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Separation of polyamines, conjugated to DNA, by reversed-phase high-performance liquid chromatography

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Abstract

Genomic DNA was isolated from the lichen *Evernia prunastri* in order to analyze by high-performance liquid chromatography the occurrence of polyamines conjugated to the macromolecule. The acid-insoluble (PH) fraction of this DNA contained mainly conjugated spermidine, although small amounts of free putrescine and spermidine were also present. The PH fraction of DNA also contained conjugated evernic acid, the main phenol produced by this lichen species. Conjugation of polyamines to calf thymus DNA was carried out under in vitro conditions. Conjugation was to spermidine and mainly to spermine and produced DNA compactation. Evernic acid enhanced the action of polyamines in order to produce DNA aggregation.

Keywords: *Evernia prunastri*; Polyamines; DNA; Evernic acid

1. Introduction

Polyamines are considered powerful effectors of cell growth in animals as well as in plants. Polyamines and some polyamine analogs exert cytostatic effects on several cell lines at micromolar concentrations [1]. Polyamines, viz. spermine and spermidine, stabilize the secondary structure of DNA and UV absorption studies indicate B-to-Z DNA transition in the presence of polyamines [2]. In addition, spermine is an efficient radioprotector of plasmid or viral DNA and of viral minichromosomes by a mechanism involving radical scavenging and the induction of compaction and aggregation of DNA [3].

Polyamines conjugate with nucleic acids by electrostatic linkages through which they induce compactation of the macromolecule [4]. According to Manning [5], strong electrostatic interactions of DNA with polycations, such as polyamines, produce a radial distribution of the counterion around the DNA molecule. Feuerstein et al. [6] hypothesize that the most favoured interaction between spermine (SPM) and DNA is related to the alternative sequences of purine/pyrimidine in the main groove of DNA. The linkage to the secondary groove seems to be less improbable. In relation to this, Snyder [7] finds that polyamines are able to displace the unspecific linkage of Hoechst 33258 to phosphate groups of the DNA molecule, but they are unable to displace the intercalating agent from the secondary groove. Delcross et al. [8] demonstrated that a portion of intercalated ethidium bromide can be removed by SPM from poly(dA-dT)·poly(dA-dT) or poly(dG-dC)·poly(dG-dC).

Conjugation of photoactive polyamine derivatives, such as ANB-SPM (azidedinitrobenzoyl spermine) and ABP-SPD (azidebenzamidin spermidine),

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changes the turn of the nucleosomal DNA helix as well as natural SPD and SPM [9]. This action is able to change the DNA-B conformation to the DNA-Z conformation [10].

Separation and quantitation of polyamines is currently achieved by high-performance liquid chromatography (HPLC) since Flores and Galston [11] developed an analytical procedure for the separation of these compounds in higher plant extracts. Marcé et al. [12] describe the separation of 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine as dansyl derivatives on an ODSA Spheri-5 column and acetonitrile–water as mobile phase in the appropriate gradient. Alternatively, Kotzabasis et al. [13] use benzoylchloride derivatives since benzoylation, in contrast to dansylation, produces only a small number of side products. Separation of benzoyl polyamines from *Vitis champani* leaves was achieved using a C_{18} narrow-bore Hypersil column and 63% aqueous methanol as mobile phase. Van Eijk et al. [14] used a new fully endcapped reversed-phase packing material, Inertsil, to separate *o*-phthalaldehyde derivatives of putrescine, cadaverine, spermidine, and spermine, whereas Legaz et al. [15] preferred the production of tosylated derivatives to separate polyamines by capillary electrophoresis

The classical separation of conjugated polyamines into SH and PH groups is based on their solubility in acid. Acid-soluble conjugated polyamines (SH) appear associated with small molecules, such as sugars, amino acids, and mainly phenols. In this last case, conjugates are produced by the action of several phenoyl-coenzyme A transferases [16], the products of which have been related to physiological events, such as cell proliferation [17], biosynthesis of pyrrolidine alkaloids [18] and the acquisition of resistance against pathogens [19]. Acid-insoluble conjugated polyamines (PH) are usually associated with macromolecules, such as nucleic acids, in which conjugation is related to DNA synthesis and mitosis [20,21], polysaccharides [22], and proteins [23,24].

However, this classification of conjugated polyamines based upon extraction procedure is not completely valid. For example, some PH-SPD have been found in sucrose-containing fractions isolated from sugarcane juices, whereas SH-SPM appear as a component of the fraction containing large-molecular-mass polysaccharides [25].

In this report, evidence of the conjugation of polyamines to DNA of a lichen species is presented and the true composition of the PH fraction analyzed.

2. Material and methods

2.1. Chemicals

All chemicals used for the preparation of the buffers, morpholinoethane sulfonic acid (MES), EDTA, tris(hydroxymethyl)aminomethane (Tris), β-mercaptoethanol, sodium dodecyl sulfate (SDS), hydrochloric acid and sodium hydroxide, were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. Water was of Milli-Q grade (Millipore, Bedford, MA, USA). Dansyl chloride, proteinase k, RNase, BUT, putrescine (PUT), SPD and SPM were obtained from Sigma (St. Louis, MO, USA). Perchloric acid, potassium acetate, sodium acetate, ethanol, isopropanol, methanol, acetonitrile, diethyl ether, ethyl acetate, chloroform, and toluene were also from Merck.

2.2. Biological material

Evernia prunastri (L) Ach, growing on *Quercus pyrenaica* Willd and collected in Valsaín (Segovia, Spain), was used throughout this work. Samples of 1.0 g of dry thallus were floated on 15 ml 20 m*M* MES buffer, pH 6.8 or 9.15 for 8 h at 26°C in the dark. Samples were then gently dried with filter paper and macerated with 20 ml acetone to extract free phenolics. The residue was dried in air and used to purify DNA.

2.3. Purification of DNA

Extraction of genomic DNA was achieved according to Dellaporta et al. [26], but the phenolization reaction was substituted by proteinase k treatment [27]. Thalline powder was extracted with 15 ml cold buffer (0.1 *M* Tris–HCl, 50 m*M* EDTA and 0.5 *M* NaCl, pH 8.0) containing 10 μl β-mercaptoethanol. After shaking, 10 ml 20% (w/v) SDS was added and the mixture was incubated for 10 min at 60°C. Then, 5 ml 5.0 *M* potassium acetate, pH 5.2, was added

and newly incubated for 20 min at 0°C. The mixture was centrifuged at 20 000 *g* for 15 min at 4°C. The pellet was resuspended in 700 μl of proteinase k solution containing 50 μg protein per ml of 10 m*M* Tris–HCl, 5 m*M* EDTA and 0.5% (w/v) SDS, pH 8.0, and incubated for 5 h at 55°C. After this, protein was precipitated by adding 150 μl 3.0 *M* potassium acetate, pH 5.2, and removed by incubation of the samples for 0 min at −20°C and centrifugation at 12 000 *g* for 20 min at 4°C. The pellet was discarded and DNA precipitated from the supernatant by adding a 0.6 volume of isopropanol. Precipitate containing DNA was resuspended in 700 μl of the extraction buffer and treated with 25 μg ml^{-1} RNase for 2 h at 37°C. DNA was recovered through a sequential precipitation with 0.1 volume of 2.0 *M* potassium acetate, 0.6 volume of isopropanol, and 0.6 volume of 70% (v/v) aqueous ethanol. The final precipitate was dissolved in 400 μl of distilled, sterile water.

2.4. DNA electrophoresis

A sample of this DNA solution (5.0 μl containing about 2.0 μg DNA) was analyzed by electrophoresis on 0.8% (w/v) agarose gel (type II, Sigma) in TAE buffer (40 m*M* Tris–HCl, 20 m*M* sodium acetate and 2.0 m*M* EDTA, pH 8.1) for 1 h at 625 V m^{-1} at 20°C. Genomic DNA bands were stained with ethidium bromide and examined under UV light [28]. DNA from λ phage, digested with *Hind*III (Boehringer), was used as molecular mass marker.

2.5. Extraction and analysis of polyamines

Polyamines were analyzed as their dansyl derivatives by HPLC modifying the method described by Escribano and Legaz [29]. Samples of 1.0 ml of DNA preparation (about 125 μg DNA) were mixed with 6.0 ml of 5% (w/v) cold perchloric acid (PCA) containing 150 μl of 5 m*M* *n*-butylamine as internal standard. The mixtures were stored overnight at 4°C in plastic tubes and then centrifuged at 48 000 *g* for 20 min at 2°C. The supernatant (first supernatant) contained free (S) and non-liberated acid-soluble (SH) polyamines, whereas the precipitate (first precipitate) contained non-liberated acid-insoluble (PH) polyamines. This first precipitate was washed four times with 5% (w/v) cold PCA, resuspended in 6.0 ml 1.0 *M* NaOH containing 150 μl 5 m*M* *n*-butylamine and, finally, stored for 12 h at 4°C. Aliquots of 2.0 ml of both first supernatant and first resuspended precipitate were hydrolyzed with 2.0 ml 12 *M* HCl for 18 h at room temperature. After this, hydrolyzates were centrifuged at 30 000 *g* for 15 min at 2°C. Pellets were discarded. Centrifugation produced two different supernatants, one called second supernatant, derived from the first, and another called third supernatant, derived from the first, resuspended precipitate. The second supernatant contained both free (S) and liberated, acid-soluble (SH) polyamines, whereas the third supernatant contained acid-insoluble polyamines. Both second and third supernatants were dried at 40°C under a stream of air and then resuspended in 1.5 ml of 5% (w/v) cold PCA to be centrifuged later at 6000 *g* for 15 min at 2°C. Precipitates were discarded and supernatants (fourth and fifth supernatants, respectively) were used for derivatization.

Aliquots of 0.2 ml of the first, fourth and fifth supernatants were dansylated 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. Then, 0.1 ml of 2.0 m*M* proline was added to destroy the excess dansyl chloride. Dansylated polyamines were extracted from the mixtures with 3×3.0 ml toluene (HPLC grade). The toluene phase was dried at 40°C under a stream of air. Upon concluding the derivatization procedure, samples were cleaned by adding 0.6 ml of 5.0 *M* KOH in methanol (HPLC grade), according to Seiler and Knödgen [30]. Mixtures were left to stand for 45 min at 40°C and then 1.5 ml of an aqueous mixture containing 200 mg KH_2PO_4 and 200 mg Na_2HPO_4 was added. Polyamines were extracted again with 3×3.0 ml toluene, as described above. The organic phase was dried and the dry residues redissolved in 200 μl methanol (HPLC grade) to be chromatographed.

Polyamines were eluted from a MicroPak MCH-5N cap Varian (Walnut Creek, CA, USA) (15 cm×4 mm) reversed-phase column at 40°C using a methanol–water gradient [29]. Detection was performed by fluorescence intensity measurements.

2.6. Phenolics analysis

The PH fraction containing polyamines was hydrolyzed for 18 h with 12 *M* HCl at room temperature. Phenolics from 1.0 ml of hydrolysate were extracted by adding 4×5.0 ml diethyl ether–ethyl acetate (65:35, v/v), shaking the mixture for 30 min at 40°C. The organic phase was recovered and the aqueous phase was mixed with 4×5.0 ml chloroform–acetonitrile (60:40, v/v), newly stirred for 30 min at 40°C, and the organic phase collected . Both organic solutions were then mixed, dried in a flow of air and stored at −13°C until required.

Phenolics were analysed by HPLC using a Varian 5060 liquid chromatograph as follows: column, reversed-phase Nucleosil C_8, 125 mm×4.0 mm I.D.; mobile phase, water–acetic acid–acetonitrile (29.7:0.3:70, v/v/v); flow, 0.7 ml min^{-1}; temperature, 25°C; pressure, 66 atm; detector, UV set at 270 nm; AUFS, 0.002; injection, 10 μl.

Quantitation of phenolics was achieved by interpolating area count values in a straight line constructed with variable concentrations of standards, purchased from Sigma.

3. Results

3.1. Analysis of polyamines in the PH fraction from lichen DNA

Fig. 1 shows the chromatographic trace obtained for polyamine standards as well as for PH polyamines isolated from *E. prunastri* thallus floated on 20 m*M* MES buffer, pH 6.8, for 2 h at 26°C in the dark, where SPD was the most abundant polyamine. All of them were eluted in about 20 min and the reproducibility of this separation was almost perfect. In addition, evernic acid was the only phenol recovered from the PH fraction obtained from the DNA preparation (Fig. 2A) and the corresponding peak was specifically increased when the mixture was loaded with standard evernic acid (Fig. 2B).

DNA isolated from *E. prunastri* thalli has a

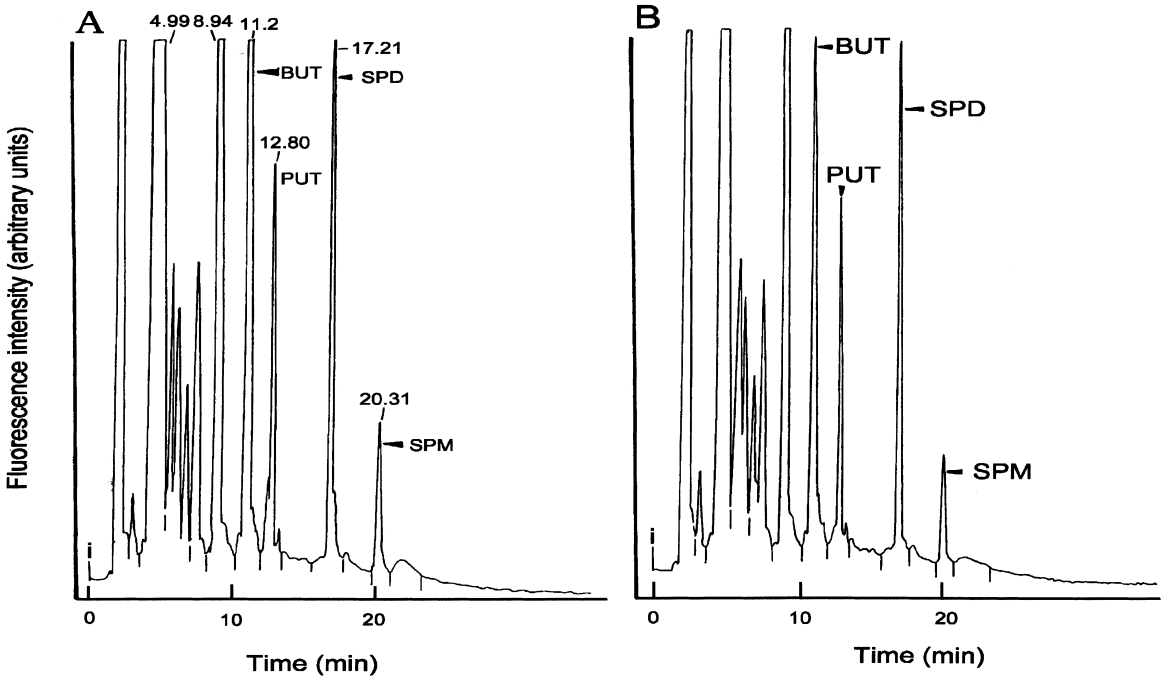

Fig. 1. Chromatographic traces of polyamines in HPLC. (A) Separation of standards and (B) separation of PH polyamines extracted from *Evernia prunastri* thalli floated on 20 m*M* MES buffer, pH 6.8, for 2 h at 26°C in the dark. Number near the peak represents the retention time (min).

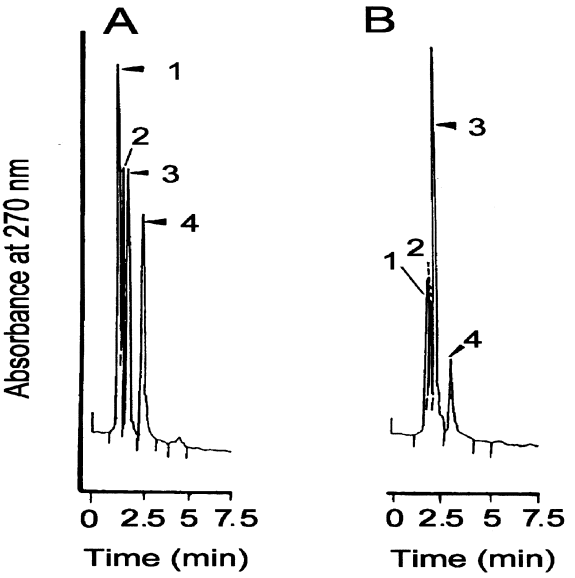

Fig. 2. Chromatographic traces of lichen phenolics in HPLC. (A) Phenols extracted from the PH fraction of polyamines obtained from *E. prunastri* thalli floated on 20 m*M* MES buffer, pH 6.8, for 2 h at 26°C in the dark. (B) The same separation after loading the solution with 20 μg standard evernic acid. 1=Orsellinic acid (retention time, 1.50 min); 2=everninic acid (retention time, 1.58 min); 3=evernic acid (retention time, 2.27 min); 4=unidentified compound (retention time, 3.22 min).

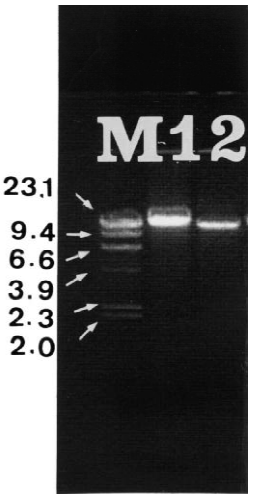

Fig. 3. Electrophoresis of genomic DNA isolated from *E. prunastri* thalli floated for 2 h on 20 m*M* MES buffer, pH 6.8 (lane 1) or pH 9.15 (lane 2). Molecular mass markers, consisting of DNA of λ phage digested with *Hind*III, are shown in lane M. Numbers indicate the molecular size of the markers (kb).

molecular size of about 9.0 kilo base pairs (kb), as shown in Fig. 3 (lanes 1 and 2). This DNA preparation obtained from thalli floated on MES buffer, pH 6.8, contained both PUT and SPD, but not SPM. Small amounts of S-PUT were recovered during the first 3 h of thalli incubation, whereas PH-PUT strongly increased from 0 to 2 h and decreased later. In contrast, only S forms of both polyamines were recovered from DNA obtained from thalli floated on MES buffer at pH 9.15 (data not shown). It can be concluded that alkaline pH values, which promote the action of arginase in order to synthesize putrescine, impeded conjugation of this polyamine, and other derivatives such as spermidine, to the DNA macromolecule.

3.2. In vitro conjugation of polyamines to calf thymus DNA

The possibility of DNA complexing the three main polyamines (PUT, SPD and SPM) has been verified under in vitro conditions using calf thymus DNA. Surprisingly, SPM, a polyamine which did not appear as a DNA conjugate form in *E. prunastri*, although it appeared as moderately abundant as S and SH forms, preferentially conjugated with DNA in vitro (Fig. 4), whereas SPD complexed DNA only from the fifth hour of DNA incubation. When conjugation of SPD to DNA was carried out in the presence of evernic acid, appreciably enhanced compactation was found (data not shown).

4. Discussion

The method used here to analyze and quantify polyamines is shown to be highly sensitive since it is possible to detect about 0.1 pmol of mass injected onto the column for standard polyamines. This method is preferred to other methods usually employed, such as precolumn derivatization with benzoyl chloride, since its feasibility is very low when applied to plant crude extracts [11]. The detection by UV absorbance of tosyl derivatives [31] is approximately 50 times lower than that of fluorescence detection, although it can be improved by capillary electrophoresis [15]. By using the method described

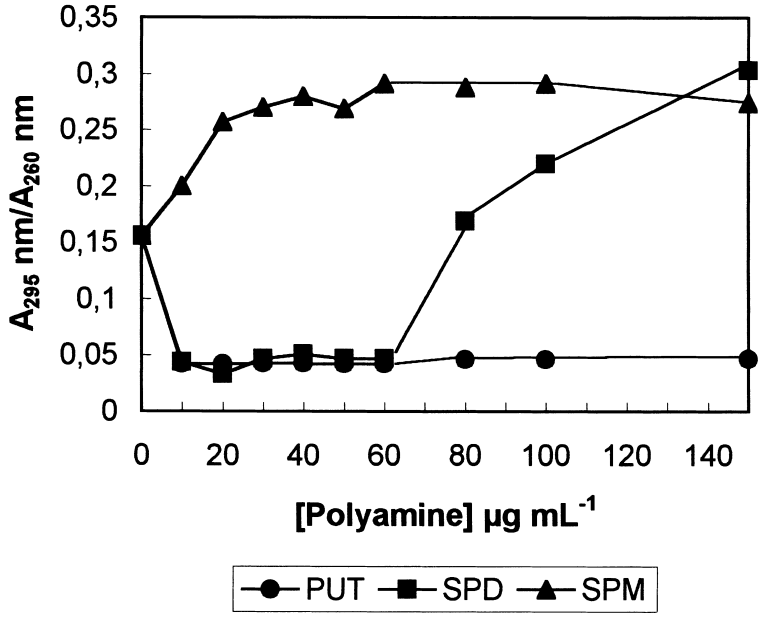

Fig. 4. DNA compactation (estimated as A_{295}/A_{260}) in a sample of calf thymus DNA incubated with polyamine concentrations varying from 0 to 150 μg ml^{-1} in a final volume of 3.0 ml^{-1}. Values are the mean of three replicates. Vertical bars give the standard error when larger than the symbols.

in this paper, polyamines were separated in about 20 min and the analysis reveals that SPD is the main polyamine in *Evernia prunastri* thallus as S and PH fractions (Fig. 1)

The amount of polyamines co-precipitated with DNA as the soluble (S) fraction or conjugated to the DNA molecule (PH) is shown to be dependent on the pH chosen to incubate the thallus samples. Hence, more S-PUT and S-SPD were obtained from DNA samples isolated from thalli floated on MES buffer, pH 9.15, than that obtained from thallus floated on MES buffer, pH 6.8. Thus, the synthesis of free putrescine seems to be an 'alkaline process' depending on the enzymes arginase (optimum pH 9.15) and ornithine decarboxylase (optimum pH 8.5) [32,33]. At neutral pH, the production of putrescine is achieved through a minor pathway which includes arginine decarboxylase [34] and agmatine amidino hydrolase [35] activity. The occurrence of two different functional pathways to synthesize putrescine has been resolved in lichen symbiosis by a compartmentalization system: arginase and ornithine decarboxylase are fungal enzymes, whereas arginine decarboxylase and agmatine amidino hydrolase are restricted to the algal partner [36]. However, conjugation of polyamines to DNA is really favoured by neutral pH during thalli incubation, since neither PH-PUT nor PH-SPD were found in the DNA fraction isolated from thallus samples floated at pH 9.15.

Nevertheless, condensation of DNA in vitro was promoted by SPD and mainly by SPM, which did not appear as conjugated polyamines to lichen DNA. PUT does not promote DNA condensation (Fig. 4). This can probably be related to a direct relationship between the charge of the polyamine molecule and the transition B-DNA to Z-DNA, since pentamines isolated from *Thermus thermophilus* aggregate DNA at concentrations lower than that of SPM required to obtain the same degree of condensation [37]. However, there is neither S-SPM nor PH-SPM in DNA isolated from *Evernia prunastri* thalli. This can

probably be explained as a result of the very low concentration of this polyamine instead of a lack of affinity for the ligand (Fig. 4).

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