetramide intermediate (McCormick, 1966). In conclusion, the evidence of the presence of

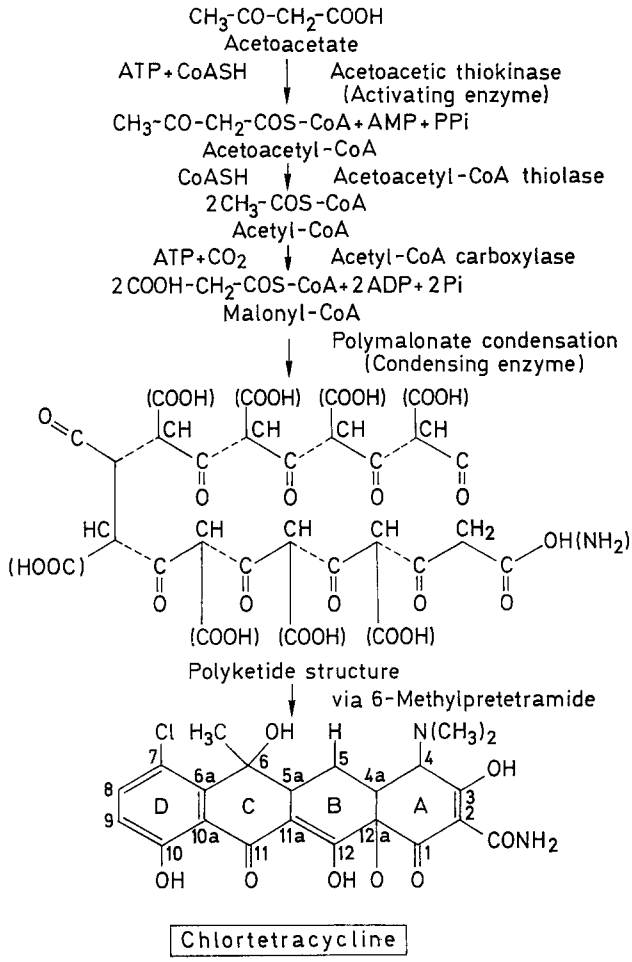

Fig. 3. Mechanism of stimulation of chlortetracycline antibiotic by L-tyrosine via p-HPPA, HGA, AA and then through polyacetate and polymalonate condensation

L-tyrosine oxidative pathway has opened up the doors of unravelling the mechanism of stimulation of chlortetracycline antibiotic produced by *S. aureofaciens.*

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References

Armstrong MD, Shaw KNF, Wall PE (1956) J Biol Chem 218: 293–303
Gatenbeck S (1962) Biochem Biophys Res Commun 6: 422–426
Hager SE, Gregerman RI, Knox WE (1957) J Biol Chem 225: 935–947

Knox WE (1955) In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol. 2. Academic Press, New York, pp 287–289
La Du BN, Zannoni VG, Laster L, Seegmiller JE (1958) J Biol Chem 230: 251–260
Lawate WV, Maladkar NK (1960) Hindustan Antibiot Bull 3: 64–65
Maladkar NK, Deshpande VN (1978) Indian J Exp Biol 16: 394
Maladkar NK (1984) Hindustan Antibiot Bull 26: 81–95; Chem Abstr (1985) 103: 19575 h
Maladkar NK (1985) Hindustan Antibiot Bull 27: 1–15; Chem Abstr (1986) 105: 39028b
McCormick JRD (1966) Biosynthesis of the tetracyclines: an integrated biosynthetic scheme. In: Herold M, Gabriel Z (eds) Proceedings of the congress on antibiotics, Prague 1964. Butterworth, London, pp 565–574
Smith I, Seakins JWT, Dayman J (1969a) In: Smith I (ed) Chromatographic and electrophoretic techniques, 3rd edn., vol 1. William Heinemann, Medical Books Ltd., London, pp 364–389
Smith I, Smith MJ (1969b) In: Smith I (ed) Chromatographic and electrophoretic techniques, 3rd edn., vol 1. William Heinemann, Medical Books Ltd., London, pp 330–341
Suda M, Takeda Y, Sujishi K, Tanaka T (1951) J Biochem (Tokyo) 38: 297–302
Zelinka Ya, Kovachichova L, Gudets M (1962) Mikrobiologiya 31: 816–818

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