Phytochemistry, Vol. 22, No. 6, pp. 1329–1333, 1983.
Printed in Great Britain.

0031–9422/83/061329–05$03.00/0

SUPPRESSION OF CATECHOL OXIDASE BY NORLEUCINE IN PLANT SUSPENSION CULTURES

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(*Revised received* 12 *November* 1982)

Key Word Index—*Nicotiana tabacum*; Solanaceae; cell suspension culture; catechol oxidase; norleucine.

Abstract—The presence of norleucine in the culture media of tobacco cell suspensions represses catechol oxidase activity by 50–70% after 5 days without affecting growth. The catechol oxidase activity present after norleucine treatment has different properties as indicated by its K_m, heat stability and pH dependence and its response to activation. In addition, certain isoenzymes disappear and others are reduced in intensity. The effect of norleucine can be reversed by addition of methionine. It is suggested that norleucine induces the specific loss of some isoenzymes and is incorporated into others thereby changing their structure and conformation and, consequently, their activity. Norleucine may be of use in controlling catechol oxidase activity in tissue and suspension cultures.

INTRODUCTION

In two previous papers [1, 2], we showed that in apple suspension cultures the amino acid analogue ethionine specifically reduces catechol oxidase (EC 1.10.3.1) activity in cells without markedly affecting viability or growth of the tissue. Ethionine is known to be highly carcinogenic [3] and is unpleasant to handle. We, therefore, sought other possible amino acid analogues of methionine having similar effect. Norleucine is known to act similarly to ethionine as an antagonist of methionine and it has been investigated in a number of systems. Norleucine inhibited methionyl tRNA formation, but less so than ethionine [4]. *Staphylococcus* extracellular nuclease contained norleucine if the cells were fed excess norleucine in the presence of methionine [5]. Barker and Bruton [6] investigated the effect of norleucine as a methionine antagonist in *Escherichia coli*. They found that the analogue can charge tRNA Met species and is incorporated into protein. Growth and viability of the bacterium were only partly reduced. It seemed that norleucine would, therefore, be a suitable substance to try and suppress catechol oxidase activity in suspension culture in a way analogous to that of ethionine. The significance of changing catechol oxidase activity specifically is clear [7], in view of the many undesirable browning reactions catalysed by this enzyme. Furthermore, we wanted to investigate the possible mechanism by which enzyme activity is reduced. The present paper reports on this problem.

RESULTS

Preliminary experiments were made to determine whether norleucine could effect browning of apple slices. Floating the slices in 5 mM norleucine with aeration produced a visually observable reduction in browning, even after 2 hr of exposure.

This encouraged us to determine catechol oxidase. When catechol oxidase activity was determined in extracts of the slices, it was found that norleucine induced a significant drop in enzyme activity over and above the drop in the controls. This drop was partly prevented when chloramphenicol was added to the slices together with the norleucine. This indicated that protein synthesis is in some way involved in the repression of catechol oxidase activity. This was further borne out by experiments in which the effect of norleucine was examined at room temperature or at 2° in the presence or absence of aeration. In the absence of aeration or at the lower temperature no effect of norleucine was noted. It seems that aerobic metabolism was needed for the norleucine to be effective. Norleucine uptake by the slices was probably also affected by lowered temperature and anaerobiosis.

Work with tissue slices is not always convenient and ageing effects are known to occur after cutting the tissue, accompanied by various metabolic changes. We, therefore, turned to suspension cultures of tobacco for continuation of the experiments. Suspension cultures were grown and then 5 mM norleucine added and growth allowed to continue for 5 days. Growth and respiration of such cultures was only slightly reduced, while enzyme activity was markedly affected (Table 1). It is important to note that the effect of norleucine on enzyme activity was completely reversed by the addition of 6 mM methionine. Thus, norleucine presumably acts in this system also in some way at a site normally occupied by methionine. The effect of norleucine on growth and catechol oxidase activity in the cell suspension with length of culture was compared. Growth was only slightly repressed even after 10 days of culture, while the inhibition of catechol oxidase activity increased with time of culture.

In suspension cultures, as in apple slices, the effect of norleucine was partially reversed or prevented by inhibitors of protein synthesis (Table 2). However, the results are far more difficult to interpret here. They tend to support the idea that reduction of enzyme activity is linked with protein synthesis.

To determine whether the effect of norleucine was a specific one or whether other amino acid analogues had similar effects, the suspension cells were cultured in the

Table 1. Growth, respiration and catechol oxidase activity of tobacco suspension cultures after exposure to 5 mM norleucine for 5 days

	Enzyme activity (μl O_2/min·ml packed cells)		Respiration (μl/O_2 hr·ml packed cells)	Growth* (packed cell vol.) ml
	20000 g ppt	Soluble fraction		
Control	70	233	133	44.2
Norleucine treatment	27	65	124	37.7
Norleucine + methionine	66	229	—	—

*Growth as packed cell vol./65 ml culture medium.

Table 2. Effect of inhibitors of protein synthesis on the norleucine induced reduction of catechol oxidase activity in suspension cultures of tobacco

Treatment*	Activity (μl O_2/min·ml packed cells)	
	20000 g ppt	Soluble fraction
None	69	154
Chloramphenicol (300 μg/ml)	72	234
Norleucine	42	82
Norleucine + chloramphenicol	73	136
Cycloheximide (30 μg/ml)	89	180
Norleucine + cycloheximide	104	116

*Exposure to norleucine for 5 days.

presence of azetidine-2-carboxylic acid (1 mg/ml) or *p*-fluorophenylalanine (2 mg/ml), both known anti-metabolites [8]. In both cases the amino acid analogues caused rapid cell death and inhibition of growth. There was no specific effect on catechol oxidase, and reduction in its activity was simply due to cell death.

It appeared that norleucine induced changes in the catechol oxidase present in the cells or partially prevented its formation. Attempts were, therefore, made to characterize the catechol oxidase in the cells and to compare its properties in normal and norleucine treated cells.

In many tissues catechol oxidase is activated by various treatments, such as detergents or trypsin [7]. We tested this property in our enzyme preparations (Table 3). Clearly, the activity of the enzyme normally present in the cells is not changed by activators to any appreciable extent. In contrast, the enzyme present in the norleucine treated cells is markedly activated by urea and by deoxycholate and also appears to be far more sensitive to trypsin treatment, being rapidly inactivated. The enzyme preparations differ in their heat stability (Fig. 1), the enzyme from the treated cells being somewhat more sensitive to raised temperatures. The pH dependence of enzymes from the treated and control cells was different. The pH optimum in the soluble fraction of treated cells was less clear cut with no peak at pH 6.5.

Table 3. Effect of potential activators on catechol oxidase activity (μl O_2/min. ml packed cells) from normal and norleucine treated tobacco suspension cells (treatment *in vitro* as in Methods)

Treatment	20000 g ppt		Soluble fraction	
	Control	+ Norleucine	Control	+ Norleucine
None	36	14	47	24
Urea 5 min	35	18	44	39
Urea 2 hr	45	45	57	52
Digitonin	21	23	36	27
Deoxycholate	10	49	34	48
SDS	10	18	35	18
Trypsin	25	19	35	2.3

A marked difference in K_m of the enzyme from the treated cells was observed. The K_m as determined from Lineweaver–Burk plots was 3.25×10^{-3} M in the controls and 2.94×10^{-4} M in the treated cells, for enzyme in the soluble fraction.

The soluble enzyme was also characterized using acrylamide gel electrophoresis, electrophoresis on cellulose acetate and by isoelectric focusing. In all cases distinct differences were observed between the pattern of the soluble enzyme from control and treated cells. On cellulose acetate, at pH 5 and 8, one band was noted moving to the cathode in both cases, but a band moving to the anode was absent in the treated cells. Fewer enzyme bands were also noted following acrylamide electrophoresis of

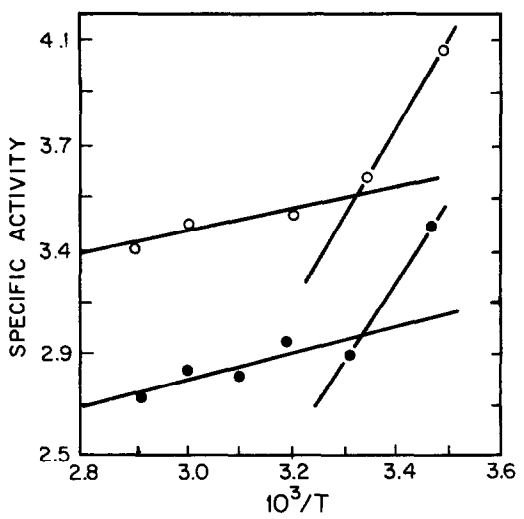

Fig. 1. Heat inactivation of catechol oxidase from normal and norleucine treated tobacco suspension cells. (Soluble fraction, length of norleucine treatment 5 days.) (○), Control; (●) norleucine treated.

extracts of the treated cells. Following isoelectric focusing a multiplicity of isoenzymes was observed with isoelectric points of 4.25, 4.6, 4.75, 4.95, 5.9 and 6.25 in the control while in the treated cells the band at 5.9 was absent, the 6.25 band was weak and instead a stronger band appeared at pH 4.95.

Estimation of the MW on thin layer Sephadex gel filtration showed that enzymes from controls migrated to a position corresponding to *ca* 60 000, while the enzyme from treated cells had an approximate MW of 30 000. Although the absolute MWs were not determined the difference between the treatments was highly significant.

All the results described might be due simply to marked changes in the subcellular location of the enzyme or due to changes in ultrastructure due to norleucine treatment. The cells in the suspension were, therefore, investigated by electron microscopy. In addition, the cells were also allowed to react with substrate in order to locate the catechol oxidase at the ultrastructural level. As can be seen from the electron micrographs in Fig. 2, the only major change resulting from norleucine treatment of the cells was a transformation of chloroplasts into amyloplasts. However, the amyloplasts retained some lamellae and catechol oxidase was clearly located in them.

DISCUSSION

The results reported here confirm our previous work on the repression of catechol oxidase activity in plant cells by the use of a methionine antagonist [1, 2]. One significant difference between the apple suspension cultures and the tobacco cells is that, in the former, short contact with ethionine was sufficient to induce a subsequent reduction in activity, while we were unable to achieve such an inductive effect with norleucine. The probable cause is that the culture medium for apples did not contain amino acids at all so that any added amino acid would be immediately taken-up and incorporated. The medium for the tobacco cells contained 600 mg/l. casein hydrolysate, and this hydrolysate contained *ca* 2.6% methionine. Therefore, the added norleucine had to compete with methionine. Anfinsen and Corley [5] used a norleucine–methionine ratio of 100 to get norleucine incorporation into nuclease. The effect of norleucine must be considered in the light of the very low methionine content of catechol oxidase, 2–5 residues per 288 amino acids [9–11] in fungal enzyme as well as in higher plant, grape, catechol oxidase [12]. The methionine concentration in the growth media was of the order of 0.1 mM, so that the ratio methionine–norleucine was *ca* 50. At this ratio norleucine was clearly non-toxic and did not affect the growth of the cells, although a change in their morphology was observed at the subcellular level. The reduction of catechol oxidase activity, which resulted from norleucine treatment, was characterized by several features. Apparently certain isoenzymes were lost entirely, presumably due to turnover not accompanied by new synthesis. Some at least of the remaining isoenzymes of catechol oxidase had altered molecular properties, in particular their heat stability and their K_m decreased. In addition, the enzyme formed in the presence of norleucine was sensitive to activation with urea and deoxycholate, which are supposed to act by changing enzyme conformation [7].

It might be suggested that all the results are simply due to supression of some catechol oxidase isoenzymes. As a result, the average properties of the remaining isozymes change. Although this could account for changed heat stability and perhaps changed K_m it cannot possibly account for the appearance of activation by urea, or increased sensitivity to trypsin. This increased trypsin sensitivity suggests a conformational change which exposes peptide bonds cleaved by trypsin. If all the results are considered together they are consistent with the view that norleucine is incorporated into the newly formed enzyme, which results in changed tertiary or quaternary structure or both. The changed enzyme has a lower activity, but higher affinity for substrates. Nevertheless, there is as yet no definite proof for this changed structure. It will be necessary to demonstrate that, indeed, catechol oxidase in the suspension cultures undergoes turnover and resynthesis and to isolate an enzyme formed in the presence of norleucine and show the presence of norleucine in it. However, already at this stage norleucine might provide a useful tool to depress the activity of an enzyme which often is troublesome while manipulating plant cell cultures. A further finding, which is of considerable importance although not apparently directly related to the effect of norleucine in catechol oxidase, is the conversion of chloroplasts in the cells to amyloplasts. This presents interesting problems in cell development in the presence of an amino acid analogue, which have not to our knowledge been reported previously.

EXPERIMENTAL

Apples. Variety 'Grand Alexander', were purchased at a local supermarket. Apple slices *ca* 5 mm thick were immersed for various periods, with aeration, in H_2O or 5 mM norleucine, at 2° or 20°. The soln contained 0.1 M sodium ascorbate, to prevent browning during immersion. After immersion the slices were dried and exposed to air, and the rate of browning followed visually.

Suspension cultures. The cultures of *Nicotiana tabacum* cv Xanthi were started from callus tissue and grown in the growth media (B5) of Gamborg *et al.* [13], without agar. The cultures were maintained in 250 ml Erlenmeyer flasks at 26° on a rotary shaker, operated at 120 cycles/min. Growth of the suspension cultures was determined by measuring their packed cell vol., following centrifugation in sedimentation tubes at 1000 *g* for 10 min.

Cell viability. Determined by staining the cells with 0.01% neutral red and observing accumulation of stain in the vacuole under the microscope.

Preparation of enzyme. Apple slices were homogenized in a blender, in 0.1 M Pi buffer, pH 7.3, containing 0.4 M sucrose and 0.1 M sodium ascorbate. The homogenate was filtered through gauze and centrifuged at 20 000 *g* for 30 min. The ppt was suspended in 0.1 M Pi buffer, pH 7.3 or pH 5.1, containing 0.4 M sucrose. The supernatant was used as source of the soluble enzyme.

Tobacco suspension cells. Disrupted and homogenized in KPi buffer, pH 7.2, containing 0.4 M sucrose and 0.1 M sodium ascorbate. The homogenate was centrifuged at 20 000 *g* for 30 min. The ppt was suspended in KPi buffer containing 0.4 M sucrose at pH 7.2 or 5.1. The supernatant was used as a source for the soluble enzyme.

Enzyme activity. Determined using a polarographic oxygen electrode using 5 mM 4-methyl catechol as substrate [14]. In some cases enzyme in the 20 000 *g* ppt was solubilized with Triton X-100. The ppt was treated with 1% Triton for 90 min at 2°, the suspension centrifuged for 30 min at 20 000 *g* and dialysed

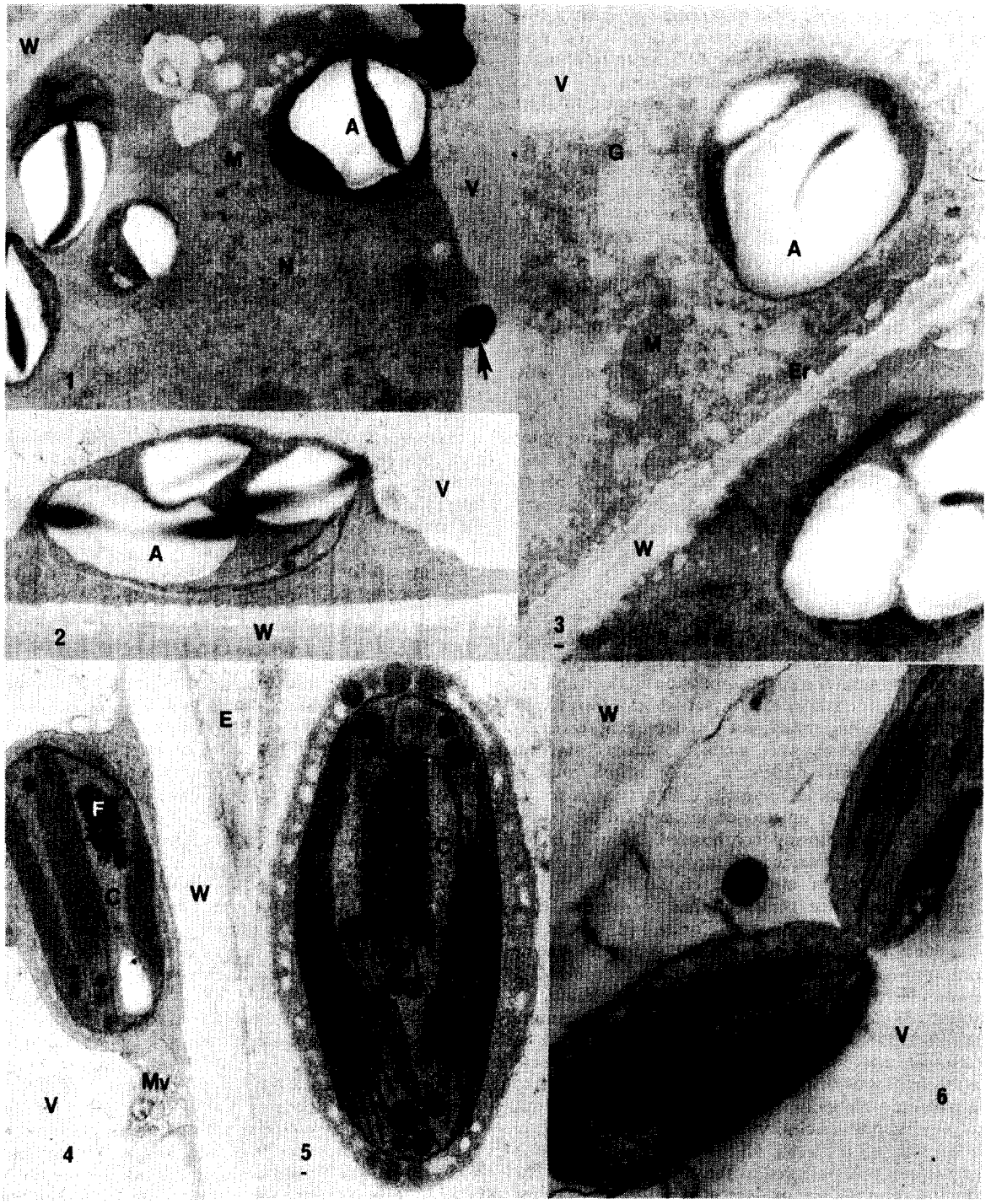

Fig. 2. (1, 2) Norleucine treated cells incubated with 3,4-dihydroxyphenylalanine (DOPA). (3) As (1, 2) but incubated in presence of DOPA together with diethyldithiocarbamate. (4, 5) Control cells incubated with DOPA. (6) As (4, 5) but incubated with DOPA + diethyldithiocarbamate. A, amyloplast; C, chloroplast; E, external environment; Er, endoplasmic reticulum; F, phytoferritin; M, mitochondria; G, Golgi body; V, vacuole; Mv, multivesicular body; W, cell wall. Magnifications: 1 × 17997; 2 × 22040; 3 × 30370; 4 × 9831; 5 × 30370; 6 × 14060.

overnight. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, the fraction ppting between 40 and 95% satn was collected, resuspended in suitable buffer and dialysed overnight and then used as enzyme source.

Enzyme activation. Suitable samples were treated with: 4 M urea for 5 min or 2 hr, or with 1% digitonin in 0.5 M NaCl, pH 7.0, for 10 min, or 1% deoxycholate for 10 min, at pH 7.3, or with 1% sodium dodecyl sulphate, pH 7.3, for 10 min, and activity was

then determined. Trypsin treatment was at pH 6.3 and 37° for 1 hr using 1 mg trypsin per 2–3 mg enzyme protein.

Heat inactivation. Determined in the usual way, taking care that samples reached the required temp. rapidly.

Electrophoresis. Carried out in acrylamide gel [15] and isoelectric focusing between pH 4 and 6 and 3 and 10 on an LKB Multiphore apparatus according to standard procedures; staining for enzyme activity being done as described by Harel and Mayer [15].

Thin layer gel filtration. On Sephadex G200 to give a rough idea of the MW of the enzyme was according to Andrews [16].

Respiration of cells. Determined by following O_2 uptake manometrically.

Electron microscopy. Carried out essentially as previously described [17]. However, when stained for catechol oxidase the glutaraldehyde fixed cell suspension was incubated overnight in 10 mM 3,4-dihydroxyphenylalanine (DOPA) in Pi–citrate buffer or DOPA + 1 mM diethyl dithiocarbamate at 4°. The cells were then rinsed and resuspended in the same solns and incubated for 2 hr at 37°. After incubation they were again rinsed, dehydrated and embedded in Spurr's embedding medium as previously described [17].

Acknowledgements—Our thanks are due to Dr. S. Trachtenberg for the electron micrographs. This work has been supported by a grant from the L. Wolfson Foundation.

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