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Improvement of the analysis of dansylated derivatives of polyamines and their conjugates by high-performance liquid chromatography☆

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Abstract

The paper described a method for improving the hydrolysis of conjugated polyamines in PH fraction, isolated from the lichen *Evernia prunastri,* as well as the optimization of dansylation procedure of these polyamines on the basis of the pH value to which derivatization is achieved. Dansylated polyamines have been later separated by high-performance liquid chromatography (HPLC) using a gradient elution. Hydrolysis of conjugates requires acid treatment at room temperature rather than at 110°C, as usually described. Dansylation is improved at high pH values, whereas removal of phenolics (mainly evernic acid), from the conjugates requires low pH values. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Evernia prunastri; Derivatization, LC; Plant materials; Polyamines; Phenolic compounds

1. Introduction

Polyamines (PAs) are biologically ubiquitous aliphatic nitrogen-containing compounds of low molecular mass and polycationic nature. They occur in the free form (S-PAs, soluble poyamines) or bound to low-molecular mass compounds (SH-PAs, acid soluble polyamines) or macromolecules (PH-PAs, acid insoluble polyamines). In the plant kingdom, the highest concentration of PAs occurred in both meristematic and actively growing tissues [1]. Conjugation of PAs to phenolic acids has been observed in many plants with flowers [2]. Conjugation consists in a covalent amide bond between the amine and, mainly, hydroxycinnamic acids. Hydroxycinnamic amides have been related to several developmental process. For example, these compounds are accumulated, after floral induction, in the apex shoot [3].

In *Pseudevernia furfuracea*, *Platismatia glauca* and *Hypogymnia physodes*, three lichen species, the concentration of PAs was similar to that of their precursor amino acids and they occur as conjugated PAs rather than in their soluble forms. Jäger and Weigel [4] postulate that PAs were bound to nucleic acids, ribosomes and membranes providing a certain stability against extreme environmental conditions. In *Evernia prunastri* thallus, Escribano and Legaz [5] demonstrated the presence of putrescine (PUT), spermidine (SPD) and spermine (SPM) as soluble as well as conjugated forms.

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PAs have routinely been analyzed by high-performance liquid chromatography (HPLC) as benzoyl or dansyl derivatives, after acidic extraction from the tissues and ulterior hydrolysis in order to obtain both free and conjugated forms. Biological samples are heated with 12 *M* HCl, at 100–120°C for 18 h before derivatization, although some authors suggest that the acidic hydrolysis may be omitted [6].

Usually, PAs are analyzed by HPLC and detected as dansyl derivatives by fluorescence emission at 500 [7] or 520 nm [8]. However, other derivatization processes are also routinely used. PAs from milk have been derivatized with 9-fluorenylmethoxycarbonyl. The excess of reagent was reacted with aspartic acid and detection was monitored at 313 nm [9]. Putrescine, cadaverine, spermidine and spermine have also been derivatized with *N*-hydroxysuccinimidyl 6-quinolinyl carbamate and detected by fluorescence emission at 398 nm [10] or with *o*-phthalaldehyde (OPA)-2-mercaptoethanol, using fluorescence detection at 445 nm [11,12].

Alternatively, polyamines were detected by UV absorbance at 254 nm as acetylated molecules [13], or at 242 nm after derivatization with 4-fluoro-3-nitrobenzotrifluoride [14]. Recently, Legaz et al. [15] were able to separate polyamines as tosyl derivatives and detection by absorbance at 200 nm in capillary electrophoresis.

The aim of the present work was to optimize the hydrolytic procedure of polyamine conjugates by using different analytical conditions (different acids, variable temperatures, and times), the dansylation procedure and to improve the recovery of phenolic acids from acid soluble and acid insoluble polyamine conjugates.

2. Material and methods

2.1. Biological material and incubation conditions

Evernia prunastri (L.) Ach. thallus growing on *Quercus pyrenaica* Lam. and collected in Valsain (Segovia, Spain) was used throughout this work. Plant material was stored in polythene bags, at 7°C, until required, no longer than 1 month. Samples of 1.0 g dry thallus were incubated on 15 ml of 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.8 for 2 h at 26°C in the dark.

2.2. Chemicals

All chemicals used for the preparation of the buffers and hydrolysis solutions, acetic acid, formic acid, hydrochloric acid, perchloric acid and MES, were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. Water was of Milli-Q grade (Millipore, Bedford, MA, USA). Butylamine, putrescine, spermidine and spermine, were purchased from Sigma (St. Louis, MO, USA), whereas diethyl ether, ethyl acetate, chloroform, methanol and acetonitrile were also from Merck.

2.3. Analysis of free and conjugated PAs by HPLC

Samples were subjected to acidic hydrolysis with 12 *M* HCl at 110°C, according to Tiburcio et al. [16], with some modifications. Thalli were rinsed, after incubation, with distilled water for 2 min, gently dried with filter paper and then macerated with 6.0 ml 5% (v/v) cold perchloric acid (PCA) containing 0.15 ml 5.0 m*M* butylamine (BUT). Homogenates were stored overnight at 4°C and after this centrifuged at 48 000 *g* for 20 min at 2°C. Supernatants (containing S- and SH-PAs) were stored at −20°C whereas precipitates (containing PH-PAs) were resuspended in 6.0 ml 1.0 *M* NaOH containing 0.15 ml 5.0 m*M* BUT and stored at 4°C for 12 h.

Aliquots of 2.0 ml of both supernatants and precipitates were mixed with: (i) 2.0 ml 12 *M* HCl for 18 h at 110°C in flame-sealed vials or, alternatively, at room temperature; (ii) 2.0 ml 1.0 *M* acetic acid for 18 h at 110°C or, alternatively, at room temperature; and (iii) 2.0 ml 90% (v/v) formic acid for 8 h at 110°C or at room temperature.

After hydrolysis, mixtures were centrifuged at 12 000 *g* for 15 min at 2°C. Supernatans were divided between two samples, one of them used to analyze polyamines and the another one to study conjugated phenols.

2.4. Derivatization of PAs

Aliquots of 0.2 ml were previously adjusted at pH 0.5, 2.0, 7.0, and 12.0 by adding sufficient volume of

NaOH solutions at different concentrations, never more than 10 μl per aliquot, and then dansylated according to Smith and Best [17] with 0.4 ml of 75 m*M* dansyl chloride in acetone in the presence of 0.4 ml of saturated sodium carbonate. Dansylation was carried out in a vial hermetically sealed for 18 h at room temperature. Proline (0.1 ml of 2.0 m*M*) was then added to destroy the excess of dansyl chloride. Dansylated polyamines were extracted from the mixtures with 3×3.0 ml of toluene (HPLC grade). The toluene phase was dried at 40°C under a stream of air. Once the derivatization procedure was concluded, samples were cleaned by adding 0.6 ml of 5.0 m*M* KOH in methanol (HPLC grade), according to Sailer and Knödgen [18]. Mixtures were left to stand for 45 min at 40°C and then 1.5 ml of an aqueous mixture containing 200 mg of KH_2PO_4 and 200 mg of Na_2HPO_4 was added. Polyamines were extracted again with 3×3.0 ml of toluene, as described above. The organic phase was dried, and dry residues were redissolved in 200 μl of methanol (HPLC grade) to be chromatographed.

2.5. Analysis of PAs

Analysis of PAs was performed by using a Varian 5060 (Walnut Creek, CA, USA) liquid chromatograph equipped with a Fluorichrom fluorescence detector (Varian) and a SpectraSystem UV2000 detector (SpectraPhysics, Freemont, CA, USA) in parallel, connected as required by using a Rheodyne valve, and a Vista CDS 401 computer (Varian). The chromatographic conditions were according to the method described by Escribano and Legaz [5]. Polyamines were eluted from a Varian MicroPak MCH-5N cap (15 cm×4 mm I.D.) reversed-phase column at 40°C using a methanol–water gradient. Gradient elution was as follows: methanol–Milli-Q water in a linear gradient from 60:40 (v/v) to 80:20 (v/v) in 15 min. This last proportion was maintained for 10 min to the end of the analysis. Simultaneously, a flow gradient was applied by increasing from 0.7 to 1.5 ml min^{-1} for 15 min and maintaining this last value to the end of the analysis. Detection was performed by fluorescence intensity measurements. PAs were quantified by interpolating values of area counts for each peak in the corresponding straight line constructed by using increasing concentrations of standard PAs from 1.0 to 100 μ*M*.

2.6. Extraction of phenolic acids associated to conjugated PAs (SH- and PH-fractions) and HPLC analysis

An aliquot of 0.2 ml of each conjugated polyamine fractions, SH and PH, was firstly extracted twice with 4×4.0 ml of chloroform–acetonitrile (60:40, v/v) and second with 4×4.0 ml diethyl ether–ethyl acetate (65:35, v/v). The extraction was performed at room temperature for 5 min or at 40°C for 30 min, as indicated. Organic phases were mixed and carried out to dryness under air flow [19]. Residues were redissolved in 0.1 ml of acetonitrile and used for HPLC analysis.

Phenolics were separated in a Nucleosil C_8 (125× 4.0 mm I.D.) column (5.0 μm of particle diameter), using as mobile phase (water–acetic acid, 99:1, v/ v)–acetonitrile (30:70, v/v). Flow rate was 0.7 ml min^{-1}, temperature was 25°C, and the separation was achieved at 6.68 MPa. Detection was performed by absorbance at 270 nm.

3. Results and discussion

3.1. Effects of the different hydrolytic treatments

Thallus samples floated for 2 h on 20 m*M* MES, pH 6.8, at 26°C in the dark were used to extract PH-PAs by acidic hydrolysis. As shown in Table 1, no PAs were extracted with acetic acid at 110°C or with acetic or formic acid at room temperature. Only 12 *M* HCl seemed to achieve a more efficient extraction of PUT, mainly at room temperature. However, evernic acid was always extracted although the best extraction was achieved by using 90% (v/v) formic acid at room teperature for 8 h.

The main part of papers published about plant PAs extraction and analysis include tissue homogeneization in cold PCA or trichloroacetic acid (TCA) [20,21]. This first step has also been used herein, but the results indicate that conjugates breakdown absolutely required acidic hydrolysis, preferently with HCl. No liberation of conjugated PAs was achieved without hydrolysis (data not shown) [6] or by using

Table 1
Polyamines and evernic acid isolated, after different hydrolytic treatments, from PH fractions

Hydrolysis	Concentration (μg g^{-1} dry thallus)			
	Evernic acid	PUT	SPD	SPM
12 *M* HCl for 18 h at room temperature	33.63±1.16 (70%)	0.40±0.036 (68.9%)	2.60±0.31 (100%)	0.35±0.027 (100%)
12 *M* HCl for 18 h at 110°C	25.13±0.25 (52.3%)	0.58±0.05 (100%)	1.10±0.09 (42.3%)	0.20±0.017 (57.1%)
1 *M* Acetic acid for 18 h at room temperature	18.39±0.37 (38.2%)	0.55±0.04 (94.8%)	2.10±0.23 (80.7%)	n.d.
90% (v/v) Formic acid for 2 h at room temperature	36.71±1.1 (76.4%)	n.d.	n.d.	n.d.
90% (v/v) Formic acid for 8 h at room temperature	48.06±1.45 (100%)	n.d.	n.d.	n.d.

Values are the mean of three replicates±standard error. Recovery, as per cent of the maximum amount obtained for each compound, is shown in brackets. n.d.=non detected.

organic acids, such as acetic or formic acids. In addition, according to these results, hydrolysis was highly efficient when it was carried out at room temperature, rather than at 100–120°C, as other authors recommended [2,7].

3.2. Derivatization with dansyl chloride is dependent on the initial pH value

Initial pH of the dansylation process was revealed as a very important parameter. Always the amount of dansylated PAs was higher at pH 12 than at low pH values (Table 2). Even at pH 0.5, no PUT was detected in S-fraction whereas at pH 12, the amount of free PUT was higher than 0.5 μg g^{-1} dry thallus. SH-fraction only contained SPD, as previously demonstrated, although the yielding of dansylation was highest at pH 7.0–12.0, whereas all PAs were efficiently dansylated at pH 12 in PH fraction.

These results were in agreement with those previously described by Cichy et al. [22]. They hypothesized that PAs are excessively protonated at acidic pH values and, in this form, dansylation is, in part, prevented. This situation is reversed at alkaline pH values, since the pK_a of PAs is higher than 8.0. Another possibility consists on the inhibition of dansylation by an excess of Cl^- anions [23].

3.3. Extraction of evernic acid, the only phenolic conjugated to pH-PAs is dependent on temperature and pH values

Evernic acid was the only bicyclic phenolic compound found in conjugated PAs fractions, extracted from thallus samples incubated for different time values on 20 m*M* MES buffer pH 6.8. *E. prunastri* also contains other bicyclic phenolics, such as atranorin, chloroatranorin and usnic acid [24], but these were never found in PH-fraction of PAs. Hydrolysis of PH-PAs was performed at room temperature with 12 *M* HCl for 18 h. Evernic acid was mainly recovered from the corresponding polyamine

Table 2
Dependence on pH value of dansylation procedure to derivatize S-, SH- and PH-PAs isolated from thallus samples floated on 20 m*M* MES buffer, pH 6.8, for 2 h

pH	Concentration (μg g^{-1} dry thallus)								
	S-PUT	S-SPD	S-SPM	SH-PUT	SH-SPD	SH-SPM	PH-PUT	PH-SPD	PH-SPM
0.5	n.d.	1.5±0.12	0.48±0.04	0.22±0.01	n.d.	n.d.	0.1±0.01	1.3±0.09	0.2±0.03
2.0	0.2±0.01	1.7±0.14	0.62±0.05	0.24±0.03	n.d.	n.d.	0.2±0.01	1.2±0.09	1.2±0.11
7.0	0.3±0.02	2.1±0.18	0.7±0.06	0.48±0.04	n.d.	n.d.	0.3±0.02	2.3±0.21	0.3±0.03
12.0	0.7±0.05	4.6±0.37	0.8±0.05	0.45±0.05	n.d.	n.d.	0.35±0.04	2.7±0.18	0.4±0.03

Values are the mean of three replicates±standard error. n.d.=non detected.

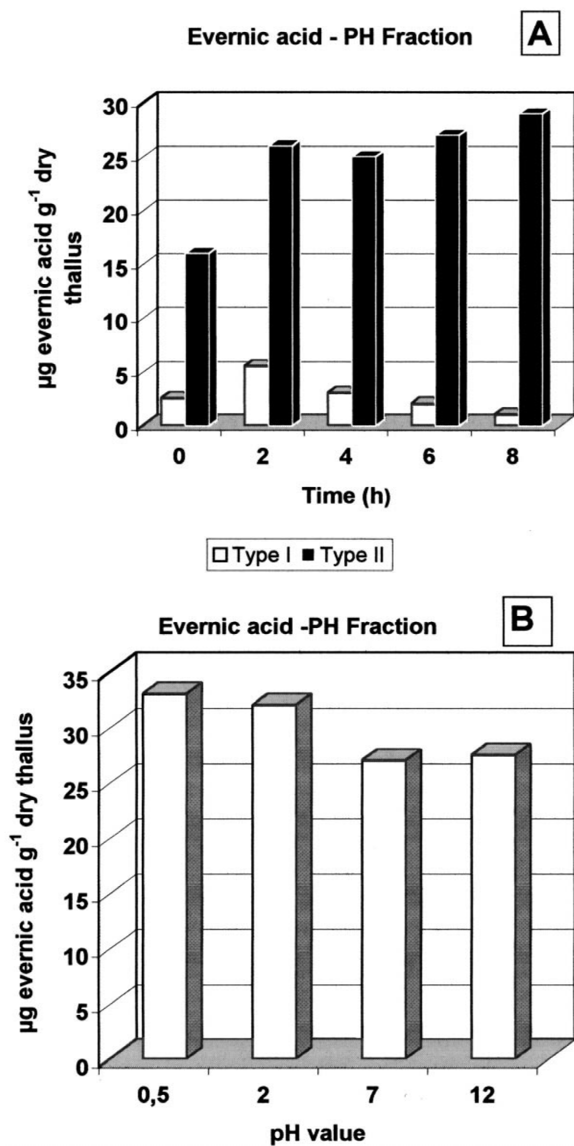

Fig. 1. (A) Time course of evernic acid extracted from PH-PA fraction from thallus samples incubated on 20 m*M* MES buffer, pH 6.8, from 2 to 8 h. Hydrolysis of conjugates PAs was performed in the presence of 12 *M* HCl for 18 h at room temperature. Extraction of evernic acid was achieved at room temperature for 5 min (type I) or at 40°C for 30 min (type II). (B) Dependence on pH value of evernic acid extraction with organic solvents from PH-PA fraction from thallus samples floated on 20 m*M* MES buffer, pH 6.8, for 2 h.

fraction when partition in organic phases was performed by shaking samples for 30 min at 40°C (extraction type II in Fig. 1A) instead of for 5 min at room temperature (extraction type I). When extraction was achived for 5 min at room temperature, only 15% of bound evernic acid was recovered into the organic phase. The thermal dependence of extraction procedures to isolate phenolic compounds from conjugated-PA fractions has also been described for feruloyl conjugates in higher plants [25].

Extraction of evernic acid was also dependent on pH value. At low pH values, 0.5 and 2.0, recovery of phenolic acid was higher than that occurred at high pH values (Fig. 1B). High pH values increased the amount of both everninic and orsellinic acids recovered from the samples. This implied that the ester bond between these two monocyclic units in evernic acid was hydrolyzed and, in this way, the amount of evernic acid recovered at these pH values was always lower than that recovered at acidic pH values during extraction.

Fig. 2 shows HPLC profiles of phenolic acids isolated from SH and PH polyamine fractions where peak 3 has been revealed as evernic acid. This peaks

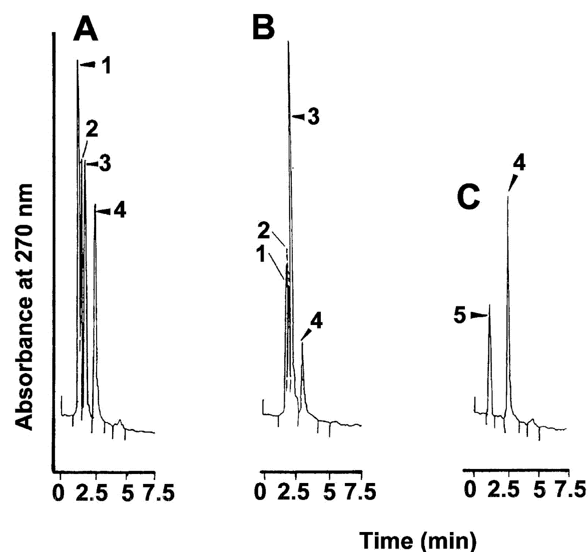

Fig. 2. HPLC profiles of evernic acid and their precursors extracted from conjugated polyamine fractions from thalli incubated on 20 m*M* MES buffer, pH 6.8, for 2 h from: (A) PH-PAs fraction; (B) PH-PA fraction loaded with 20 μg evernic acid; and (C) S-fraction. 1=Orsellinic acid; 2=everninic acid; 3=evernic acid; 4 and 5=unidentified.

is specifically increased after loading the sample with 20 μg pure evernic acid. The method proposed herein is a modification of that described by Pedrosa and Legaz [26] according to which, it is possible to separate monocyclic precursors and, in a general way, the retention time values of lichen phenolics have been significantly decreased.

4. Conclusions

Hydrolysis of conjugated polyamines is mainly produced with 12 *M* HCl for 18 h at room temperature. The pH value of polyamine dansylation must be adjusted to 12.0. Recovery of evernic acid conjugated to polyamines was temperature and pH dependent. Maximun extraction was produced at pH 0.5 by shaking samples for 30 min at 40°C with a mixture of organic solvents.

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