Phytochemistry, 1969, Vol. 8, pp. 1407 to 1413. Pergamon Press. Printed in England.

PHENYLALANINE AND TYROSINE AMMONIA LYASE ACTIVITY IN *SPOROBOLOMYCES ROSEUS*

E. L. CAMM and G. H. N. TOWERS

Botany Department, The University of British Columbia, Vancouver 8, B.C., Canada

(*Received* 11 *March* 1969)

Abstract—Peak production of phenylalanine and tyrosine ammonia lyase activity occurs during late logarithmic phase in the growth of batch cultures of the yeast, *Sporobolomyces roseus*. There is some evidence that two enzymes are involved although the production and activity of each enzyme appears to be under common control. Replacement media containing either phenylalanine or tyrosine stimulate production of both enzymes whereas cinnamic acid represses their formation. The activities of both enzymes are also inhibited by cinnamic or *p*-coumaric acids.

INTRODUCTION

A CONSIDERABLE amount of information is now available concerning the ammonia lyases for phenylalanine and tyrosine (PAL and TAL respectively), enzymes which appear to be confined to higher plants and to certain fungi. In higher plants these enzymes appear to be a link between aromatic amino acid metabolism and much of the secondary biosynthetic activity of the plant. In fungi they appear to be involved in catabolic processes as well.[1] Factors affecting the stimulation or repression of enzyme activity or synthesis have been studied in plants[2–6] and in a yeast.[7] Methods for the preparation of purified phenylalanine ammonia lyases from a number of sources have also been published.[6, 8–14]

In much of the work on phenylalanine ammonia lyase it is not clear whether the enzymes under discussion are specific for phenylalanine or whether tyrosine may also serve as substrate. In the yeast, *Rhodotorula glutinis*, the inducible PAL and TAL activity appears to be due to a single enzyme. On the other hand, the activity of purified PAL from sweet potatoes[6] and from *Ustilago hordei*[13] is restricted to phenylalanine. There is also evidence for a tyrosine specific enzyme in barley,[12] sweet potatoes[6] and in wheat.[14] Indirect evidence that separate enzymes are involved comes from measurements of PAL and TAL activities in various parts or organs of higher plants.

We have studied some of the factors controlling the levels of these enzymes in the yeast,

[1] K. MOORE, P. V. SUBBA RAO and G. H. N. TOWERS, *Biochem. J.* **106**, 507 (1968).
[2] L. L. CREASY, *Phytochem.* **7**, 441 (1968).
[3] H. SCHERF and M. H. ZENK, *Z. Pflanzenphysiol.* **56**, 203 (1967).
[4] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).
[5] G. ENGELSMA, *Planta* **75**, 207 (1967).
[6] T. MINIMIKAWA and I. URITANI, *J. Biochem.* **57**, 678 (1965).
[7] K. OGATA, K. UCHIYAMA and H. YAMADA, *Agr. Biol. Chem.* **31**, 200 (1967).
[8] K. OGATA, K. UCHIYAMA, H. YAMADA and T. TOCHIKURA, *Agr. Biol. Chem.* **31**, 600 (1967).
[9] E. HAVIR and K. R. HANSEN, *Biochemistry* **7**, 1896 (1968).
[10] H. V. MARSH, JR., E. HAVIR and K. R. HANSEN, *Biochemistry* **7**, 1915 (1968).
[11] J. KOUKOL and E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[12] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[13] P. V. SUBBA RAO, K. MOORE and G. H. N. TOWERS, *Can. J. Biochem.* **45**, 1863 (1967).
[14] M. R. YOUNG and A. C. NEISH, *Phytochem.* **5**, 1121 (1966).

Sporobolomyces roseus. This report provides additional indirect evidence that PAL and TAL are distinct enzymes in this organism.

RESULTS

The stationary phase of growth of *Sporobolomyces roseus* in batch culture was achieved in about 72 hr. After this time the formation of buds and pseudomycelia made absorbance measurements and cell counts meaningless. Maximum PAL and TAL activity of cells (expressed as units per milligram of protein in buffer extracts) occurred just after the beginning of the stationary phase (Fig. 1).

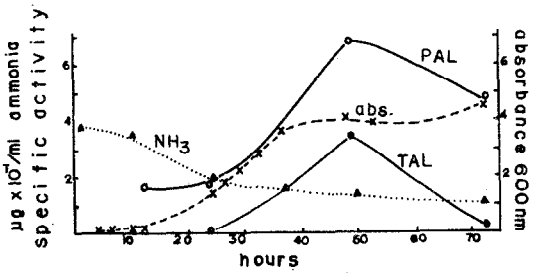

FIG. 1. ENZYME PRODUCTION, AMMONIA UTILIZATION AND INCREASE IN CELL NUMBER IN *Sporobolomyces roseus* GROWN ON 1% GLUCOSE AND SALTS.

PAL activity —○— (units per mg protein); TAL activity —●— (units per mg protein); absorbance at 600 nm – – × – –; ammonium ion . . . ▲ . . . ($\mu g \times 10^{-1}$ per ml).

TABLE 1. EFFECT OF GLUCOSE CONTENT OF GROWTH MEDIUM ON THE SPECIFIC ACTIVITY OF PAL AND TAL IN *Sporobolomyces roseus* CELLS*

Original glucose content of medium (%)	Specific activity†	
	PAL	TAL
0·1	44·3	19·8
1	6·7	3·4
2	1·1	0·0

*Cells harvested in early stationary phase.
† Specific activity—units per mg protein.

Table 1 shows that the highest specific activities of PAL and TAL were obtained from cells grown in the presence of a low concentration of glucose. The addition of L-tyrosine to the medium at concentrations up to saturation (about 0·1 per cent) increased the production of PAL and TAL (Fig. 2). The stimulation of enzyme production was proportionately lower at higher tyrosine concentrations and the ratio of specific activities also varied.

Cultures which had reached the stationary phase resumed growth when washed and resuspended in fresh media containing glucose plus some other carbon source. Figure 3 shows that the levels of PAL and TAL increased significantly when cells were resuspended in media which included low levels of either tyrosine or phenylalanine. The addition of *p*-coumarate to the medium had no effect on enzyme levels while cinnamic acid caused a marked decrease in the activity of both PAL and TAL (Fig. 4).

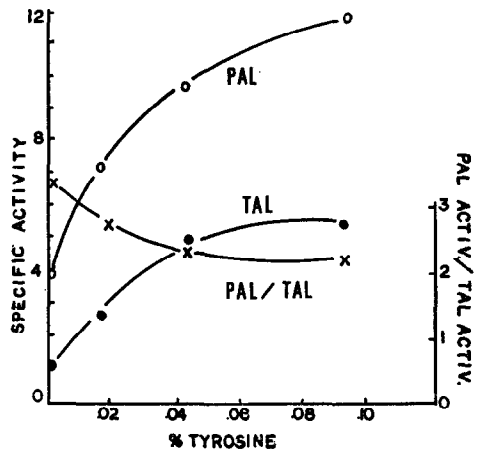

FIG. 2. ENZYME PRODUCTION AND RATIOS OF SPECIFIC ACTIVITIES IN 48-hr *Sporobolomyces roseus* CELLS GROWN ON 1% GLUCOSE AND SALTS WITH VARIOUS LEVELS OF ADDED TYROSINE.

PAL activity —○— (units of activity per mg protein); TAL activity —●— (units of activity per mg protein); ratio of the two specific activities —×—.

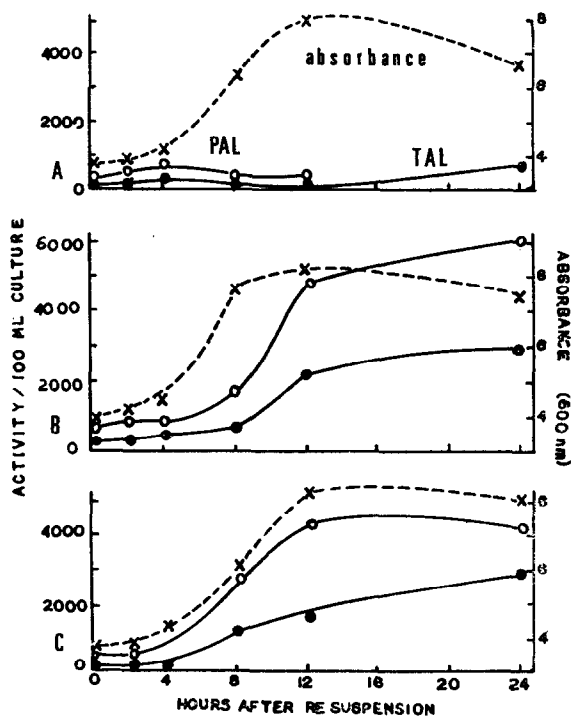

FIG. 3. GROWTH AND ENZYME ACTIVITY OF *Sporobolomyces roseus* CELLS IN REPLACEMENT MEDIUM OF 1% GLUCOSE AND SALTS.

A: glucose and salts alone; B: glucose and salts plus 0·05% tyrosine; C: glucose and salts plus 0·05% phenylalanine. (PAL activity —○—; TAL activity —●—; absorbance – – × – –.)

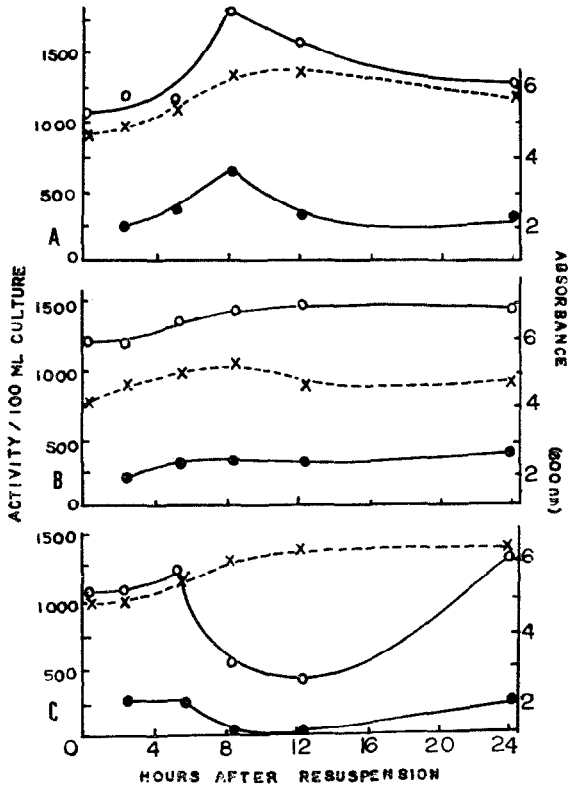

FIG. 4. GROWTH AND ENZYME CONTENT OF *Sporobolomyces roseus* CELLS IN VARIOUS REPLACEMENT MEDIA.

A: 0·05% glucose plus salts; B: 0·05% *p*-coumaric acid plus salts; C: 0·05% cinnamic acid plus salts. (PAL activity —○—; TAL activity —●—; absorbance – – × – –.)

TABLE 2. SUBSTRATE COMPETITION AND PRODUCT INHIBITION OF PAL AND TAL FROM *Sporobolomyces roseus*

	Compound added to reaction mixture	Enzyme activity as % of control
PAL assay	Control (no inhibitor)	100
	L-Tyrosine (5 μmoles)	51
	p-Coumaric acid (4 μmoles)	21
	Cinnamic acid (4 μmoles)	31
TAL assay	Control (no inhibitor)	100
	L-Phenylalanine (5 μmoles)	27
	p-Coumaric acid (4 μmoles)	17
	Cinnamic acid (4 μmoles)	0

Table 2 shows the effect of substrate competition and product inhibition of PAL and TAL. *p*-Coumarate and cinnamate were strongly inhibitory. TAL was inhibited by phenylalanine and PAL by tyrosine. Two attempts at purification of the enzymes are presented in Table 3. This Table shows that the ratio of specific activities of PAL to TAL varied in the ammonium sulfate fractions.

TABLE 3. AMMONIUM SULFATE FRACTIONATION OF PAL AND TAL FROM *Sporobolomyces roseus*

Step	Specific activity		Ratio TAL/PAL
	PAL	TAL	
Experiment 1			
1. Crude extract	1·22	0·31	0·26
2. After protamine sulfate	1·33	0·61	0·46
3. Ammonium sulfate (%)			
30–40	0·84	0·33	0·39
40–50	5·0	2·3	0·46
50–60	9·4	3·1	0·33
60–70	4·5	1·3	0·29
Experiment 2			
1. Crude extract	7·7	2·7	0·35
2. After protamine sulfate	—	—	
3. Ammonium sulfate (%)			
30–40	11·0	8·2	0·74
40–50	52·7	16·4	0·31
50–60	21·4	16·4	0·76
60–70	7·9	1·6	0·20

DISCUSSION

In *Sporobolomyces roseus*, the ratio of specific activities of PAL to TAL in buffer extracts varies according to the growth medium and to the stage of growth of the batch culture. The ratio of PAL to TAL was also different in partially purified preparations. It would appear therefore that the enzymes responsible for the deaminations of tyrosine and phenylalanine in *S. roseus* are different proteins.

On the other hand, Ogata *et al.*[8] found that the enzymes from *Rhodotorula glutinis* were not separated and that the ratio of specific activities remained constant during purification. They suggested that perhaps the PAL and TAL reactions were carried out by the same protein. In this respect, then, the *Sporobolomyces* enzymes do not resemble the *Rhodotorula* enzymes as much as they do those of sweet potato, which have been resolved into two protein fractions. The two red yeasts, *Sporobolomyces* and *Rhodotorula*, show some similarities, however, in that the peak production of the enzymes PAL and TAL occurred just as the batch culture was entering the stationary phase.

In a batch culture, the shift of growth from the logarithmic phase to the stationary phase is often correlated with the exhaustion of some nutrient. A change in metabolic activity often occurs at this time. In *Penicillium islandicum*, for example, the shift in metabolic

activity is correlated with exhaustion of ammonia in the medium.[15] In *Sporobolomyces*, however, the cultures entered stationary phase even when ammonia levels in the medium were as high as two-thirds of the original concentration, and enzyme levels rose and then fell during a time when the ammonia levels appeared to change very little (Fig. 1).

The levels of carbon as energy source (in this case glucose) in the medium had a marked effect on the levels of enzymes produced by the cells. A similar repression of catabolic enzymes by high levels of carbon and energy sources in the medium is well documented in microorganisms.[16] Most reports discuss the phenomenon as a repression of inducible enzymes. Since PAL and TAL appear in cells that have grown on glucose and salts alone, they are certainly not inducible enzymes in the classical sense, even though they are subject to catabolic repression.

Cells grown in the presence of tyrosine produced greater amounts of both PAL and TAL than did cells grown on minimal medium. However, the presence of high levels of tyrosine resulted in relatively less stimulation of enzyme production than did lower concentrations. There is a possibility that at the higher levels, the tyrosine was acting as an additional carbon source. On the other hand, the production of enzymes by the cell may be approaching the highest levels of which the cell is capable.

The experiments with cells resuspended in media containing tyrosine or phenylalanine showed that either aromatic amino acid stimulated the production of both ammonia lyases in these cells. During the growth of the culture, the enzyme content increased in terms of the whole culture, on a per cell basis or when expressed as a fraction of total cell protein. Experiments similar to these on *Rhodotorula*[7] showed a sharp increase in the amount of enzyme 4 hr after resuspension in phenylalanine followed by a slow decline.

In *Sporobolomyces*, PAL and TAL activities were effectively inhibited by cinnamic acid and to a lesser extent by *p*-coumaric acid. This is in contrast to purified PAL from *Ustilago hordei*[13] which is inhibited by cinnamic acid but not by *p*-coumaric acid or L-tyrosine. Barley PAL, on the other hand, is inhibited by both cinnamic and *p*-coumaric acids.[12]

In nature, the above fungus grows on the surfaces of leaves and other organs of higher plants. The concentrations of *p*-coumaric and cinnamic acids in this substratum are not known so that we do not know whether the fungal ammonia lyases would be repressed. Certainly during balanced growth, free phenylalanine and tyrosine would not be produced by the plant in large amounts; thus the substrates for the enzymes would be present only in low concentrations. Lee and Aronoff[17] have shown that a result of boron deficiency in higher plants is an increased production of phenolic acids. Perhaps it is in such cases of unbalanced growth of the host organism (resulting in high levels of the products of the ammonia lyases) that the control system over these enzymes becomes effective. In any event, the production of these enzymes in saprophytic fungi in relation to the metabolism of the host plant would appear to be worthy of study. The purification of a tyrosine specific ammonia lyase from either a higher plant or a fungal source is also necessary in order to clarify these problems.

EXPERIMENTAL

Culture of the Organism

Sporobolomyces roseus Kluyver and van Neil (UBC 901) was grown in shake culture at 25° in a medium containing 1% glucose and Vogel's salts.[18] Cells were also grown on the same medium with the addition of

[15] S. GATENBECK, and S. SJOLAND, *Biochim. Biophys. Acta* **93**, 246 (1964).
[16] W. F. LOOMIS and B. MAGASANIK, *J. Mol. Biol.* **8**, 417 (1964).
[17] S. LEE and S. ARONOFF, *Science* **158**, 798 (1967).
[18] H. J. VOGEL, *Microbiol. Gen. Bull.* **13** (1956).

tyrosine. Growth was followed by changes in absorbance at 600 nm. The ammonia content of the medium was measured by adding Nessler's reagent to a 1/100 dilution of the cell-free supernatant from a growing culture and reading absorbance at 500 nm. Cells were harvested by centrifugation, washed twice with distilled water and stored at $-20°$ until needed.

Enzyme Assay

The assay procedure was similar to that used for *Ustilago* PAL.[17] One unit of PAL is the amount of enzyme which catalyzes the conversion of 0·01 μmoles of L-phenylalanine to cinnamic acid in 1 hr at 37°. Similarly, one unit of TAL converts 0·01 μmoles of L-tyrosine to *p*-coumaric acid under the same conditions. Frozen cells were ground by hand with powdered alumina and extracted with twice the volume of 0·05 M tris buffer, pH 8·0. The slurry was centrifuged at 10,000 × *g* for 20 min and the supernatant was used as the crude enzyme. The assay system contained 0·5 ml of crude buffer and 5 μmoles of L-tyrosine or L-phenylalanine in 0·05 M tris buffer, pH 8·8 (total volume 3·0 ml). After incubation at 37° for 1 hr, the reaction was stopped with 1 ml of 1 N HCl. The mixture was extracted with 5 ml of peroxide-free ether, and an aliquot of this was evaporated to dryness and then taken up in 0·05 N NaOH. The cinnamic or *p*-coumaric acid formed was determined spectrophotometrically.[15,16] The method of Lowry *et al.*[19] was used for the estimation of protein.

The crude enzyme was treated with 2% protamine sulfate, and the centrifuged supernatant was used for $\delta(NH_4)_2SO_4$ fractionation. The most active fractions were de-salted on a Sephadex G-25 column. These de-salted fractions were used for studies of inhibition and competition. The usual assay was set up with the addition of 0·5 μc uniformly labelled ^{14}C L-phenylalanine or L-tyrosine to the unlabelled substrate. Potential competitors and inhibitors were added (see Table 2). After incubation, radioactivity in the ether-soluble fraction was measured with a scintillation counter and compared with the radioactivity in controls, to which no inhibitors had been added.

Studies Using Replacement Media

Cells were grown in the normal manner in 1% glucose and salts for 48 hr. The cells were harvested aseptically, washed twice with sterile distilled water and resuspended in fresh medium containing salts and one or more of several compounds as carbon source. The cultures were again incubated with shaking and aliquots were removed at various times for analysis.

Acknowledgements—We thank the National Research Council of Canada for financial support of this work and for a scholarship to one of us (E. L. Camm).

[19] O. H. LOWRY, N. ROSEBROUGH, A. FARR and R. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).