Phytochemistry, Vol 23, No 7, pp 1379–1381, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

THE CONCENTRATIONS OF LUNULARIC ACID AND PRELUNULARIC ACID IN LIVERWORTS

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(*Received* 9 *December* 1983)

Key Word Index—*Marchantia polymorpha*, *Conocephalum conicum*, *Marchantia paleacea* var *diptera*, Hepaticae, suspension culture, lunularic acid, prelunularic acid

Abstract—The amounts of lunularic acid in suspension cultured cells of *Marchantia polymorpha* and thallus of *Conocephalum conicum* were carefully re-examined taking into account the presence of prelunularic acid, a possible labile precursor of lunularic It was found that the true content of lunularic is 2–4 μg/g dry wt while very much larger amounts of prelunularic are present

INTRODUCTION

Lunularic acid (LNA) was first isolated from the thalli of *Lunularia cruciata* [1, 2] and has been detected in more than 70 species of liverworts in concentrations ranging from 1 to 650 μg/g fresh wt [3] It has been suggested that LNA may be an endogenous growth regulator on the basis of its inhibitory activity on the growth of liverworts

Recently we isolated prelunularic acid (preLNA) from the suspension cultured cells of *Marchantia polymorpha* and also showed the presence of this compound in some intact liverworts [4, 5] PreLNA was easily converted into LNA under acidic or basic conditions and appears to be a direct precursor of LNA Since preLNA is very labile, we suspected that the analytical procedure for LNA previously employed might overestimate the true LNA content In this experiment, we determined the true content of LNA in cultured cells of *M polymorpha* and also in thallus of *Conocephalum conicum*, taking the instability of preLNA into consideration

RESULTS AND DISCUSSION

Extraction methods for quantitative determination of LNA

Prelunularic acid has been shown to be converted into LNA under acidic or basic conditions [4, 5] Even under neutral conditions, this compound is slowly converted into LNA For example, approximately 0 3% of preLNA in water was converted in one day at room temperature, and 9% on boiling for 30 min This suggests that a part of LNA which has been extracted and determined in the previous papers might be derived from preLNA Thus the amount of LNA in the suspension cultured cells of *M polymorpha* was re-examined using different extraction methods (A, B and C) In method A, the cells were extracted with boiling methanol containing 1% acetic acid, and boiling methanol without acetic acid was used in method B In method C, the cells were sonicated in 60% acetonitrile in water at 4° Subsequently portions of these extracts were treated with 1 M sodium hydroxide as described in the Experimental in order to convert preLNA into LNA The amounts of LNA in these extracts before (a) and after (b) the alkaline treatment were determined, and the results are summarized in Table 1 Since the authentic preLNA was converted completely into LNA by this treatment, the amount of LNA (b) should be considered as the sum of preLNA and LNA which had been originally present in the cells

The content of LNA determined by the method A was 4 42 μg/mg dry wt before the alkaline treatment This value was much higher than that obtained by method B However, the amounts of LNA after the alkaline treatment were the same, 4 94 μg/mg dry wt, in both extracts, indicating that most of the preLNA had been converted into LNA during the extraction with acidic solvent in method A Since the same method was employed in our previous experiment [6], the amount of LNA reported represents the total of preLNA and LNA The LNA content determined by method C was 0 12 μg/mg dry wt, which was 3 6% of the sum of these two compounds These results show that preLNA is the dominant constituent

Similar results were also obtained for the intact thalli of *C conicum* and *M paleacea* Segments of the thalli were extracted by soaking in methanol overnight at 4° As shown in Table 1, the amount of LNA before the alkaline treatment (a) was 0 046 μg/mg dry wt (8 7 μg/g fr wt) in *C conicum* and 0 016 μg/mg dry wt (6 2 μg/g fr wt) in *M paleacea*, respectively These values are 2–4% of the sum of preLNA and LNA (b), indicating that the amount of LNA in the intact liverworts is also smaller than that of preLNA, as in the cultured cells of *M polymorpha*

The previously reported values of LNA content in the thalli of *C conicum* were 200–650 μg/g fresh wt [3] and 60 μg/g fresh wt [7] Such large values, in light of our results, could be due to their drastic extraction conditions using 2 M hydrochloric acid in boiling alcohol The amounts of LNA determined under such drastic conditions do not represent the actual content of LNA in the liverworts, but the sum of the LNA and preLNA contents

Table 1 Lunularic acid content in liverworts

	Extraction method	LNA content before NaOH (a)	LNA content after NaOH (b)	a/b × 100 (%)
		μg/mg dry wt (μg/g fr wt)		
Marchantia polymorpha				
Cultured cells	A	4 4±0 1	4 9±0 0	89 8
	B	0 39±0 01	4 9±0 2	8 0
	C	0 12±0 01	3 37±0 08	3 6
Conocephalum conicum				
Thallus		0 046±0 001 (8 7±0 2)	1 01±0 02 (193±3)	4 6
Marchantia paleacea var *diptera*				
Thallus		0 016±0 003 (6 2±1 1)	0 81±0 02 (309±7)	1 9

Cultured cells of *M polymorpha* were extracted by three methods A, boiling MeOH containing 1% HOAc, B, boiling MeOH, C, sonication in 60% MeCN The thallus of *C conicum* and *M paleacea* were extracted by soaking in MeOH at 4° These extracts were assayed for LNA content before (a) and after (b) alkaline treatment (1 M NaOH at room temperature for 10 min) Each value is the mean of four samples with s e

The true content of LNA in the liverworts

Determination of the true content of LNA is essential for understanding its growth regulating effects The results obtained above may not indicate the true amount of this compound because the conversion of preLNA into LNA is still possible even under the conditions employed in method C Instead, preLNA was reduced with sodium borohydride to the corresponding diols, which do not form LNA, simultaneously with extraction [cf 4, 5] When 40 μg authentic preLNA was treated with 0 4 g sodium borohydride, complete disappearance of preLNA was shown on HPLC On the other hand, the authentic LNA was completely recovered after the same treatment with sodium borohydride So the amount of LNA determined in the presence of sodium borohydride represents the true content of LNA in the liverworts The cultured cells of *M polymorpha* and thallus segments of *C conicum* were frozen and ground in ethanol in the presence of a large excess of sodium borohydride After quenching the reaction and centrifugation, the amount of LNA in these homogenates were determined before and after alkaline treatment, as described in the Experimental The same extraction without sodium borohydride was performed as the control

As shown in Table 2, only 4 μg/g dry wt of LNA was detected in the cultured cells of *M polymorpha* and 2 μg/g dry wt in the thallus of *C conicum*, respectively The fact that there was no increase of LNA after the alkaline treatment excluded the possibility that the plant materials contained a conjugate of LNA such as a glycoside which also produces LNA under basic conditions These values were about 0 1–0 2% of the total of preLNA and LNA determined in the control experiment (b') This result shows that the real amount of LNA in the liverworts is much smaller than those determined by the usual extraction procedures, including the results shown in Table 1 In addition, preLNA, the possible *in vivo* precursor of LNA, actually accumulates in much larger amounts in the liverworts

Table 2 Lunularic acid content determined in the presence of $NaBH_4$

	LNA content				
	$NaBH_4$ treatment		Control		a/b' × 100 (%)
	(a)	(b)	(a')	(b')	
	μg/mg dry wt				
Marchantia polymorpha					
Cultured cells	0 004	0 005	0 127	3 97	0 1
Conocephalum conicum					
Thallus	0 002	0 002	0 044	1 09	0 2

Data are the means from two experiments The cultured cells or thallus were ground with or without (control) $NaBH_4$ LNA contents in these homogenates were determined before (a and a') and after (b and b') alkaline treatment

Biosynthetically, this accumulation of precursor is unusual It suggests that the conversion of preLNA, accumulated in a pool of large size, into LNA is strictly regulated to keep the concentration of LNA at a low level On this basis, we can now reinterpret the results obtained in the *in vivo* experiment by Gorham [8] In his experiment using thallus of *C conicum*, he reported a discrepancy between the metabolism of exogenously supplied ^{14}C-LNA and that of ^{14}C-LNA biosynthesized from labelled phenylalanine *in situ* Knowing that the endogenous ^{14}C-LNA which was extracted with 2 M hydrochloric acid in methanol was actually preLNA, we can see that his results showed that a large amount of preLNA, biosynthesized from phenylalanine, is pooled in the thallus without conversion into LNA and that excess LNA, when supplied exogenously, is rapidly metabolized Such a strict control of LNA level in liverworts may be related to the growth inhibitory activity of this compound

EXPERIMENTAL

Plant materials The cells of *M polymorpha* were cultured in MSK-2 liquid medium supplemented with 2% glucose and 1 mg/l of 2,4-D as reported previously [9, 10] Intact thalli of *C conicum* and *M paleacea* var *diptera* were collected at Shaku-dai in Osaka prefecture and washed well with running water

Extraction and determination of LNA and preLNA The cells of *M polymorpha*, cultured for 10–14 days, were harvested and extracted three times with boiling MeOH containing 1% HOAc (A) or with boiling MeOH (B) After removal of the solvent *in vacuo*, the residue was resuspended in 1 ml of 60% MeCN in H_2O and passed through a Waters Sep-pak C_{18} cartridge using the same solvent The eluate (4 ml) was analysed for LNA (a) A portion of this eluate was evaporated *in vacuo* and the residue was treated with 1 ml of 1 M NaOH for 10 min in order to convert preLNA into LNA The mixture was acidified with 6 M HCl and extracted with EtOAc After evaporation of the solvent, the residue was redissolved in 60% MeCN in H_2O and analysed for LNA (b)

The cells were also homogenized by sonication in 60% MeCN in H_2O (C) to shorten the extraction time and to keep the temperature low (4°) The homogenate was centrifuged (3000 rpm, 10 min) and the supernatant was passed through a SEP-PAK C_{18} cartridge and analysed for LNA

Fresh thalli of *C conicum* and *M paleacea* were cut into small pieces and extracted with MeOH by soaking overnight at 4° Subsequent procedures were same as in the case of cultured cells

The amount of LNA in the samples were determined on an HPLC fitted with a Partisil ODS column (4 × 250 mm) The elution solvent was 50% MeCN in H_2O containing 20 mM ammonium acetate adjusted to pH 3 6 with HOAc The absolute amount of LNA was determined by comparing the peak height monitored at 285 nm with that of standard soln PreLNA was analysed according to ref [5], when authentic preLNA was treated with NaOH and $NaBH_4$

Determination of LNA in the presence of $NaBH_4$ The suspension cultured cells (*ca* 40 mg in dry wt) were harvested by filtration and frozen in a deep freezer (−70°) The frozen cells were ground in EtOH in the presence of 0 4 g $NaBH_4$ and 1 g quartz sand (150–200 mesh) in a mortar for about 15 min A saturated aq soln of NH_4Cl was slowly added to terminate the reaction The reaction mixture was centrifuged and the supernatant evaporated to dryness *in vacuo* The residue was dissolved with EtOAc and partitioned with 5% $NaHCO_3$ soln After acidification, the aq phase was extracted with EtOAc to obtain the strong acid fraction EtOAc was substituted by 60% MeCN in H_2O for the assay of LNA The same extraction without $NaBH_4$ was performed as a control The thallus segments of *C conicum* (*ca* 160 mg in dry wt) were also examined in the same way as the cultured cells In this case, 1 6 g $NaBH_4$ and 2 g sea sand (20–30 mesh) were utilized in the extraction step Recovery of LNA was checked by treating 25 μg authentic LNA with 0 4 g $NaBH_4$

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