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THE BIOSYNTHESIS OF FERULOYLTYRAMINE IN *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; biosynthesis; feruloyltyramine; hypersensitivity; tobacco mosaic virus; hydroxycinnamoyl-CoA thioesters; arylamines; amide bond; coenzyme A.

Abstract—The biosynthesis of feruloyltyramine in *Nicotiana tabacum* Xanthi n.c. leaves is achieved through the action of the enzyme feruloyl-CoA tyramine *N*-feruloyl-CoA transferase. Its activity is increased 5- to 8-fold following infection by tobacco mosaic virus at 20°. The enzyme is soluble, its MW is 45 000, and it can synthesize a wide range of amides due to its low specificity for cinnamoyl-CoA thioesters and aromatic amines. Its affinity for feruloyl-CoA fragments is also described.

INTRODUCTION

In higher plants, the widespread nature of hydroxycinnamic acid amides of the di- and polyamines is now recognized [1]. However, few reports describe the occurrence of cinnamic acid amides of tyramine or related arylamines (e.g. octopamine, dopamine) [2]. *p*-Coumaroyltyramine has been identified in the bark of *Evodia bellahe* [3] and was later found in tobacco anthers [4]. Tobacco seeds also contain feruloyltyramine [4], whereas both amides are present in the roots of eggplant and bell pepper, together with feruloyl- and *p*-coumaroyloctopamine [5, 6]. Flowers are also known to contain a variety of hydroxycinnamic acid amides [7] and although these are mainly polyamine conjugates, some of them have been found to contain tyramine derivatives [7, 8].

The postulated involvement of cinnamoyl-CoA thioesters in hydroxycinnamic acid amide synthesis [9] was confirmed by the discovery of the enzyme agmatine coumaroyl-transferase (ACT) in the shoots of barley seedlings [10, 11]. In tobacco leaves, feruloylputrescine and *p*-coumaroylputrescine are synthesized at 20° during the hypersensitive reaction to tobacco mosaic virus (TMV) [12]. This synthesis follows a strong increase of the activity of ornithine and tyrosine decarboxylases 48 hr after inoculation [13]. Attempts to detect a putrescine feruloyl-CoA transferase activity were unsuccessful, but crude extracts from 72 hr inoculated leaves were found to catalyse the formation of feruloyltyramine. Although, this amide has not been identified in tobacco leaves, it seemed of interest to study this enzyme and its possible relationship with the hypersensitive reaction.

RESULTS

In vitro *biosynthesis and identification of feruloyltyramine*

After centrifugation, crude extracts of 72 hr infected Xanthi n.c. leaves catalysed the synthesis of feruloyltyramine from feruloyl-CoA and [2-^{14}C]tyramine at pH 7.5. No amide synthesis was detected when a boiled extract or no enzyme extract was used. No chemical formation of feruloyltyramine could be detected even at pH 10. This contrasts with the non-enzymatic formation of monoacetylputrescine from putrescine and acetyl-CoA at pH 8 and above [14]. *In vitro* synthesized feruloyltyramine was identified by co-chromatography with the synthetic product on TLC in solvents (1) and (2) (see Experimental). Solvent (2) separated the *cis* and *trans* forms. The amide synthesized *in vitro* was mainly in the *trans* form although trace amounts of the *cis* form were always present. The *cis-trans* isomerization after irradiation under UV light was also used in the identification of the products. No metabolism of [2-^{14}C]tyramine could be detected *in vitro* when it was incubated with the enzyme extract without feruloyl-CoA. Most of the activity was found in the supernatant (10 000 *g*, 10 min) after grinding in 3 vols. of buffer. However, residual activity was still detected in the resuspended pellet even after repeated washing in the extraction medium.

Optimal requirements for tyramine feruloyl-CoA transferase (TFT) activity

The activity detected in the crude extract is stable when buffers containing mercaptoethanol (ME) are used. No loss was detected after dialysis for 16 hr at 4°. The activity can be concentrated with ammonium sulfate. 70% of the initial activity was recovered in the fraction precipitated between 45 and 65% saturation. At this stage, the concentrated enzyme is very stable and can be kept in solution at 4° for 1 week with only a 10% loss in activity. The UV spectra of hydroxycinnamoyl-CoA thioesters and hydroxycinnamic acid amides are pH-dependent in the pH range 7.5 to 10. Thus the pH required for optimum activity of the transferase was determined using an isotopic assay with [2-^{14}C]tyramine. A saturating concentration (1 mM) of tyramine was used and the reaction was stopped after 5 min in order to retain a linear reaction rate. After incubation, feruloyltyramine was separated from tyramine by TLC. [^{14}C]Feruloyltyramine formation could be detected between pH 6 and 10, the optimum pH being 7.5 with half-activity at pH 6.5 and 9. This procedure was repeated with caffeoyl, *p*-coumaroyl and sinapoyl-CoA thioesters. The results are reported in

Table 1. Relative activity of CoA thioesters in the tyramine transferase assay

Substrate	Optimum pH	λ* (nm)	Extinction (M^{-1} cm^{-1})	K_m (μM)	Relative activity†
Cinnamoyl-CoA	8.5	313	20000	10	100
p-Coumaroyl-CoA	9	350	12000	6.7	61
p-Hydroxyphenyl-propionyl CoA	n.d.‡	—	—	n.d.	1§
Caffeoyl-CoA	—	—	—	—	0
Feruloyl-CoA	7.5	356	16000	6.2	41
Sinapoyl-CoA	8	358	14200	50	48

*Wavelength used in the spectrophotometric assay.
† V_{max} relative to the V_{max} of cinnamoyl-CoA.
‡n.d. = not determined.
§Estimated using the isotopic assay.

Table 1. The reliability of the assay was checked using the spectrophotometric assay with cinnamoyl-CoA, since its UV spectrum does not change with pH.

Spectrophotometric assay. The assay used for the determination of agmatine coumaroyltransferase activity [11] was adapted for TFT. Pyrophosphate buffer (0.1 M) was used to inhibit the enzymatic phosphohydrolysis of hydroxycinnamoyl-CoA thioesters [15]. The wavelengths used for the assay corresponded to the maxima of the difference spectra between the thioesters and the corresponding amides, and were slightly longer than the λ_{max} of the thioesters. The data are collected in Table 1. The apparent K_m of TFT for feruloyl-CoA at pH 7.5 and 1 mM tyramine was 6.2 μM in partially purified extracts. The apparent K_m for tyramine was 20 μM at 25 μM feruloyl-CoA. When the spectrophotometric assay was used, 1 mM EDTA, 20 mM $(NH_4)_2SO_4$ and 0.1 M PPi were found not to inhibit TFT activity *in vitro*. $MgCl_2$ (0.1–10 mM) also did not affect TFT activity.

MW estimation. TFT activity co-chromatographed with ovalbumin (MW 45000) on an Ultrogel column. After the gel filtration step, the specific activity of the enzyme was increased 10-fold compared with the crude extract. The kinetic properties of the enzyme remained unchanged.

Substrate specificity. Several arylamines were used as substrates (Fig. 1) but no activity was detected with tyrosine, tryptamine, serotonin or the polyamines putrescine, spermidine and spermine. The structure–activity relationships showed that a phenolic group is needed for maximal activity whereas further hydroxylation of the ring or the side chain leads to a decrease in activity. The *in vitro* formation of feruloylphenethylamine was confirmed using [^{14}C]phenethylamine. The activity of synephrine is based only on spectrophotometric evidence. Amides formed from secondary amines and hydroxycinnamic acids are known to occur in plants [1, 2] and the activity of synephrine is therefore not surprising although it needs to be confirmed by physical methods or by using [^{14}C]synephrine. *N*-Methyltyramine is another potential substrate for TFT.

Table 1 shows the relative activity of several CoA thioesters in the tyramine transferase assay. The enzyme had a high affinity for feruloyl-CoA and *p*-coumaroyl-CoA. However, the greatest activity is obtained using cinnamoyl-CoA as substrate, whereas substitution in the phenolic ring leads to a decrease in activity when it introduces asymmetry in the molecule. Caffeoyl-CoA was not used as the substrate: no activity could be detected between pH 5 and 10. *p*-Hydroxyphenylpropionyl-CoA was used only very slowly indicating that the enzyme recognizes the double bond of cinnamic acids. This could be important for the enzyme in order to differentiate between the two substrates, which are both phenolics.

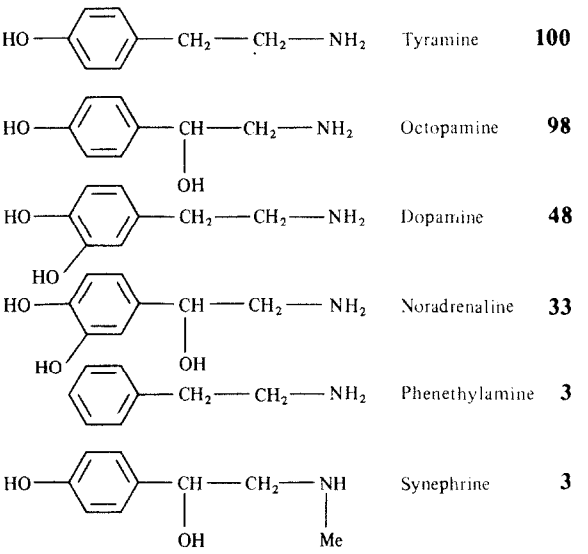

Fig. 1. Relative activity of aromatic amines in the tyramine feruloyl-CoA transferase assay. DL-Octopamine, L-noradrenaline and DL-synephrine were used. The relative activities of the arylamines did not change when *p*-coumaroyl-CoA was used as the substrate instead of feruloyl-CoA.

Table 2 shows the relative activity of feruloyl-CoA fragments in the TFT assay. Hydroxycinnamoyl-CoA thioesters are rapidly hydrolysed through the action of phosphatases in crude plant extracts [15]. In order to determine the relative activity and affinity of the different feruloyl-CoA fragments in the TFT assay, it was necessary to quantify precisely the different thioesters in solution. We therefore studied the possible interactions [15] between the hydroxycinnamic moiety and adenine in the molecule. Radiationless energy transfer has been found to occur in synthetic substrates for phosphodiesterases in which the fluorophore 1-aminonaphthalene-5-sulfonate

Table 2. Relative activity of feruloyl-CoA fragments in the tyramine transferase assay

Substrate	K_m (μM)	Relative activity
Feruloyl-CoA	6.2	100
Dephosphoferuloyl-CoA	n.d.*	67
Feruloyl-4′-phosphopantetheine	12.5	94
Feruloylpantetheine	n.d.	9†

*n.d. = not determined.

†The activity of feruloylpantetheine based on spectrophotometric evidence was not confirmed with the isotopic assay.

had been coupled to nucleotides [16]. Furthermore, NMR spectroscopy has shown that benzoyl-CoA exists in solution in a specific intramolecular complex involving interaction between the adenosyl and benzoyl moieties [17]. The phosphohydrolysis of feruloyl-CoA in 0.1 M Tris–HCl buffer at pH 9 by alkaline phosphatase resulted in a bathochromic shift from 350 to 400 nm (Fig. 2). However, the UV spectrum remained unchanged when the phosphohydrolysis was carried out at pH 7, suggesting that the interaction between the ferulic moiety and adenine is pH-dependent. At pH 7, the hydrolysis by alkaline phosphatase was slow but nevertheless went to completion. Thus the thioesters can be quantified precisely using the same extinction coefficient [9] at pH 7.

Intact coenzyme A is not essential to catalyse the amide bond formation. Dephosphoferuloyl-CoA is less active than feruloyl-CoA, but surprisingly is also less active than feruloyl-4′-phosphopantetheine. In order to minimize the effect of the phosphohydrolysis, already greatly inhibited by the inorganic pyrophosphate buffer [15], tyramine was added to the spectrophotometric assay only a few seconds after feruloyl-CoA. This was possible because there is no shift in the baseline before adding the amine when feruloyl-CoA is used as the substrate (see Experimental)

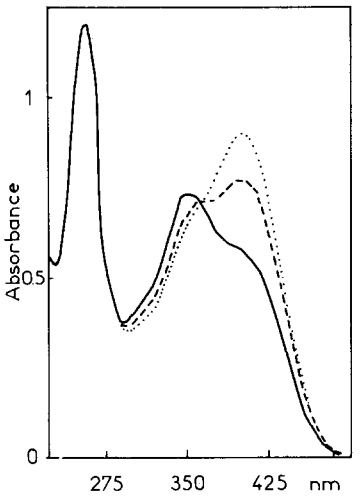

Fig. 2. Bathochromic shift in the UV spectrum of feruloyl-CoA following phosphohydrolysis by alkaline phosphatase in 0.1 M Tris–HCl buffer at pH 9. (——) Feruloyl-CoA; spectrum during (– – –) and after (· · ·) phosphohydrolysis.

TFT activity in TMV inoculated or healthy leaves of Nicotiana tabacum *var. Xanthi n.c. or Samsun NN*

Figure 3 shows the time course of changes in TFT activity extracted from Xanthi n.c. leaves during the hypersensitive reaction at 20°. TFT was also detected in Samsun NN leaves 3 days after inoculation by TMV at 20°. It is difficult to detect any activity in the extracts of healthy leaves and for this reason, the crude enzyme extract was concentrated with ammonium sulfate prior to the enzyme assay. At 20° local necrotic lesions appeared 36–48 hr after inoculation. TFT activity reached a maximum 24 hr later, i.e. after the death of most of the infected cells. The fact that TFT activity is not enhanced in the living cells which are going to die was shown by temperature shift experiments. One mature leaf was inoculated at 20° and the plant was then kept at 32° for 72 hr. At this temperature, TMV multiplication is systemic [18, 19] and when the plant is transferred back to 20° the part of the plant in which the virus is multiplying, i.e. the apical part, and the inoculated leaf collapse within 7–9 hr [19]. No increase in TFT activity could be detected in the apical leaves during such experiments. The activity remained at levels similar to those found in healthy leaves at 20° (data not shown).

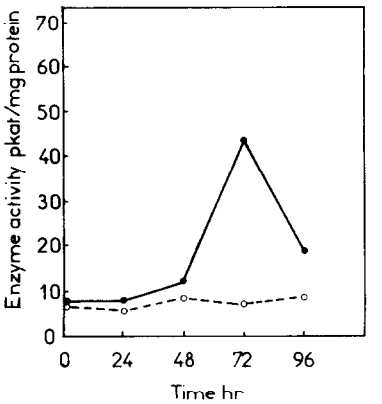

Fig. 3. TFT activity during the hypersensitive reaction to TMV in Xanthi n.c. mature leaves at 20°. (●——● TMV, o– – –o water inoculated control).

DISCUSSION

This is the first report of the occurrence of the enzyme feruloyl-CoA tyramine *N*-feruloyl-CoA transferase (TFT) (EC 2.3.1.—). TFT is a soluble enzyme of MW 45 000 and is therefore comparable to ACT found in barley seedlings [11]. Both enzymes have a high affinity for their substrates and a low specificity for the cinnamic moiety. However, ACT is specific for agmatine whereas TFT can use at least six different aromatic amines as substrates. More purification work is needed to determine whether there is only one enzyme responsible for the observed activities or several isoenzymes. The fact that the different cinnamoyl-CoA derivatives require a different pH for optimum activity could be an indication of the existence of such isoenzymes, although it could also be explained as a consequence of the substitution of the cinnamic moiety on the thioester reactivity.

ACT and TFT are both very stable in solution, providing a thiol is added to the buffers used for the extraction and subsequent purification. Both enzymes can use fragments of CoA [15]. CoA, however, is probably the natural substrate (higher V_{max}, lower K_m). The fact that feruloyl-4'-phosphopantetheine is more active than dephosphoferuloyl-CoA could indicate that the 4'-phosphate group can substitute for the 3'-phosphate of intact CoA in the active site of the enzyme.

The increase of ACT activity during the germination of barley [20] and of TFT activity during the hypersensitive reaction in tobacco are transient. The two enzymes remain very active only for 1–2 days. This implies that the regulation of their synthesis (or activation) must be strictly controlled. One must notice, however, that mature healthy tobacco leaves contain a weak but detectable TFT activity. The specific activity of TFT extracted from TMV-inoculated leaves is about four-fold less important than the specific activity of ACT in the shoots of barley seedlings [11]. However, tobacco leaves on which local necrotic lesions have developed are histologically very heterogeneous. We have shown, using temperature shift experiments, that TFT activity is not enhanced in the cells which are going to die, but this of course does not imply that it is homogeneously distributed in the remaining living tissues.

Attempts to detect feruloyltyramine or feruloyloctopamine in healthy or TMV-infected Xanthi n.c. leaves were unsuccessful. Even 3 or 4 days after inoculation at 20°, i.e. during or after TFT activation, they were not present in sufficient amounts to be detected by fluorescence after TLC. This result could indicate that these amides undergo rapid metabolism. Like most phenolics, hydroxycinnamic acid amides have been found to occur in plants as glucosides [21] and can undergo oxidation [6, 21, 22]. Dimers of *p*-coumaroylagmatine and feruloyltyramine have been identified in barley (hordatines [21]) and bell pepper (grossamide [6]). Moreover, infection of potato tuber tissues by *Phytophthora infestans* leads to the synthesis of cinnamoyltyramine and cinnamoyloctopamine, together with *p*-coumaroyl conjugates [23]. These amides have been detected by fluorescence around the inoculation site but have been found to leave the cells and bind to the cell wall [23]. The hypersensitive reaction to TMV could therefore trigger a similar mechanism in tobacco.

The enhanced synthesis of phenolics is a common feature of plant defence reactions following pathogen infection [24]. From recent studies, it appears that lignification plays a part as a defence mechanism in plants reacting by hypersensitivity to pathogens [25, 26]. One may wonder whether hydroxycinnamic acid amides, which are good substrates of peroxidases *in vitro* [6, 21, 22], could form insoluble polymers *in vivo* and play a role in host–pathogen interactions. A biochemical study of the fate of feruloyltyramine and related amides *in vivo* during the hypersensitive reaction is, however, necessary to confirm the preliminary observations [23] mainly based on microscopic evidence using the natural fluorescence of the amides.

EXPERIMENTAL

Plant material. Nicotiana tabacum cv Xanthi n.c. and Samsun NN were grown in a greenhouse for 7 weeks and then transferred 3 days before inoculation to a controlled-growth room (16 hr photoperiod, 16 klx, 70% relative humidity at 20°, 50% at 32°). Fully expanded leaves were inoculated with a purified TMV soln (10 μg/ml) to give 4–5 lesions per cm^2 using carborundum as abrasive. Plants inoculated with H_2O were used as blanks.

TLC was on Kieselgel using the following solvents: (1) $CHCl_3$–EtOH (1:1) (R_f tyramine 0.05; cinnamoyl, *p*-coumaroyl, feruloyl and sinapoyltyramine 0.9); (2) $CHCl_3$–MeOH (24:1) (R_f tyramine 0; cinnamoyltyramine (*cis* and *trans*) 0.62; sinapoyltyramine *cis* 0.44, *trans* 0.38; *p*-coumaroyltyramine *cis* 0.27, *trans* 0.19; feruloyltyramine *cis* 0.15, *trans* 0.11; feruloylphenethylamine 0.9; *p*-hydroxyphenylpropionyltyramine 0.1).

Hydroxycinnamoyl-CoA thioesters were prepared by transesterification [27] of hydroxycinnamoyl-*N*-hydroxysuccinimide esters, synthesized as described in ref. [15].

p-*Hydroxyphenylpropionyl-CoA* was prepared by transesterification of *p*-hydroxyphenylpropionyl-*N*-hydroxysuccinimide ester, synthesized as described in ref. [28]. The CoA thioester was purified by prep. TLC on cellulose plates in BuOH–EtOH–H_2O (4:1:2) (R_f 0.28), and detected using diazotized sulfanilic acid (λ_{max} 260 nm in 0.1 M Na Pi buffer pH 7). The extinction coefficient (λ_{260} 16000) was determined by measuring the amount of Pi liberated after hydrolysis by alkaline phosphatase at pH 9 [15].

Dephosphoferuloyl-CoA, feruloyl-4'-phosphopantetheine and feruloylpantetheine were synthesized from feruloyl-CoA as described in ref. [15].

Cinnamic acid amides of tyramine were synthesized from *N*-hydroxysuccinimide esters and tyramine in a 1:1 mixture of Me_2CO and H_2O, adjusted to pH 8 with $NaHCO_3$ [22]. They were purified by prep. TLC in solvents (1) and (2). Isotope dilution was used in order to estimate the extinction coefficients of the amides.

Extraction of feruloyltyramine. 72 or 96 hr infected Xanthi n.c. leaves (10 g fr. wt) were extracted in 5 vols. MeOH. The homogenate was then centrifuged (5000 *g*, 10 min) and the ppt. was washed twice with MeOH. The supernatants were collected and evapd to *ca* 4 ml. H_2O was then added and the aq. phase extracted twice with 1 vol. EtOAc. The EtOAc phase was then evapd *in vacuo* under red. pres. and redissolved in 5 ml MeOH. Aliquots (10–100 μl) were analysed by TLC in solvent (2). Feruloyltyramine, *p*-coumaroyltyramine and feruloyloctopamine could not be detected by fluorescence on the plate even after spraying with aq. Na_2CO_3.

Enzyme preparation. Leaves were extracted in a mortar in 3 vols. 0.1 M Na PPi buffer, pH 7.5 (10 mM ME, 1 mM EDTA, 0.5% ascorbic acid w/v). The homogenate was centrifuged at 10000 *g* for 10 min. The supernatant was stirred, and protamine sulfate (2 mg/10 mg protein) was then added dropwise. After centrifugation, TFT activity was partially purified by $(NH_4)_2SO_4$ fractionation. The proteins precipitated between 45 and 65% saturation were redissolved in the extraction buffer (1 ml/g fr. wt) and dialysed in 0.01 M PPi buffer, pH 7.5 (10 mM ME, 1 mM EDTA) for 16 hr at 4°. This extract was used to determine the kinetic properties of TFT. In order to measure TFT activity during the hypersensitive reaction, the crude extract after centrifugation was precipitated directly with solid $(NH_4)_2SO_4$ (65% saturation). The pellet was redissolved in the extraction buffer (1 ml/g fr. wt) and dialysed.

Detection of TFT activity. 60 μl of the crude extract (after centrifugation) obtained from a leaf which had been inoculated 72 hr earlier at 20° was mixed with feruloyl-CoA (20 nmol, 20 μl) and [2-^{14}C]tyramine (50.4 mCi/mmol, 10 μl, 20 μCi/ml). The mixture was incubated at 30° for 1 hr. The reaction was stopped with 10 μl HOAc. 10 μl aliquots were spotted on TLC plates and chromatographed in solvent (2).

Isotopic assay. This was conducted as follows: 10 μl [2-^{14}C]tyramine (10 μCi/ml) was mixed with 25 nmol feruloyl-CoA (10 μl), 40 μl 0.1 M PPi buffer (pH 7.5), 30 μl enzyme extract and 10 μl 10 mM unlabelled tyramine. The mixture was incubated at 30° for 5 min. The reaction was stopped with 10 μl 17 M HOAc and 20 μl aliquots were analysed by TLC. Unlabelled feruloyltyramine, used as a carrier, was also spotted on the plate and detected by fluorescence.

Spectrophotometric assay. 100 μl enzyme extract was mixed with 400 μl 0.1 M PPi buffer, pH 7.5 (10 mM ME), in a 0.5 ml microcell at 30°. The reference cell contained the same mixture. Feruloyl-CoA (12.5 nmol, 5 μl) was added. No drift in the baseline was observed with feruloyl-CoA and sinapoyl-CoA, whereas a marked drift was always found with cinnamoyl-CoA and, to a lesser extent, with *p*-coumaroyl-CoA. The drift occurred only in the presence of the enzyme extract and was not due to the pH used for the incubation mixture: it also occurred at pH 7.5. After 1 min, tyramine (500 nmol, 5 μl) was added and the decrease of A_{356} recorded for 2 to 3 min. The reaction rate was linear for 4–10 min, depending on the activity. The spectrophotometric assay was very sensitive and the low activity with phenethylamine could be detected unambiguously.

The MW of TFT was estimated by gel filtration on an Ultrogel AcA 54 column (50 cm × 2.8 cm). 2.5 ml fractions were collected using a flow rate of 4 ml/cm^2 per hr. TFT was eluted in the 153–172 ml fraction. The column was calibrated with bovine serum albumin, ovalbumin, myoglobin and cytochrome *c* [29]. TFT was detected after the column using feruloyl-CoA and tyramine as substrates.

Radioactivity was detected on Kieselgel plates by autoradiography. It was quantified with a Geiger counter on the plate and, after elution with MeOH, by liquid scintillation.

Alkaline phosphatase from bovine intestine containing 12% pyrophosphatase activity was from Sigma.

Protein was determined according to the method of ref. [30].

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