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L-Tyrosine β-naphthylamide is a potent competitive inhibitor of tyramine *N*-(hydroxycinnamoyl)transferase in vitro

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Abstract

L-Tyrosine β-naphthylamide, a synthetic substrate designed to measure tyrosine aminopeptidase activity, is a potent inhibitor of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT) purified from elicited tobacco cell-suspension cultures. The inhibition is competitive, with the inhibitor binding reversibly to the tyramine binding site of the enzyme. Similar results were obtained with THT extracted from elicited potato cell-suspension cultures. K_i values were found to be 0.66 μM for the enzyme from tobacco and 0.3 μM for the enzyme from potato. L-Tyrosine 7-amido-4-methylcoumarin, a fluorogenic substrate for tyrosine aminopeptidases, the structure of which is close to that of L-tyrosine β-naphthylamide, was also a powerful inhibitor, but slightly less effective with K_i values of 0.72 and 0.42 μM for tobacco and potato THT, respectively. L-Tyrosine β-naphthylamide was rapidly hydrolysed when fed in vivo to tobacco or potato cell cultures or when incubated in crude enzymic extracts prepared from these cultures. This hydrolysis, which is presumably catalysed by aminopeptidases, precludes the use of L-tyrosine amides as inhibitors of THT in vivo.

Keywords: *Nicotiana tabacum*; *Solanum tuberosum*; Hydroxycinnamoyl transferase; Inhibition; L-Tyrosine β-naphthylamide; L-Tyrosine 7-amido-4-methylcoumarin; Tyrosine aminotransferase

1. Introduction

The enzyme hydroxycinnamoyl-CoA: tyramine *N*-hydroxycinnamoyl transferase, (THT, EC 1.2.2.110) catalyses the condensation of various hydroxycinnamoyl-CoA esters with tyramine. The amides formed by THT have repeatedly been found associated with cell wall fractions and their deposition together with other phenolics in the cell wall is believed to create a barrier against pathogens by reducing cell wall digestibility (Negrel et al., 1996; Facchini et al., 1999; McLusky et al., 1999). THT is a rational target to attempt to inhibit the synthesis of hydroxycinnamoyltyramines to evaluate the role played by these amides in the reinforcement of cell walls. THT has so far been purified from potato, tobacco, and opium poppy cell-suspension cultures (Hohlfeld et al., 1996; Negrel and Javelle, 1997; Yu and Facchini, 1999). Both potato and tobacco THT cDNA clones have been isolated, creating the opportunity to generate THT-downregulated plants using an antisense RNA strategy (Farmer et al., 1999; Schmidt et al., 1999). In a parallel approach, we have been screening for THT inhibitors which might be used in vivo. We previously reported that various L-tyrosine esters are competitive inhibitors of tobacco THT, the most active being L-tyrosine benzyl ester (Negrel and Javelle, 1997). In this paper we report that L-tyrosine β-naphthylamide (L-Tyr-NA, Fig. 1), a commercially available substrate used to measure tyrosine aminopeptidase activity, is a remarkably powerful inhibitor of both tobacco and potato THTs. However, when fed to tobacco or potato cell-suspension cultures, L-Tyr-NA was rapidly hydrolysed, most likely by aminopeptidases. This hydrolysis precludes the use of L-Tyr-NA as a THT inhibitor in vivo.

2. Results and discussion

2.1. Inhibition of THT in vitro

During the course of our research on the inhibition of tobacco THT by L-tyrosine derivatives, we found that L-Tyr-NA, a synthetic substrate for tyrosine aminopeptidase, was a very potent inhibitor of the transferase

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(Table 1). THT catalyses the transfer of ferulic acid from feruloyl-CoA to tyramine in an ordered bi bi mechanism that initially involves binding of feruloyl-CoA to the enzyme (Hohlfeld et al., 1995; Negrel and Javelle, 1997). The inhibition pattern observed in the presence of L-Tyr-NA was similar to that previously described with L-tyrosine benzyl ester (Negrel and Javelle, 1997). The inhibition was completely reversed in the presence of high concentrations of tyramine (Fig. 2A), whereas uncompetitive inhibition was observed when increasing concentrations of feruloyl-CoA were added in the presence of fixed concentrations of tyramine (Fig. 2B). These results show that L-Tyr-NA does not inhibit the initial binding of feruloyl-CoA to the transferase, but instead competes with tyramine for the same binding site. This was further confirmed by analysing the structure–activity relationships of several amino acid naphthylamides. L-Tyr-NA was much more active than phenylalanine β-naphthylamide, whereas the arginine derivative was totally inactive (Table 1). As expected, L-Tyr-NA was also a powerful inhibitor of the recombinant tobacco transferase, in displaying 92% inhibition at 50 μM in the presence of 25 μM feruloyl-CoA and 125 μM tyramine. Interestingly, L-tyrosine 7-amido 4-methyl coumarin (L-Tyr-AMC, Fig. 1), a sensitive fluorogenic substrate for tyrosine aminopeptidase (Zimmerman et al., 1977), was also a strong inhibitor of tobacco THT, although slightly less potent than L-Tyr-NA. Both compounds inhibited potato THT, and were about 10-fold

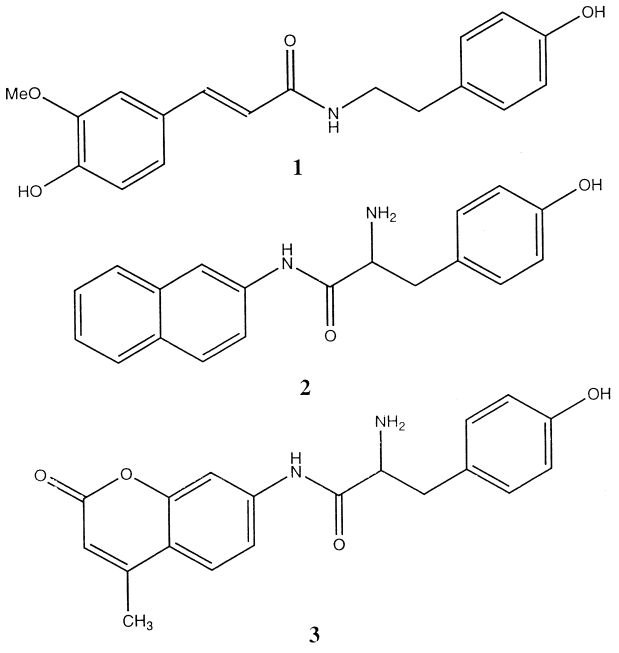

Fig. 1. Structures of feruloyltyramine (**1**), L-tyrosine β-naphthylamide (**2**) and L-tyrosine 7-amido-4-methylcoumarin (**3**).

Table 1
Inhibition of tobacco or potato THT by various amino acid amides or esters

Inhibitor	K_i (μM)[a]	
	Tobacco	Potato
L-Tyrosine β-naphthylamide	0.66	0.3
L-Tyrosine 7-amido 4-methylcoumarin	0.72	0.42
L-Tyrosine benzyl ester	3	3.4
L-Phenylalanine β-naphthylamide	112	91
L-Arginine β-naphthylamide	–[b]	–

[a] K_i values were calculated from Dixon plots, after measuring the inhibition of THT at different inhibitor concentrations, from 10 to 200 μM.

[b] No detectable inhibition.

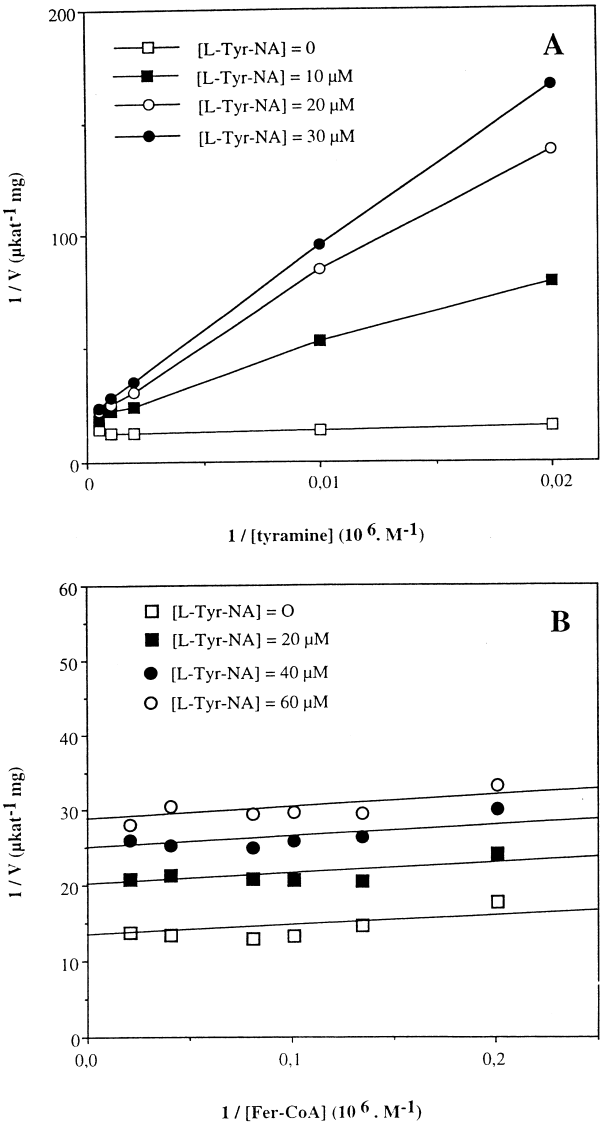

Fig. 2. Lineweaver–Burk plots of THT activity showing the variations of 1/V at constant and saturating concentration of one substrate (**A**: [feruloyl-CoA] = 25 μM; **B**: [tyramine] = 250 μM), with varying concentrations of the second substrate, in the absence or presence of various concentrations of L-Tyr-NA.

more potent than L-tyrosine benzyl ester (Table 1). The fact that the highest inhibition was obtained with the naphthylamine derivative is interesting because it provides a clue about the mode of action of this family of inhibitors. The structure of L-Tyr-NA is notable because it is intermediate between tyramine and a feruloyltyramine analogue (Fig. 1). Taking the mechanism of action of THT into account, it is likely that the naphthylamide moiety of the inhibitor, which is comparable to a rotationally constrained analogue of ferulic acid, can interact with the feruloyltyramine binding site after L-Tyr-NA has bound to the tyramine binding site of the enzyme-feruloyl-CoA complex. This likely explains the high affinity of THT for L-Tyr-NA and the fact that L-Tyr-AMC is less effective than L-Tyr-NA. Thus the mode of action of these inhibitors seems different from that of other specific inhibitors which have been designed to inhibit acyl-CoA amine acyltransferases, such as nonhydrolysable acyl-CoA analogues (Paige et al., 1990, Rudnick et al., 1991) or multisubstrate analogues (Cullis et al., 1982, Erwin et al., 1984).

2.2. Metabolism of L-Tyr-NA

We next considered the possibility of using L-Tyr-NA as an inhibitor of THT in vivo. Since amino acid naphthylamides are known substrates of aminopeptidases (Bodansky, 1971), we first tried to incubate L-Tyr-NA in crude protein extracts to detect a possible tyrosine aminopeptidase activity. Both L-Tyr-NA and L-Tyr-AMC were rapidly hydrolysed when incubated in enzyme extracts from tobacco or potato cell-suspension cultures. The hydrolysis of L-Tyr-NA was monitored photometrically whereas hydrolysis of L-Tyr-AMC was detected fluorometrically. Aliquots of the enzyme extracts were analysed by HPLC at regular intervals to check the nature of the products formed during the reaction. As expected, tyrosine and β-naphthylamine were the only products formed from L-Tyr-NA (Fig. 3) whereas tyrosine and 7-amino-4-methylcoumarin were rapidly formed from L-Tyr-AMC. The specific activities of tyrosine aminopeptidase in crude enzyme extracts prepared from potato or tobacco cell-suspensions one week after sub-culture were 6 and 1 nkat/mg protein, respectively. The rate of hydrolysis of L-Tyr-NA was similar in extracts from elicited and non-elicited cells. L-Tyr-NA was also hydrolysed when fed in vivo to tobacco or potato cell-suspension cultures. When added at a concentration of 10^{-4} M to the culture medium of tobacco cell suspensions, Tyr-NA was almost completely taken up after 3 h (Fig. 4). It was then rapidly metabolised inside the cells and became undetectable after 8 h. At that time β-naphthylamine could be detected in cell extracts but it was also quickly metabolised and became undetectable after 24 h (Fig. 4). Similar results were obtained with potato cell cultures (data not shown). Aminopeptidases are widespread enzymes in higher plants (Taylor, 1993; Chao et al., 1999). The rapid hydrolysis of L-Tyr-NA and L-Tyr-AMC therefore precludes their use in vivo as THT inhibitors. However, it may be possible to synthesize L-Tyr-NA analogues which would be resistant to aminopeptidases. Potent inhibitors of choline acetyltransferase resistant to cholinesterase have been obtained by synthesizing keto analogues of acetylcholine (Rama Sastry et al., 1988). Substitution of a ketone group for the amide bond of L-Tyr-NA may prevent the hydrolysis by aminopeptidases while keeping the inhibitory properties of the molecule. The preliminary results presented in this

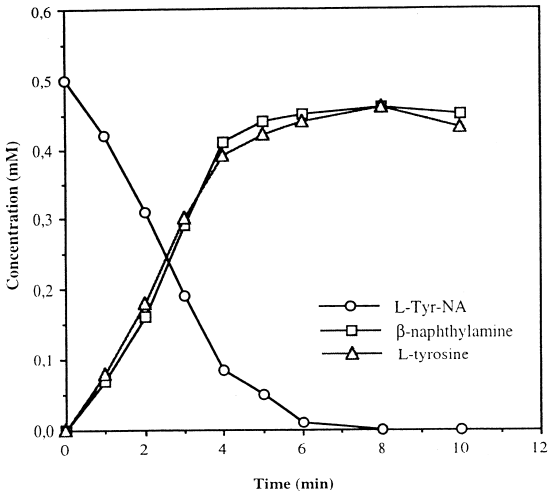

Fig. 3. Time course of hydrolysis of L-Tyr-NA in a crude enzyme extract from tobacco cells. The incubation mixture contained 1.14 mg protein and 0.5 μmol L-Tyr-NA in a total volume of 1 ml.

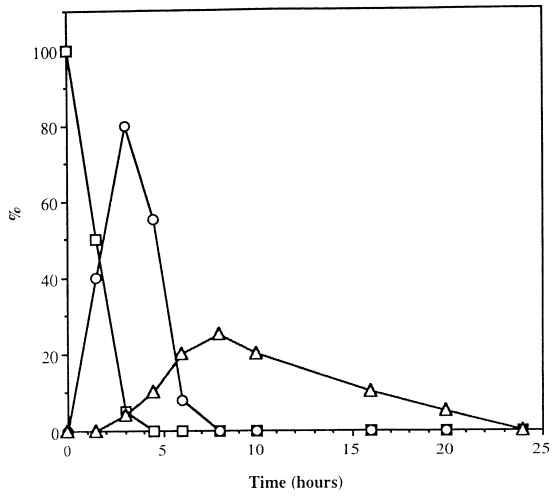

Fig. 4. Uptake and metabolism of L-Tyr-NA in tobacco cell-suspension cultures. Results are expressed as a percentage of the initial quantity of L-Tyr-NA (25 μmol) added to the culture medium: L-Tyr-NA in the culture medium (□), L-Tyr-NA (○) and β-naphthylamine (△) extracted from the cells.

paper show the usefulness of amino acid naphthylamides as tools to study the mechanism of action of amine *N*-hydroxycinnamoyltransferases and to identify the three-dimensional structure of the active site of these enzymes.

3. Experimental

3.1. Chemicals and substrates

L-Tyr-NA, L-Tyr-AMC, L-phenylalanine β-naphthylamide, L-arginine β-naphthylamide and L-tyrosine benzyl ester were all purchased from Sigma. Feruloyl-CoA was prepared by transesterification of feruloyl *N*-hydroxysuccinimide ester as previously described (Negrel and Javelle, 1997).

3.2. THT purification

The inhibition of tobacco THT was studied using an enzyme purified to apparent homogeneity from cell-suspension cultures of tobacco (*Nicotiana tabacum* L. cv Xanthi), as previously described (Negrel and Javelle, 1997). The potato enzyme was partially purified using the same protocol from pronase-elicited cell-suspension cultures of *Solanum tuberosum* L. (cv BF15), originally initiated from tuber callus tissue culture. The specific activity of the potato enzyme was 58 nkat/mg protein. It was completely free of tyrosine aminopeptidase activity. Inhibition of the recombinant tobacco enzyme was studied using a crude bacterial extract obtained after lysis of the XLOLR E. coli transformant (clone 10) in 0.1 M Tris–HCl buffer pH 7.5 containing 1 mg ml^{-1} lysosyme and 10 mM ME (Negrel and Javelle, 1997).

3.3. Enzyme assays

THT activity was measured photometrically as previously described (Negrel and Javelle, 1997). Addition of inhibitors did not interfere with the assay at 356 nm. None of the inhibitors tested was used as substrate by THT, using feruloyl-CoA as acyl donor. Tyrosine aminopeptidase activity was measured photometrically at 328 nm using L-Tyr-NA as substrate. The incubation mixture contained 200 μl protein extract, 800 μl 0.1 M Tris–HCl buffer pH 7.5 and 10 μl L-Tyr-NA 50 mM. Hydrolysis of L-Tyr-NA occured over a wide range of pH (5–10) with an optimum at pH 7.5. When L-Tyr-AMC was used as a substrate, the activity was measured in the same conditions but with a fluorometer, using activation and emission wavelengths of 380 and 460 nm, respectively.

3.4. HPLC

Products were separated on a Waters Novapack C18 column (3.9×300 mm, 4 μm) and detected using a two-channel UV detector at 280 and 328 nm. The following elution system was applied: linear gradient elution within 45 min from 10 to 85% MeOH in water acidified at pH 2 with 4% HCO_2H and 2 g/l ammonium formiate, using a flow rate of 0.8 ml/min. Retention times: L-tyrosine 5.45 min, β-naphthylamine 25.65 min, 7-amino-4-methylcoumarin 29.46 min, L-tyrosine benzylester 30.98 min, L-Tyr-AMC 32.50 min, L-Tyr-NA 36.43 min.

3.5. Metabolism of L-Tyr-NA

To monitor the degradation of L-Tyr-NA in vivo, 9.2 mg L-Tyr-NA dissolved in 2 ml MeOH 50% were added to 250 ml tobacco or potato cell-suspension cultures 1 week after sub-culture. Samples (25 ml) were withdrawn for analysis by pipetting from the treated cultures. Cells were then collected by centrifugation, frozen in liquid nitrogen and kept at −80°C until analysed. Aliquots of the supernatant were also frozen in order to monitor the uptake of L-Tyr-NA from the culture medium. Cells were extracted twice in MeOH acidified with 1% acetic acid (2 ml/ g fr. wt.). After centrifugation the MeOH extracts were pooled, evaporated to dryness, and taken up in 5 ml MeOH–H_2O–AcOH (3:1:1). Aliquots (20 μl) were then analysed by HPLC.

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