Pergamon

PII: S0031-9422(97)00743-7

Phytochemistry, Vol. 47, No. 7, pp. 1241–1246, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

GERANYLGERANIOL-18-HYDROXYLASE: THE LAST ENZYME ON THE PLAUNOTOL BIOSYNTHETIC PATHWAY IN *CROTON SUBLYRATUS*

PIMPIMON TANSAKUL* and WANCHAI DE-EKNAMKUL†

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

(*Received in revised form* 3 *August* 1997)

Key Word Index—*Croton sublyratus*; Euphorbiaceae; geranylgeraniol-18-hydroxylase; plaunotol; biosynthesis; cell-free extract.

Abstract—The activity of geranylgeraniol-18-hydroxylase, a novel enzyme catalysing the C-18 hydroxylation of geranylgeraniol (GGOH) to plaunotol, has been demonstrated in a cell-free extract prepared from *Croton sublyratus* leaves. This enzyme is involved in the final step of the biosynthetic pathway of plaunotol, an anti-peptic ulcer constituent accumulated in this plant. The enzymatic formation of plaunotol was correlated with both incubation time and the amount of protein. The enzyme activity could be increased by adding NADPH and by heating the 20000 *g* pellet fraction prior to the incubation. The pH optimum for the enzyme activity was 5.0. The enzymatic product was identified as plaunotol by TLC, IR and GC-MS.

INTRODUCTION

Plaunotol (**1**), the active ingredient of a commercial drug named Kelnac®, is a mucosal protective factor-enhancing antiulcer agent [1]. It was originally found in the leaves of *Croton sublyratus* Kurz. [2], a tropical plant distributed throughout southeast Asia. Although the structure of plaunotol has been known for almost 20 years, very little is known about its biosynthetic pathway. Based on its structure, however, the biosynthesis of plaunotol in this plant should be simple since the compound is an 18-hydroxy derivative of geranylgeraniol (GGOH), a common precursor of all natural diterpenoids. It is well documented that GGOH is biosynthesized *via* the terpenoid pathway and its immediate precursor is geranylgeranyl diphosphate (GGPP) [3]. Therefore, it is reasonable to propose that plaunotol is biosynthesized from GGPP by two enzymatic reactions (Fig. 1). First, GGPP is hydrolysed by a phosphatase to form GGOH. Second, GGOH is hydroxylated at C-18 by a specific 18-hydroxylase to form plaunotol. Until now, there has been no report to support this proposed pathway. The reason may be due to the lack of suitable starting material for the study. Attempts have been made to establish *C. sublyratus* callus and cell cultures producing high plaunotol content but these have not been successful [4, 5].

Due to the availability of *C. sublyratus* plants in Thailand, we decided to use the whole plant as a source of GGOH-18-hydroxylase. The leaf part was chosen as the material for the study since it accumulates plaunotol and is potentially the site of plaunotol biosynthesis. The present report describes detection of GGOH-18-hydroxylase activity, product identification and some properties of the enzyme.

RESULTS

Detection of geranylgeraniol-18-hydroxylase activity

Different centrifugation fractions of the crude enzyme extract prepared from *C. sublyratus* leaves were examined for GGOH-18-hydroxylase activity. [1-^{3}H] GGPP was used as substrate and TLC-radioscanning was used for the detection of enzymatic reaction products. As shown in Fig. 2, it appeared from the TLC-radiochromatogram of the reaction mixture containing the 3000 *g* pellet showed very low radioactivity at the positions of both GGOH and plaunotol [Fig. 2(A)]. The chromatogram of the reaction mixture containing the 20000 *g* pellet fraction, on the other hand, clearly showed radioactive peaks for both com-

* Present address: Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla, Thailand.

† Author to whom correspondence should be addressed.

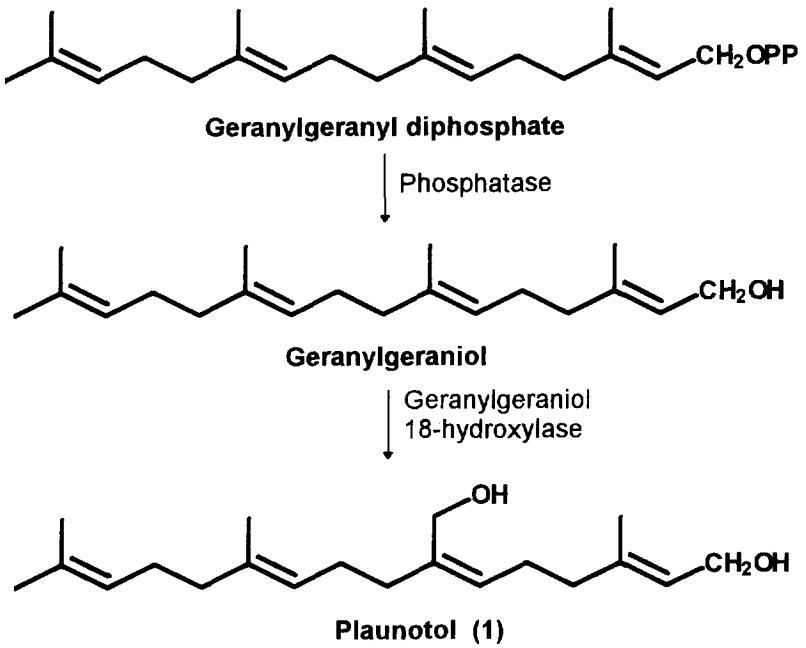

Fig. 1. Proposed biosynthetic pathway for the formation of plaunotol.

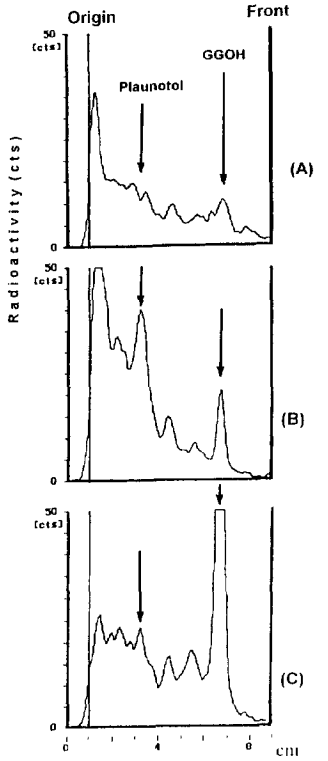

Fig. 2. TLC radiochromatograms of the reaction mixtures containing [1-^{3}H] GGPP and either the 3000 *g* pellet (A), the 20 000 *g* pellet (B), or the 20 000 *g* supernatant (C).

pounds [Fig. 2(B)]. In the mixture containing the 20 000 *g* supernatant, GGOH was the main product [Fig. 2(C)]. These results clearly suggested that phosphatase activities were present in both the 20 000 *g* pellet and the 20 000 *g* supernatant fractions whereas the activity of GGOH-18-hydroxylase was present primarily in the 20 000 *g* pellet fraction. The 20 000 *g* pellet fraction was therefore used for study the activity of this enzyme throughout this work and is hereafter called the 'cell-free extract'.

In order to confirm the proposed biosynthetic sequence for the formation of plaunotol from GGPP shown in Fig. 1, a time-course study of the conversion of [1-^{3}H] GGPP to GGOH and plautenol by the cell-free extract was carried out with an aliquot of the incubation mixture being taken for analysis after 5, 30, 60 120 and 180 min. As shown in Fig. 3, it appeared that some [1-^{3}H] GGPP was rapidly converted within 5 min to GGOH and a few minor compounds with lower R_f values. The appearance of the minor peaks was presumably due to the presence in the cell-free extract of other enzymes capable of using GGOH. A number of diterpenoids derived from GGOH have been reported to be present in *C. sublyratus* [2]. The [1-^{3}H] GGOH increased up until 30 min and then declined, until almost disappearing at 180 min. Simultaneously, the radioactive content of plaunotol showed a continuous increase during the whole time-course of the incubation. These results suggested that GGPP in the incubation mixture was first hydrolysed by the phosphatase enzyme to form GGOH followed by C-18 hydroxylation of GGOH catalyzed by GGOH-18-hydroxylase to form plaunotol and, thus, confirmed the proposed plaunotol biosynthetic pathway shown in Fig. 1.

Development of enzyme assay

The technique of TLC-densitometry was developed for determining the catalytic activity of GGOH-18-

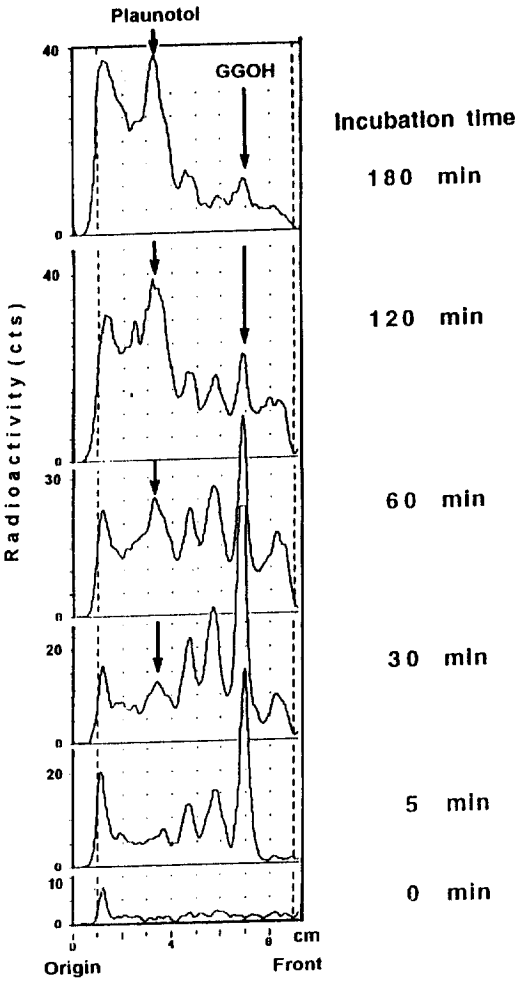

Fig. 3. Time-course of the conversion of [1-^{3}H] GGPP to [1-^{3}H] GGOH and [1-^{3}H] plaunotol by a 20 000 *g* pellet fraction of *C. sublyratus* leaves.

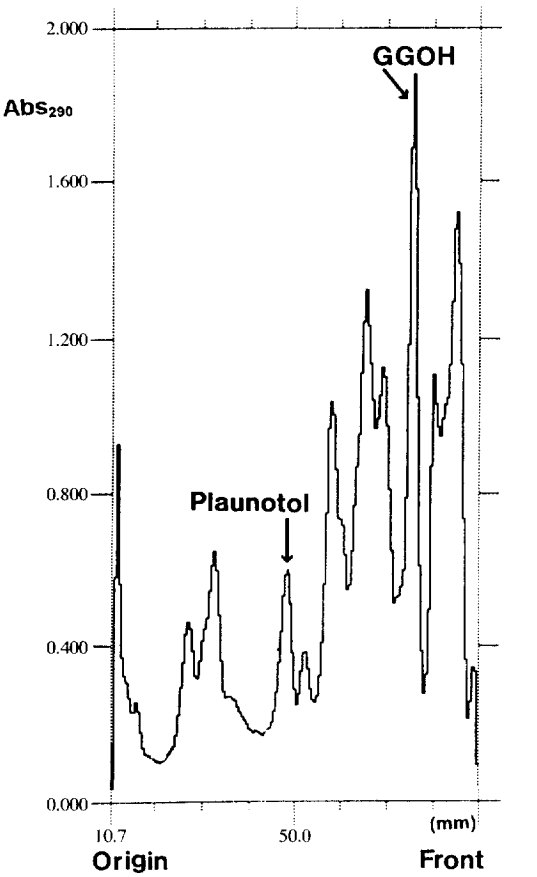

Fig. 4. A typical TLC-chromatogram of the reaction mixture separated by TLC and scanned by TLC scanner (λ = 210 nm). The R_f values of plaunotol and geranylgeraniol are 0.46 and 0.81, respectively.

hydroxylase. Non-radioactively labelled GGOH was used as substrate and the reaction was terminated by ether extraction. Both the substrate and reaction product of plaunotol were extracted into the ether phase but subsequently separated by TLC followed by densitometric scanning of the plate (using λ_{210} nm) to produce a chromatogram. Figure 4 shows a typical TLC-chromatogram of the reaction mixture containing a 20 000 *g* pellet fraction with GGOH-18-hydroxylase activity. It shows a clear separation of the plaunotol peak from other compounds. The substrate, GGOH, appeared at R_f 0.81 whereas the plaunotol was at 0.46. Based on the area under the plaunotol peak and its stardard curve which showed linearity between 0.5 and 15 nmol plaunotol, the enzyme activity of GGOH-18-hydroxylase could be determined.

Product identification

By using the enzyme assay, the formation of plaunotol was found to increase with time and with increased amounts of enzyme protein (data not shown). These findings indicated that the formation of plaunotol was the result of an enzymatic reaction. In order to identify the enzymatic reaction product, a large-scale (250-fold) incubation of the cell-free extract with GGOH was carried out. The resulting incubation mixture was worked up by ether extraction and preparative TLC to obtain the reaction product. The product was then identified by direct comparison with authetic plaunotol by both GC-MS and IR. On GC, the R_t of the enzymatic product (9.25 min) was very close to that of the standard plaunotol (9.31 min). Analysis of both peaks by CIMS showed identifical mass spectra with $[M+1-H_2O]^+$ at 289 and $[M+1-2H_2O]^+$ at 271. The IR spectra, for both the product and plaunotol showed characteristic absorption bands ν^{CHCl_3} cm^{-1}: 3350, 2923, 1645, 1379 and 1016. These results confirmed that the enzymatic product was plaunotol and that the enzyme catalysing the reaction was GGOH-18-hydroxylase.

Some properties of GGOH-18-hydroxylase

Effect of heating on enzyme activity. This study was performed as a result of the observation that the boiled

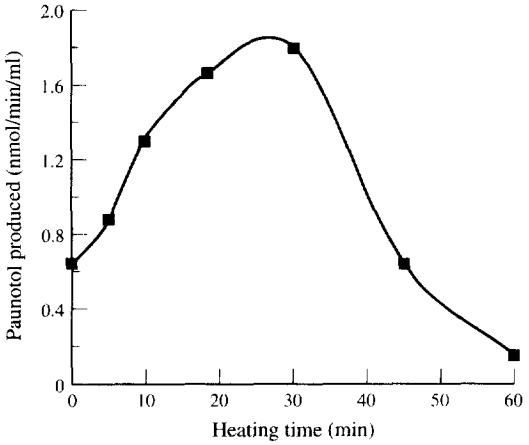

Fig. 5. Effect of heating on the activity of the 20 000 *g* pellet fraction with respect to the conversion of geranylgeraniol to plaunotol.

control frequently showed higher enzyme activity than the normal sample. The cell-free extract was heated at 100° for 5, 10, 20, 30, 45 and 60 min before being put into the reaction mixture. Each mixture was incubated for 30 min and the amount of plaunotol produced measured. It was found that the GGOH-18-hydroxylase activity in the heated cell-free extract increased steadily during the first 30 min of the treatment (Fig. 5). Thereafter, the enzyme activity declined rapidly and was almost undetectable after 60 min. These results clearly showed the positive effect of heating (30 min) on enhancing the enzyme activity.

pH. The normal pH used in the standard incubation mixture was 7.8. However, a study of the effect of pH on the enzyme activity showed that the pH optimum of the enzyme was 5.0. Above or below this pH value, the enzyme activity decreased rapidly.

Coenzymes. The hydroxylation reaction was found to depend on NADPH which was the best electron donor tested. NADH could also substituted for NADPH with an activity of about 70% of that of NADPH. However, the hydroxylation of GGOH by the cell-free extract could also be detected in the absence of these cofactors with an activity of about 54% that of NADPH.

Substrate specificity. The substrate specificity of GGOH-18-hydroxylase in the heated cell-free extract was investigated. Of the substrates tested (GGOH, farnesol and geraniol) only GGOH could be utilized by the enzyme.

Electron micrographs of the heated 20 000 g *pellet fraction*

The 20 000 *g* pellet fraction was first heated for 30 min. The fraction was then centrifuged at 20 000 *g* for 20 min to separate the supernantant and pellet. Detection of the enzyme activity in each fraction showed that most of the activity was present in the supernatant part (Table 1). The supernatant was then concentrated using Centriton-10 concentrator before being used for specimen preparation for electron microscope observation. The resulting electron micrographs revealed the presence of particles with diameters ranging from 20 to 40 nm.

DISCUSSION

We have demonstrated in this study, the presence of the enzyme GGOH-18-hydroxylase in the leaves of *C. sublyratus*. The enzyme is presumably involved in the last biosynthetic step of plaunotol formation. There has been no previous report of GGOH-18-hydroxylase in the literature. One of the crucial steps in this work was the optimization of the conditions for enzyme extraction which allowed the enzyme activity be detected. This was accomplished by using a complex extraction buffer which was slightly modified from the one reported to be used successfully for geraniol-10-hydroxylase extraction from the seedings of *Catharanthus roseus* [6]. Another crucial step was to unequivocally demonstrate that the product of this enzymatic reaction is plaunotol.

By applying the TLC-densitometric enzyme assay developed in this study, the enzyme activity of GGOH-18-hydroxylase was found to be increased with time and related to the amount of enzyme protein. It exhibited a pH optimum at 5.0. This is relatively low as compared with other cytochrome P-450 related monoterpenoid hydroxylases [6–8]. Interestingly, the enzyme activity appeared to increase about 3-fold when the 20 000 *g* pellet fraction was heated for 30 min prior assay. The reason behind this observation is still not clear. It might be possible that some-heat labile inhibitors of the enzyme are present in the cell-free extract.

Study on substrate specificity of GGOH-18-hydroxylase revealed that the enzyme was highly specific to the diterpene GGOH (C-20). No hydroxylation reaction was observed with the two shorter carbon-length substrates tested i.e. farnesol (C-15) and geraniol (C-10). Thus, there can be no doubt that this highly substrate specific 18-hydroxylase enzyme catalyses the last step of the biosynthetic pathway of plaunotol.

EXPERIMENTAL

Chemicals. Authentic plaunotol was obtained from Kelnac® soft gelatin capsules which were manufactured by Sankyo Co., Ltd, Japan; [1-^{3}H] geranylgeranyl diphosphate, triammonium salt, (specific activity = 19.3 Ci mmol^{-1}, 0.5 mCi ml^{-1}) was purchased from Du Pont. GGOH, farnesol, geraniol, NADPH, NADH and DTT were purchased from Sigma.

Plant materials. The leaves of *C. sublyratus* used in this study were obtained from plants growing in an

Table 1. Enzyme activity and protein content in the fractions obtained on 20000 *g* centrifugation of the heated 20000 *g* pellet fraction

Fraction	Enzyme activity (nmol min^{-1} ml^{-1})	Total protein (mg ml^{-1})
Unheated cell-free extract	0.55	0.32
Heated cell-free extract	1.65	0.36
20000 *g* pellet of the heated extract	0.25	0.23
20000 *g* supernatant of the heated extract	0.96	0.19

open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Preparation of cell-free extract. Fresh leaves (30 g) of *C. sublyratus* were snap frozen using liquid N_2 and ground in a pre-cooled mortar. The resulting fine powder was overlayed with 60 ml extraction buffer and allowed to thaw and stirred for 20 min at 4°. The extraction buffer was slightly modified from the grinding buffer used previously for geraniol-10-hydroxylase extraction from the seedlings of *Catharanthus roseus* [6]. It consisted of 83 mM tricine-NaOH, pH 7.8, containing 5 mM β-mercaptoethanol, 0.4 M sucrose, 10 mM EDTA, 1 mM DTT 10 mM $MgCl_2$ and 10 mg ml^{-1} BSA. The suspension was pressed through four layers of cheese-cloth and the filtrate was centrifuged at 3000 *g* for 10 min. The 3000 *g* supernatant was further centrifuged at 20000 *g* for 20 min. The 20000 *g* pellet obtained was resuspended in 10 ml of 0.1 M tricine-NaOH, pH 7.8, containing 5 mM β-mercaptoethanol, 1 mM DTT, 0.2 M sucrose, 1 mM EDTA and 15% glycerol. To prepare the cell-free extract, the 20000 *g* pellet fraction prepared as described above was exposed to boiling water for 30 min before being used.

Detection of enzymatic products of [1-^{3}*H*] *GGPP.* A reaction mixt. containing 0.045 nM [1-^{3}H] GGPP (280000 dpm), 83 mM tricine, pH 7.8, 0.8 mM NADPH and 100 μl enzyme soln in a total vol. of 150 μl was incubated at 30° for 30 min or various time intervals. The reaction was terminated by Et_2O extraction and subjected to TLC (Silica gel 60 F254) using the solvent system of $CHCl_3$–*n*-PrOH (24:1). The TLC plate was then scanned for radioactivity by means of a TLC-radioscanner.

Enzyme assay. The enzyme activity of GGOH-18-hydroxylase was determined by means of TLC-densitometry. The cell-free extract was incubated in the standard reaction mixt. containing 1 mM GGOH, 0.8 mM NADPH, 83 mM tricine buffer, pH 7.8, and 100 μl of the enzyme soln, in a total vol. of 300 μl. After 30 min, the reaction mixt. was extracted with Et_2O and subjected to TLC (Silica gel G 60 F254) in $CHCl_3$–*n*-PrOH (24:1) with triple developement. The TLC plate was then scanned a TLC densitometer (λ_{max} = 210 nm). The amount of enzymatic product formed was estimated from the area under the peak of plaunotol (R_f 0.46) and the calibration curve of authentic plaunotol which showed linearity between 0.5 to 15 nmol of plaunotol. The regression analysis and the correlation coefficient was found to be 0.996994 and its linear slope was 1.928. For the study of the pH optimum of the enzyme, the following buffers were used: 0.1 M sodium acetate buffer for pH 4, 4.5 and 5.0, 0.1 M sodium phosphate buffer for pH 6.5, 0.1 M tricine buffer for pH 7.0, 7.8 and 8.0 and 0.1 M glycine buffer for pH 9.0 and 10.0.

Product identification. The hydroxylation product was identified by direct comparisons with authentic plaunotol by GC, CI-MS and IR. GC (capillary column): DB-5 (30 × 0.25 mm), temp. of detector and injector was 250°. Initial column temp. 150° for 3 min. then increased at 10° min^{-1} until 300° which was then held for 20 min. CIMS: CH_4 as reagent gas; IR the enzymatic product and authentic plaunotol were dissolved in $CHCl_3$. Each soln was placed in a KBr liquid cell and the solvent was evapd before operation.

Electron micrograph of the heated 20000 g *pellet fraction.* The 30-min heated 20000 *g* pellet fr. was centrifuged at 20000 *g* for 20 min. The supernatant showing 18-hydroxylase activity was then concd by Centricon-10 concentrator. The concd boiled enzyme was examined by TEM.

Acknowledgements—The assistance of Mr Phitsanu Soonthornrattanaruk in the preparation of this manuscript is greatly appreciated. This work was supported by the Thailand Research Fund, Chulalongkorn University and Sankyo Co., Ltd, Japan.

REFERENCES

1. Ogiso, A., Kitazawa, E., Kobayashi, S., Komai, T., Matsunuma N. and Kataumi, S., *Sankyo Kenkyusho Nempo*, 1985, **37**, 1.
2. Ogiso, A., Kitazawa, E., Kurabayashi, M., Sato, A., Takahashi, S., Noguchi, H., Kuwano, H., Kobayashi, S. and Mishima, H., *Chemical and Pharmaceutical Bulletin*, 1978, **26**, 3117.
3. Loomis, D.W. and Croteau, R., in *The Biochemistry of Plants, A Comprehensive Treatise*, Vol.

7, ed. E. E. Conn. Academic Press, New York, 1981, p. 364.
4. Morimoto, H. and Murai, F., *Plant Cell Reports*, 1989, **8**, 210.
5. Kitaoka, M., Nagashima, H. and Kamimura, S., *Sankyo Kenkyusho Nempo*, 1989, **41**, 169.
6. Madyasta, K. M., Meehan, T. D. and Coscia, C. J., *Biochemistry*, 1976, **15**, 1097.
7. Karp, F., Mihaliak, C. A., Harris, J. L. and Croteau R., *Archives of Biochemistry and Biophysics*, 1990, **276**, 219.
8. Hallahan, D. L., Nugent, J. H. A., Hallahan, B. J., Dawson, G. W., Smiley, D. W., West, J. M. and Wallsgrove, R. M., *Plant Physiology*, 1992, **98**, 1290.
9. Taniguchi, H., Imai, Y. and Sato, R., *Archives of Biochemistry and Biophysics*, 1984, **232**, 585.