Phytochemistry, Vol. 22, No. 9, pp. 1917–1920, 1983.
Printed in Great Britain.

0031-9422/83 $3.00 + 0.00
Pergamon Press Ltd.

LUNULARIC ACID IN CELL SUSPENSION CULTURES OF *MARCHANTIA POLYMORPHA*

SETSUKO ABE and YOSHIMOTO OHTA

Suntory Institute for Bio-organic Research, Wakayama-dai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

(*Received* 6 *January* 1983)

Key Word Index—*Marchantia polymorpha*; Hepaticae; liverwort; cultured cells; bibenzyls; lunularic acid.

Abstract—Lunularic acid (LNA) was isolated from the cultured cells of the liverwort *Marchantia polymorpha*. Quantitative analysis by reverse phase HPLC showed that the content of LNA in the cells changed markedly during their growth, ranging from 1 to 7 μg/mg dry wt. The accumulation of LNA was greatly enhanced by a deficiency of phosphate in the culture medium.

INTRODUCTION

Lunularic acid (3,4'-dihydroxybibenzyl-2-carboxylic acid) (LNA) was first isolated as a dormancy factor from an Israeli strain of the liverwort, *Lunularia cruciata* [1, 2] and has been detected in more than 70 species of liverworts examined so far [3]. It has been suggested that LNA may control drought resistance in liverworts as an endogenous growth inhibitor [1] and this inhibitory activity has been compared with that of abscisic acid in higher plants [4]. However, studies on LNA are rather limited compared to those on ABA and are not quantitative enough to discuss its physiological role in liverworts. A cell suspension culture of a liverwort, therefore, seemed to be an appropriate experimental system not only for the study of LNA accumulation in the cells under various environmental conditions, but also for studies on cellular localization and metabolism of this compound. This paper describes the identification and quantitative determination of LNA in suspension cultured cells of *Marchantia polymorpha* and an examination of the growth factors which affect the content of LNA.

RESULTS AND DISCUSSION

Isolation of LNA in suspension cultured cells

Sephadex LH-20 CC of the strong acid fraction of an acetone extract of cultured *M. polymorpha* cells gave a crystalline compound which was identified as LNA by comparing its NMR and IR spectra and R_f on TLC with those of the authentic sample. LNA was detected not only in the cultured cells of *M. polymorpha* but also in those of other species of liverworts as described below. The production of LNA is thought to be ubiquitous to the cultured cells of liverworts as well as to the intact plants.

Improvement of a quantitative analytical method

The analytical method employed by Gorham [3] included the derivatization of a strong acid fraction containing LNA into the volatile TMSi derivative followed by GC determination. This procedure is rather time-consuming and inconvenient for the analysis of a large number of small samples. In our experiment, a methanol extract of plant materials was, after pre-treatment using a Waters' Sep-pak C_{18} cartridge, directly applied to a reverse phase HPLC monitored by a UV detector at 285 nm. LNA was eluted at *ca* 4 min under the conditions employed. Recovery of LNA in the cells was shown to be over 99% by examining the additivity of a known amount of the acid to the cells. LNA can be determined reliably at the ng level by this improved procedure.

Determination of LNA content in cultured cells of liverworts

The LNA content in the intact thallus of *M. polymorpha* and cultured cells of the same liverwort grown under various culture conditions are shown in Table 1. Also included are LNA contents in the cultured cells of some other liverworts of the Jungermanniales. Both the callus and the suspension cultured cells of *M. polymorpha* contain a larger amount of LNA than the intact thallus. The LNA content in the dark grown cells is lower than in the light grown cells, indicating that photosynthetic output may be essential for the production of this compound, as suggested by Gorham [5]. The callus or the suspension cultured cells of the other liverworts contain smaller quantities of LNA than that of *M. polymorpha* which belongs to the order Marchantiales. This reflects the similar relationship which has been observed in the intact plants [3, 6].

The LNA contents are considerably higher than those expected for a growth regulatory substance, such as ABA. In order to discuss the physiological role of such abundant LNA in liverworts, it is essential to know its intracellular distribution.

Growth and LNA content in M. polymorpha *cells*

The amount of LNA in *M. polymorpha* cells in suspension culture showed a characteristic change corresponding to the growth cycle of the cells (Fig. 1). The cell dry wt increased exponentially for 7 days after inoculation into

Table 1. LNA content in the cultured cells of some liverworts

Species	Culture condition		LNA content (μg/mg dry wt)
Marchantia polymorpha	intact thallus		1.19 ± 0.04
	suspension	light	4.78 ± 0.23
		dark	0.70 ± 0.09
	callus	light	10.8 ± 1.2
Jungermannia subulata	suspension	light	0.06 ± 0.01
Lophocolea heterophylla	suspension	light	0.04 ± 0.00
Calypogeia tosana	callus	light	0.15 ± 0.01

Each value is the mean of three or four samples with s.e. The cells in suspension culture were grown in the light or dark, and LNA content obtained on the cells at the stationary phase. *Ca* 1-month-old callus was used for the analysis.

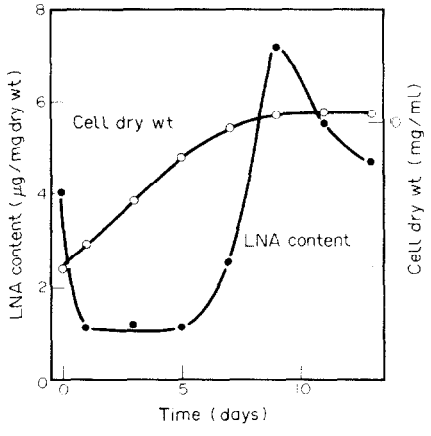

Fig. 1. Growth and changes in LNA content of *M. polymorpha* cells cultured in MSK-2 medium. Data are the means from two experiments.

fresh medium and reached the stationary phase at *ca* day-9. The amount of LNA per cell dry wt decreased immediately after the initiation of the culture, remained low and increased rapidly after the exponential phase to reach the initial value. The total amount of LNA per flask also decreased to *ca* 40% of the initial value at the early phase of the culture. This suggested that a considerable part of the acid accumulated in the cells was metabolized upon transfer of the cells into the fresh medium.

The above results show that the actively growing cells contain a smaller amount of LNA in comparison to the non-growing cells at the stationary phase. This relationship may support the concept that LNA serves as a growth regulating substance in liverworts. However, since the major nutrients in the medium had been consumed when the cells entered the stationary phase [7], it is also possible that such an undesirable environmental condition as the depletion of nutrients in the medium may have caused the cessation of cell growth and increased the LNA content.

In order to examine the influence of nutrient limitation on LNA content, the cells were grown for 10 days in the modified MSK-2 media lacking one of inorganic nitrogen, inorganic phosphate or glucose, and the LNA content of these cells was compared with the control value obtained with the cells grown in complete MSK-2 medium (Table 2). Growth of cells was considerably reduced when cultured in the media which lack one of these major nutrients. The amount of LNA accumulated in the cells grown in nitrogen or glucose deficient medium was also reduced to 54% or 27% of the control values, respectively. In contrast, in phosphate deficient medium, the accumulation of LNA was greatly enhanced to 160% of the control. The time courses of LNA content and phosphate consumption from media were examined when the cells were cultured in the media including various amount of phosphate. As shown in Fig. 2, the LNA content started to increase when phosphate was depleted from the media. For example, LNA in the cells cultured in the medium containing 1 mM phosphate started to accumulate after day-6 when phosphate was depleted, and reached a value of *ca* 12 μg/mg dry wt.

Table 2. Influence of removal of major nutrients from MSK-2 medium on growth and LNA content of cultured *Marchantia polymorpha*

Removed	Dry wt		LNA content	
	mg/ml	%	μg/mg dry wt	%
None (Control)	12.9 ± 0.1	100	6.63 ± 0.28	100
PO_4^{3-}	8.2 ± 0.3	64	10.6 ± 0.4	160
NO_3^-, NH_4^+	4.9 ± 0.2	38	3.58 ± 0.13	54
Glucose	1.1 ± 0.1	9	1.77 ± 0.15	27

Each value is the mean of four cultures with s.e. Determinations were carried out 10 days after transfer of the cells into the indicated media.

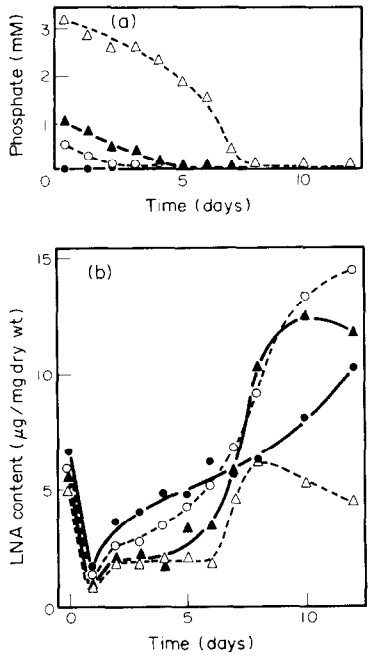

Fig. 2. Phosphate consumption from the media including various amount of phosphate (a), and changes in LNA content of *M. polymorpha* cells cultured in the above media (b). (●) 0 mM; (○) 0.5 mM; (▲) 1 mM; (△) 3 mM phosphate. Data are the means from two experiments.

Such results clearly indicate that the accumulation of LNA is inversely correlated with the amount of phosphate in the medium, and age-dependent accumulation of LNA as shown in Fig. 1 can be explained as the result of depletion of phosphate in the medium.

There have been reported similar results, that the accumulation of some phenolic compounds in the suspension cultured cells of higher plants was susceptible to the supply of major nutrients, such as nitrogen, phosphate or carbon [8–10]. An antagonistic regulation mechanism between primary and secondary metabolism has been postulated to explain the discrepancy between the pattern of cellular growth and accumulation of secondary metabolites [11, 12]. The formation of LNA in *M. polymorpha* cells in suspension culture also seems to be regulated by a similar mechanism.

Some other control mechanism in the formation of LNA as a growth regulator may operate in intact liverworts and it should be worthwhile accumulating fundamental knowledge concerning the metabolism and the intra-cellular localization of LNA using the cell culture system. Further studies on these problems are now in progress.

EXPERIMENTAL

Plant materials. The cell suspension culture of *M. polymorpha* was grown in MSK-2 medium supplemented with 2% Glc and 1 mg/l. 2,4-D according to refs. [7, 13]. The same medium solidified with 1% agar was utilized for the callus culture of *M. polymorpha*. Callus of *Jungermannia subulata* was cultured as described previously [14]. Cells of *Lophocolea heterophylla* were grown in the same manner as those of *J. subulata*, and the callus of *Calypogeia tosana* was kindly offered by Mr. Katoh of this Institute. Intact thalli of *M. polymorpha* were collected in July 1982 in the backyard of this Institute in Osaka. Measurement of the growth of the cultured cells was carried out according to ref. [13]. In the expt to examine the nutritional effects on LNA content, *M. polymorpha* cells were cultured in the modified MSK-2 medium without each of the following major nutrients; inorganic nitrogen (NO_3^-, NH_4^+), Pi or Glc, and the cells in the complete MSK-2 medium were employed as control. The cells were also cultured in the same medium containing 0, 0.5, 1 or 3 mM Pi to examine the effect on LNA production. Pi in the medium was determined by the method of ref. [15].

Isolation and identification of LNA. Cultured cells of *M. polymorpha* were harvested by filtration and extracted with Me_2CO. After removal of Me_2CO under red. pres., the residue was suspended in 5% $NaHCO_3$ soln and extracted with *n*-hexane to remove non-polar compounds. The aq. phase was acidified to pH 3 with 6 M HCl and extracted with Et_2O. The strong acid fraction obtained was chromatographed on a Sephadex LH-20 column using EtOAc–$CHCl_3$–HOAc (15:5:1). Fractions which contained a compound showing the same R_f value as authentic LNA on TLC were combined and evaporated to give a crystalline residue. Recrystallization of the residue from EtOH–H_2O yielded needles, mp 192°. This compound was identified as LNA by comparing its NMR and UV spectra and R_f value on Si gel TLC (EtOAc–$CHCl_3$–HOAc, 15:5:1) with those of the authentic sample.

Quantitative determination of LNA. For quantitative determination of LNA, cultured cells (1–5 mg dry wt) were extracted with hot MeOH containing 1% HOAc. After removal of MeOH *in vacuo*, the residue was redissolved in 1 ml of 60% MeCN in H_2O and passed through a Waters' Sep-pak C_{18} cartridge using the same solvent system in order to remove non-polar compounds. The eluate (4 ml) containing LNA was analysed by HPLC using a Partisil ODS column (4 × 250 mm) using 50% MeCN in H_2O containing 0.1% HOAc as solvent and monitoring with UV at 285 nm. The absolute amount of LNA in the eluate was determined by comparing the peak area with those of standard solns.

Acknowledgements—We thank Dr. Pryce, University of London, for NMR of LNA, and Dr. Kamikawa, Osaka City University, for supplying an authentic sample of this compound. We are also grateful to Mr. Katoh of this Institute for supplying cultured cells of *C. tosana*.

REFERENCES

1. Valio, I. F. M., Burdon, R. S. and Schwabe, W. W. (1969) *Nature (London)* **223**, 1176.
2. Valio, I. F. M. and Schwabe, W. W. (1970) *J. Exp. Botany* **21**, 138.
3. Gorham, J. (1977) *Phytochemistry* **16**, 249.
4. Pryce, R. J. (1972) *Phytochemistry* **11**, 1759.
5. Gorham, J. (1980) in *Progress in Phytochemistry* (Reinhold, L., Harborne, J. B. and Swain, T., eds.) Vol. 6, p. 203. Pergamon Press, Oxford.
6. Gorham, J. (1978) *Bull. Br. Bryol. Soc.* **31**, 11.
7. Katoh, K., Ishikawa, M., Miyake, K., Ohta, Y., Hirose, Y. and Iwamura, T. (1980) *Physiol. Plant.* **49**, 241.
8. Knobloch, K.-H. and Berlin, J. (1981) *Planta Med.* **42**, 167.
9. Zenk, M. H., El-Shagi, H. and Schulte, U. (1975) *Planta Med. (Suppl.)* 79.
10. Westcott, R. J. and Henshaw, G. G. (1976) *Planta* **131**, 67.

11. Phillips, R. and Henshaw, W. W. (1977) *J. Exp. Botany* **28**, 785.
12. Knobloch, K.-H., Beutnagel, G. and Berlin, J. (1981) *Planta* **153**, 582.
13. Ohta, Y., Katoh, K. and Miyake, K. (1977) *Planta* **136**, 229.
14. Ohta, Y., Ishikawa, M., Abe, S., Katoh, K. and Hirose, Y. (1981) *Plant Cell Physiol.* **22**, 1533.
15. Allen, R. J. L. (1945) *Biochem. J.* **161**, 83.