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Changes in free and conjugated polyamines during starvation of sugarcane juices as analyzed by high-performance liquid chromatography

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Abstract

Changes of the polyamines (PAs) titer in high- and mid-molecular-mass carbohydrates (HMMCs and MMMCs, respectively) obtained from sugarcane juices stored for 72 h at pH 5.2 or clarified at pH 8.0 have been studied. Cadaverine (CAD) is the most abundant free (S) PA in the MMMC fraction from juices at pH 5.2, whereas putrescine (PUT) was revealed as the main PA at pH 8.0. A slight increase in the free PUT titer can be noted at pH 5.2 for 72 h of juice starvation. PAs from MMMC were mainly conjugated to acid-insoluble (PH) molecules. Accumulation of PH-PAs with the time of starvation was especially significant for PUT and CAD. However, CAD has also been detected in the acid-soluble (SH) fraction and its concentration increases with the time of starvation at pH 5.2. The accumulation pattern of free and conjugated PAs from HMMCs is similar to that found for MMMCs although some differences can be observed. For instance, the increase in free PUT with the time at pH 8.0 was 2.7-times higher in the HMMC fraction than in the MMMC fraction. Conjugated PAs associated to acid-soluble macromolecules (SH fraction) achieved a level in HMMC fractions higher than that observed in the MMMC fraction. Moreover, the reported increase with time that was observed in PH-CAD from the MMMC fraction was not observed in the HMMC fraction, and, finally, the increase in PH-PUT with the time was lower for the HMMC fraction than for the MMMC fraction.

Keywords: Sugarcane juices; Food analysis; Polyamines; Carbohydrates

1. Introduction

Polyamines (PAs) are biologically ubiquitous aliphatic nitrogen-containing compounds of low molecular mass and polycationic nature. The diamine putrescine (PUT) and the triamine spermidine (SPD) are probably synthesized by all organisms, while eukaryotes contain the tetramine spermine (SPM) as well [1]. Cadaverine (CAD) is less widely distributed in the plant kingdom than PUT [2]. In the plant kingdom, PUT is formed by two alternative pathways, one direct, via ornithine decarboxylase (ODC) from ornithine, and the other, indirect, through a series of intermediates, following arginine decarboxylation. Arginine decarboxylation, producing agmatine, is catalyzed by arginine decarboxylase (ADC); agmatine is then hydrolyzed to *N*-carbamoyl putrescine (NCP) by agmatine iminohydrolase. Beyond this, there is evidence for two apparently unrelated pathways for the synthesis of PUT from NCP. One major pathway in which the carbamoyl moiety of NCP is metabolized to CO_2 and NH_3 by NCP amidohydrolase, directly yielding PUT. An alternative mechanism of PUT synthesis, via a

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multifunctional enzyme, putrescine synthase, has been proposed in which agmatine is deiminated to NCP which is then converted to PUT and citrulline. SPD and SPM are generally synthesized from PUT and methionine. An aminopropyl group must be added to convert PUT into SPD. This aminopropyl moiety is derived from methionine, which is first converted into *S*-adenosylmethionine (SAM) and then decarboxylated, in reactions catalyzed by SAM synthase and SAM decarboxylase, respectively. The resulting decarboxylated *S*-adenosylmethionine (dSAM) is used as an aminopropyl donor in a manner analogous to the use of SAM itself as a methyl donor. The transfer of the aminopropyl group from dSAM to PUT, forming SPD, is catalyzed by spermidine synthase. Another aminopropyl group, which also comes from dSAM, is needed to convert SPD into SPM, in a reaction catalyzed by a second aminopropyl transferase (spermine synthase). Little is known about CAD biosynthesis as compared to the major path. CAD arises directly from lysine via lysine decarboxylase [3] (Fig. 1).

Most references to PAs in plants are less than two decades old [4]. It is therefore not surprising that their role in the physiology of the plant if any, is still uncertain. They occur in the free form or bound to low-molecular-mass compounds or macromolecules [5]. Their titer is very responsive to external conditions, such as light, temperature, and physical or chemical stress agents [6]. PAs, especially SPD, are generally abundant in young non-senescent organs, and decline to a lower titer as organs age and senescence [7], as well as the decline in ADC activity. However, this fact not always occurs since PUT accumulation has been described in stressed oat leaves [8].

There are only two reports about PAs titer in sugarcane plants. One of them described the existence of free and conjugated to phenolic acids PA in juice [9]. The phenolic acids hydroxycinnamic and hydroxybenzoic acids are commonly found in vegetable and fruit juices and increase during biological ageing or impairment [10]. The other report described the increase in PA concentration after infection with *Ustilago scitaminea* [11,12]. On the other hand, sugarcane accumulates sucrose in its stalks, which reaches a maximum in a particular phase of the vegetative growth, the maturation [13], whereas fructose and glucose significantly decreases [14]. During this phase, stalks are cut and then crushed to produce the juice which is immediately alkalinized to pH 8 in order to form a flocculate, which traps impurities. Then, this clarified juice is subjected to different steps for crystallizing sucrose [15]. It had been previously demonstrated that in those juices that contained high concentrations of both high-molecular-mass carbohydrates (HMMCs) and mid-molecular-mass carbohydrates (MMMCs), sucrose crystallization process was affected, producing abnormal crystals of low quality [16]. In general, the titer of HMMCs and MMMCs increases during post-collection deterioration, thereby affecting the crystallization process [17]. Recently, it has been demonstrated that these polysaccharides are really a complex mixture of true polysaccharides and glycoproteins [18,19] whose titer increases under any stress situation.

In the present work, we have reproduced, under laboratory conditions, the clarification process in order to compare the pattern of HMMC and MMMC accumulation in two classes of juices, one natural juice (J juice) and the other clarified juice (Jc juice) after 72 h of storage under continuous light. Many of the countries which produce sugar from sugarcane, such as Cuba, suffer from lack of fuel and this fact implies that cut canes remain on the ground, or juices are not immediately processed. Such a situation, which could be defined as a general stress, could, in parallel, produce an alteration in polyamine metabolism. So, the aim of this work was to establish whether in fact a general stress situation as has been imposed on cane juice under continuous light for 72 h, produces a concomitant increase in PAs and carbohydrates titers, and also, whether a binding between those two molecules could be possible. Since freshly obtained juices are acidic (pH 5.0–5.5) whereas the conditioning, industrial process is achieved at alkaline pH (8.0), starvation and deterioration of juices have been reproduced in laboratory conditions at both pH values in order to attempt to minimize chemical variations occurring in these juices.

2. Experimental

2.1. Plant material

Stalks from 14-month-old field grown plants of

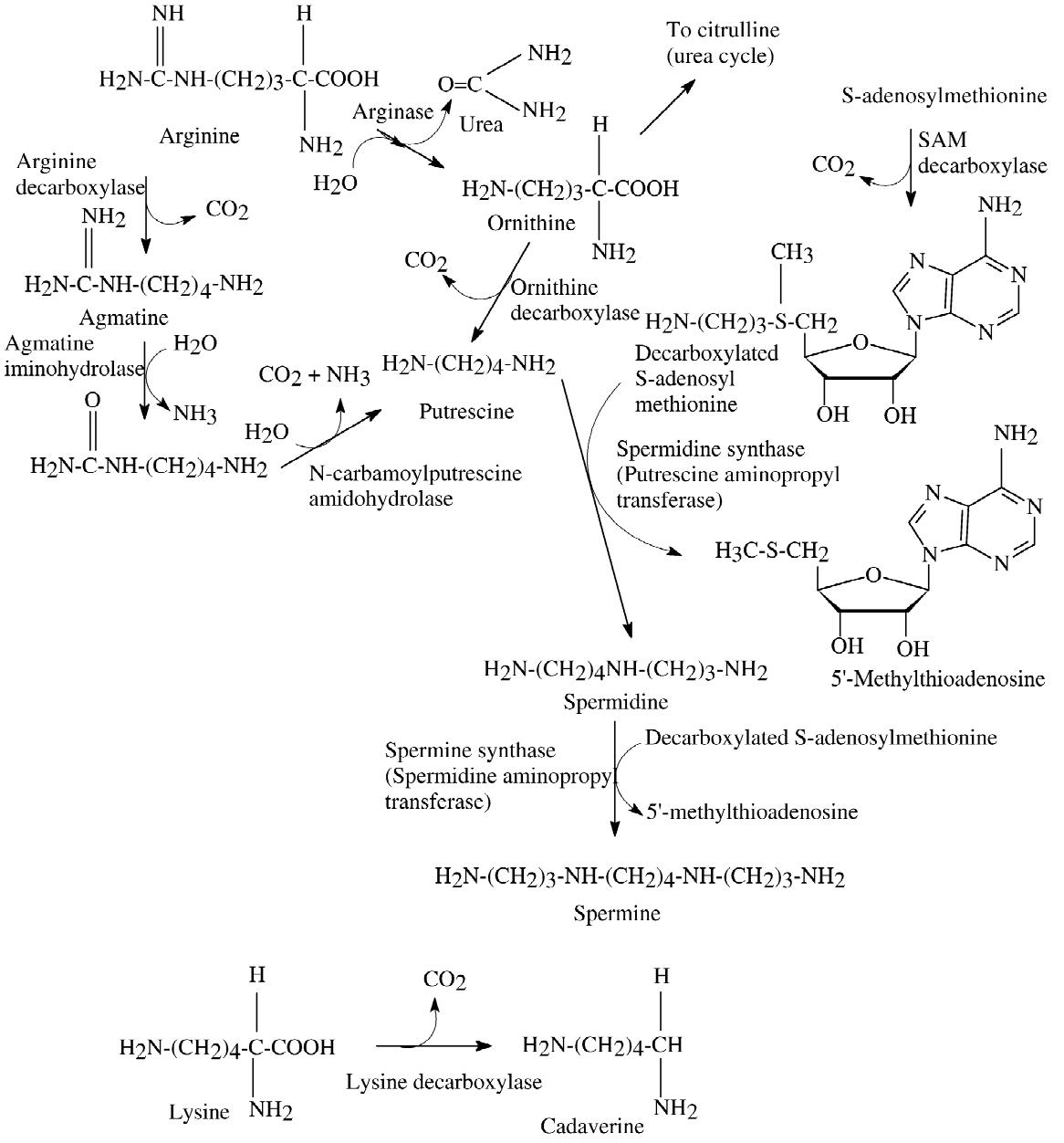

Fig. 1. A scheme of polyamine biosynthesis in plants.

Saccharum officinarum L., var. Jaronu 60-5, were used throughout this work.

2.2. Chemicals

All chemicals used here, such as sodium carbonate, potassium phosphate, sodium phosphate, 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, butylamine (BUT), PUT, CAD, SPD and SPM were obtained from Sigma (St. Louis, MO, USA).

Perchloric acid (PCA), methanol, acetonitrile and toluene were also from Merck.

2.3. Preparation of sugarcane juice: isolation of carbohydrate fractions

Sugarcane stalks were mechanically crushed immediately after being cut and the crude juice was filtered through filter paper. To this filtered juice, sodium azide was added to obtain a final concentration of 0.02% (w/v) and the pH value of this juice was about 5.3. From this acidic juice, two aliquots were prepared; the first one was centrifuged at 2500 *g* for 30 min at 4°C. This aliquot was defined as centrifuged juice (J). The second aliquot was adjusted to pH 8.0 by adding a sufficient volume of a saturated solution of sodium carbonate and then centrifuged at 20 000 *g* for 30 min at 4°C. This aliquot was defined as clarified juice (Jc). Both centrifuged (J) and clarified (Jc) juices were maintained at 30°C under continuous light of 125 μmol m^{-2} s^{-1} for 24, 48 or 72 h. After these periods of time, all the juices were filtered through a column of Sephadex G-10 (15×2.5 cm I.D.) pre-equilibrated with distilled water at pH 5.3 (for those juices from J) or, alternatively, with distilled water at pH 8.0 (for those juices from Jc). Distilled water used for pre-equilibration of columns contained 0.02% (w/v) sodium azide to avoid any microbiological contamination. Elution was carried out with distilled water at pH 5.3 or 8.0 for filtration of J or Jc juices, respectively. Fractions (1.0 ml) 1 to 20 were discarded. Fractions 21 to 35 were collected and considered as a mixture of both HMMCs and MMMCs. Fractions 36 to 65, mainly composed of sucrose, considered as low-molecular-mass carbohydrates (LMMCs) were discarded. After this separation, fractions 21 to 35 (14 ml) collected from the Sephadex G-10 column were filtered through a Sephadex G-50 column (30×2.5 cm I.D.), pre-equilibrated as above. The first 40 fractions were discarded whereas fractions 41 to 80, referred to as HMMCs and fractions 81 to 120, referred to as MMMCs, were collected [20]. Eluted fractions were monitored for carbohydrates according to Dubois et al. [21].

2.4. Extraction and analysis of polyamines

PAs from HMMCs and MMMCs obtained from J or Jc juices were analyzed as their dansyl derivatives by high-performance liquid chromatography (HPLC) using the method described by Escribano and Legaz [22]. Briefly, samples of 2.5 ml of both HMMC and MMMC fractions eluted from the Sephadex G-50 column were mixed with 6.0 ml of 5% (w/v) PCA, which contained 150 μl of 5 m*M* *n*-butylamine as an 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, resuspendend 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 the first supernatant and the 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 the second supernatant, derived from the first one, and another one called the 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 later centrifuged 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 hermetically sealed vial for 18 h, in darkness, at room temperature. Then, 0.1 ml of 2.0 m*M* proline was added to destroy the excess of dansyl chloride. Dansylated polyamines were extracted from the mixtures with 3×3.0 ml toluene (HPLC-grade). The toluene phase was collected and 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 [23]. 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 dry residues redissolved in 200 μl methanol (HPLC-grade) and 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 [23]. Detection was performed by fluorescence intensity measurements. As an example, a chromatographic trace of polyamines obtained from the S fraction of MMMCs from a centrifuged juice is shown in Fig. 2.

The recovery was previously evaluated by using [1,4-^{14}C]putrescine, 111 mCi mmol^{-1}, from Amersham (Buckinghamshire, UK), as 85% after the complete procedure of dansylation, cleaning and extraction [24].

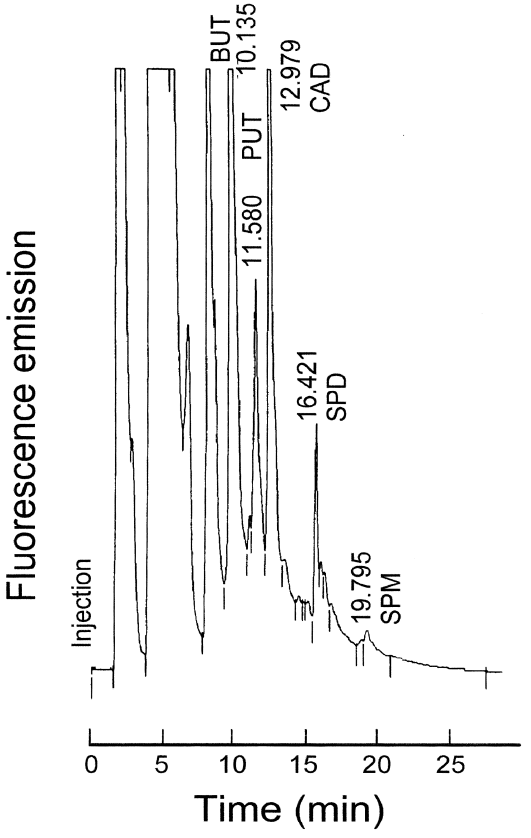

Fig. 2. Chromatographic trace in HPLC of dansylated polyamines from MMMCs obtained from a centrifuged juice. Numbers near the peaks are the retention time values in min.

3. Results and discussion

Polyamine metabolism is extremely sensitive to change in the external environment, especially ionic type stresses [25–27]. In our experiments, in which sugarcane juice has been subjected to an imposed deterioration, polyamine metabolism can be altered.

Fig. 3 shows the variation in free and conjugated PAs of the MMMC fraction of sugarcane juice with the time of incubation in light at two pH values. A general view permits one to observe a higher PA titer from juices at pH 5.3 (Fig. 3A, C and E) than from those at pH 8.0 (Fig. 3B, D and F), both in free and conjugated fractions. CAD is the most abundant S-PA in the MMMC fraction from juices at pH 5.3 (Fig. 3A), but CAD is greatly reduced at pH 8.0 (Fig. 3B). The diamine CAD has been widely found in the family *Leguminosae* and other higher plants [28–30]. In radicles or hypocotyls of 3-day-old soybean seedlings, only CAD was detected in high concentrations (2.37 and 5.09 m*M*, respectively). Clarification process that occurred at pH 8.0 could produce co-precipitation of CAD by the mere action of the pH change. Incubation did not apparently affect the pattern of accumulation of free PAs since similar concentrations were found at different times either at pH 5.3 as at pH 8.0. However, at pH 8.0, a slight increase of the free PUT titer can be noted (Fig. 3B). This PA increased from 1.45 μg ml^{-1} to 2.68 μg ml^{-1} after 72 h, although a similar increase did not occur for the accumulation patterns of SPD and SPM. Flores and Galston [31] demonstrated that the incorporation of label from [^{14}C]arginine into PUT in osmotically stressed cereal leaves was not accompanied by a comparable increase in the labels of SPD and SPM. They further suggested that the stress response involved not only a rise in PUT biosynthesis but also a block in PA synthesis. PA accumulation at pH 8.0 can be explained on the basis of a higher ornithine decarboxylase activity. Heimer and Mizrahi [32] reported an optimum pH value of 8.5 for barley ODC. Thus, at pH 5.3, the synthesis of PUT would occur via arginine decarboxylase where-

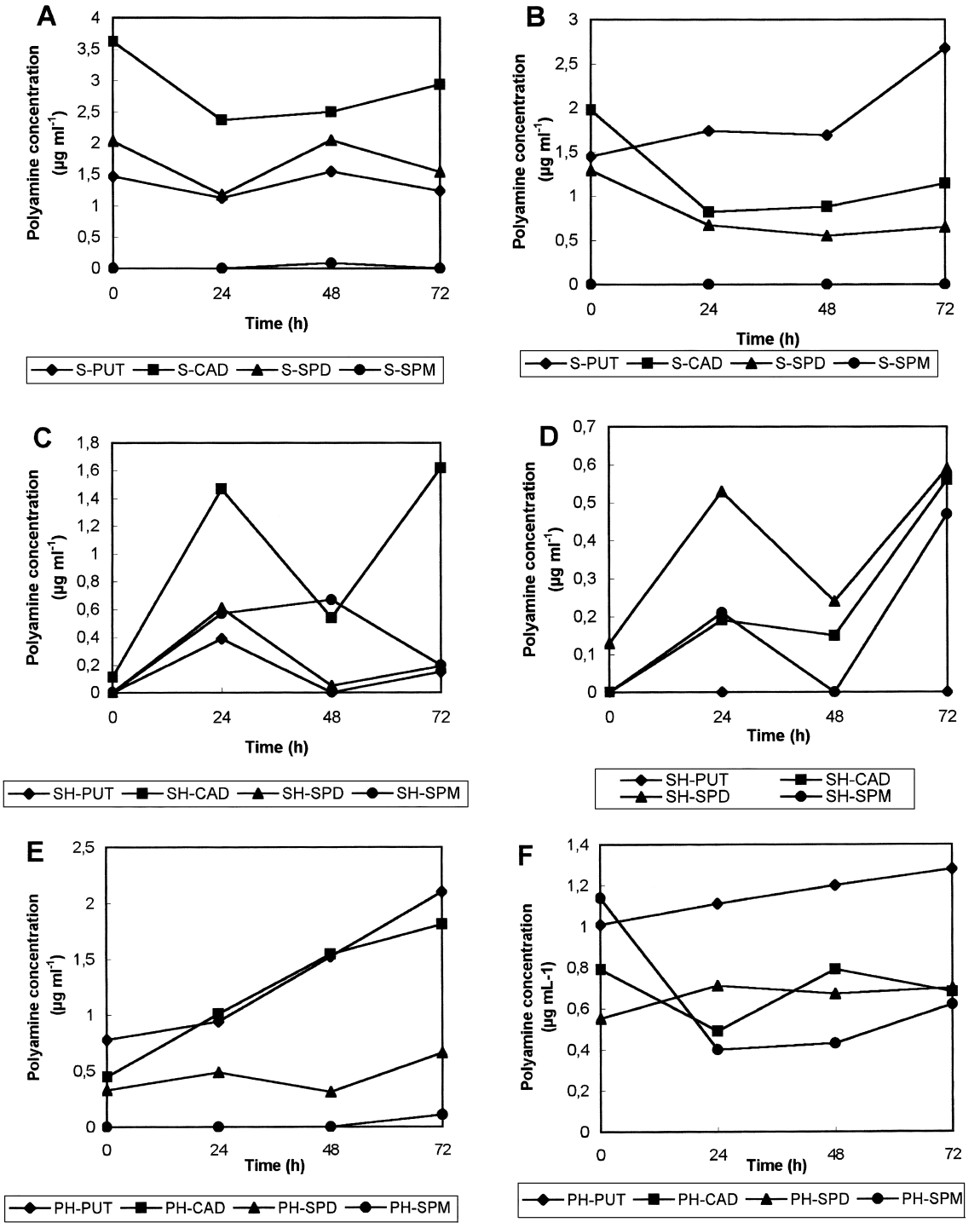

Fig. 3. Time course of PA changes in the MMMC fraction obtained from sugarcane juices stored in the light at pH 5.3 for 72 h after centrifugation (A, C and E) or stored in the light at pH 8.0 after clarification with sodium carbonate (B, D and F). PA content was estimated by HPLC as (A, B) free (S fraction), (C, D) acidic-soluble (SH fraction), or (E, F) acidic-insoluble (PH fraction) PAs. Values are the mean of three replicates. Mean standard error ≤ 0.05.

as both enzymes, ADC and ODC would be active at pH 8.0.

PAs from MMMCs were mainly conjugated to acid-insoluble molecules since concentrations of these PAs recovered as PH fraction (Fig. 3E and F) were always higher than these recovered as SH fraction (Figs. 3C and D). Accumulation of PH-PAs with the time of starvation was especially significant for PUT and CAD (Fig. 3E) while PH-SPD and PH-SPM did not increase with the time. That is, PUT and CAD bind to MMMCs, a pool of glycoproteins [19] through two different classes of molecules, one acid-soluble (Fig. 3C) and the other acid-insoluble (Fig. 3E) at pH 5.3. Balestreri et al. [33] reported the binding of PUT or its derivatives to proteins by ionic linkages in *Medicago sativa* leaves. It seems to be that conjugation of PAs to acid-insoluble molecules is favored at acidic pH values whereas alkaline conditions favored conjugation of CAD, SPD and SPM to acid-soluble molecules (SH fraction, Fig. 3D). However, CAD has also been detected in the SH fraction and its concentration increases with the time of starvation at pH 5.2 (Fig. 3C).

Table 1 analyzes the pattern of total PA variation from the MMMC fraction with the time of starvation. Whole amounts of PUT, SPD and SPM (that have the same biosynthetic pathway) as well as the amount of CAD (which forms from lysine via lysine decarboxylase) either in starved juices at pH 5.3 or at pH 8.0 have been compared. During starvation of juices at pH 5.3, there was no significant increase in total PA titer although transformation between free and conjugated PUT, SPD and SPM occurred. The time course of CAD is similar to that observed for the other PAs. However, it is important to point out that the total concentrations of PUT, SPD and SPM are very similar to that of CAD; this is, CAD is the main PA in sugarcane juice. Clarification of sugarcane juice at pH 8.0 may co-precipitate CAD resulting in lower levels of CAD detection. A decrease in free PUT, SPD and SPM, concomitant to the increase in their conjugated forms that occurred at pH 5.2, was not observed at pH 8.0. It has been reported that part of the macromolecules were removed during the clarification process [9]. If CAD binds to these macromolecules, an appreciable amount of it would be removed since at pH 8.0, ionic interactions between PAs and proteins increases [34] and they co-precipitate together.

Free PAs associated with HMMCs as well as their conjugated forms under starvation of juices at pH 5.3 and 8.0 are shown in Fig. 4. The accumulation pattern of free and conjugated PAs from both MMMCs (Fig. 3) and HMMCs (Fig. 4) is similar although some differences can be observed. For instance, the increase in free PUT over time at pH

Table 1
A comparison of the time course of the amount of free PUT, SPD and SPM against conjugated (SH+PH fractions) or total PUT, SPD and SPM, and free CAD against conjugated or total CAD in the MMMC fraction obtained from centrifuged (pH 5.3) and clarified (pH 8.0) sugarcane juices

Time (h)	Polyamines (μg ml^{-1})					
	S-(PUT+SPD +SPM)	S-CAD	SH+PH-(PUT+SPD+SPM)	SH+PH-CAD	S+SH+PH-(PUT+SPD+SPM)	S+SH+PH-CAD
MMMCs from centrifuged (pH 5.3) sugarcane juice[a]						
0	4.22	3.62	1.11	0.56	5.33	4.18
24	2.30	2.37	3.00	2.48	5.30	4.85
48	3.69	2.50	2.55	2.08	6.24	4.58
72	2.78	2.94	3.41	3.43	6.19	6.38
MMMCs from clarified (pH 8.0) sugarcane juice[b]						
0	2.74	1.98	2.83	0.79	5.57	2.77
24	2.41	0.82	2.96	0.68	5.37	1.50
48	2.24	0.88	2.54	0.94	4.78	1.82
72	3.33	1.14	3.66	1.24	6.99	2.38

[a] Values are the mean of three replicates. Standard error was never higher than ±0.28.
[b] Values are the mean of three replicates. Standard error was never higher than ±0.11.

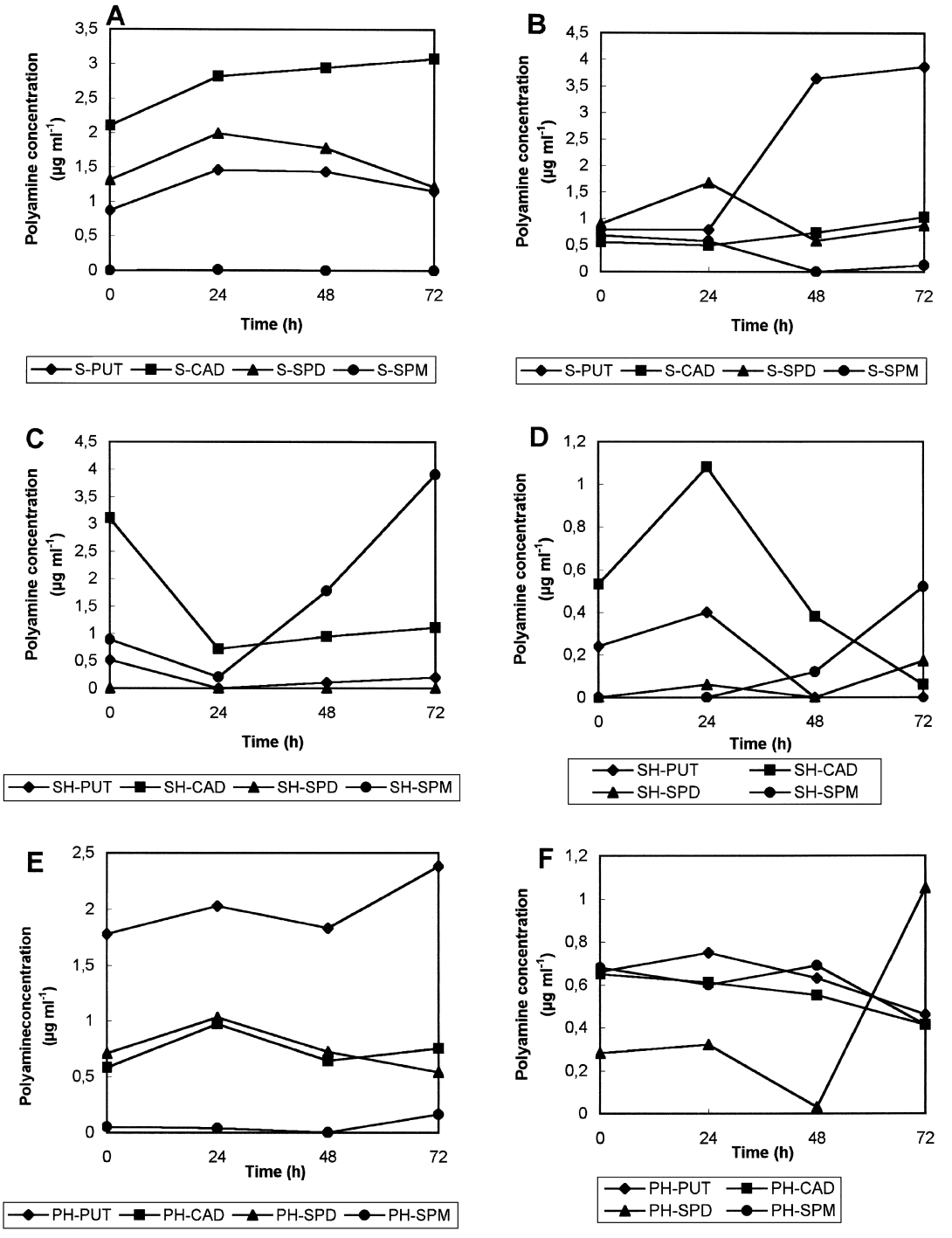

Fig. 4. Time course of PA changes in the HMMC fraction obtained from sugarcane juices stored in the light at pH 5.3 for 72 h after centrifugation (A, C and E) or stored in the light at pH 8.0 after clarification with sodium carbonate (B, D and F). PA content was estimated by HPLC as (A, B) free (S fraction), (C, D) acidic-soluble (SH fraction), or (E, F) acidic-insoluble (PH fraction) PAs. Values are the mean of three replicates. Mean standard error ≤0.04.

8.0 was 2.7-times higher in the HMMC fraction (Fig. 4B) than in the MMMC fraction (Fig. 3B). Conjugated PAs associated to acid-soluble macromolecules (SH fraction) achieved a higher level in the HMMC fractions (Fig. 4C and D) than in the MMMC fractions (Fig. 3C and D). Moreover, the reported increase with time that was observed in PH-CAD (Fig. 3E) from the MMMC fraction was not observed in the HMMC fraction (Fig. 4E), and, finally, the increase in PH-PUT with the time was lower for the HMMC fraction (Fig. 4E) than in the MMMC fraction.

Table 2 shows the variation in free and conjugated PAs from the HMMC fraction taken as the sum of PUT, SPD and SPM compared to CAD accumulation at pH 5.2 and pH 8.0. In a way similar to that described for the MMMC fraction (Table 1), an increase in the amount of PAs with the time of juice starvation can be detected at pH 5.2 and 8.0. This increase seems to be due, preferentially to the increase in free forms rather than to an increase in conjugated PAs. The accumulation with the time described for PUT, SPD and SPM was not observed for CAD (Table 2), although we can point out, as happen in the MMMC fraction, that clarification process co-precipitated CAD.

Table 3 summarizes the pattern of PA accumulation in the whole juice subjected to a starvation process at two pH values, 5.3 and 8.0. While a certain increase in PAs derived from PUT during the time of starvation in light at both pH values can be observed, this situation does not occur with CAD.

PUT accumulation, especially in cereals, has been shown to occur as a response to such varied stresses as water deprivation, high external osmolarity, high external concentration of ammonium, anaerobiosis, etc. In all the cases, where the pathway of extra PUT formation has been investigated, ADC activation has been implied. It thus appears valid to refer to ADC as a general stress enzyme and to PUT accumulation as a general symptom of stress-induced ADC activation [6]. ADC activity increases during light exposure to excised oat leaves [35]. Our experiments demonstrated that PUT accumulation occurs during deterioration when sugarcane juices have been previously clarified at an alkaline pH value (Figs. 3 and 4). This implies that PUT synthesis during impairment is mainly achieved via ODC or by chemical hydrolysis of agmatine if ADC remains active at pH 8.0.

Acknowledgements

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Table 2
A comparison of the time course of the amount of free PUT, SPD and SPM against conjugated (SH+PH fractions) or total PUT, SPD and SPM, and free CAD against conjugated or total CAD in the HMMC fraction obtained from centrifuged (pH 5.3) and clarified (pH 8.0) sugarcane juices

Time (h)	Polyamines (μg ml^{-1})					
	S-(PUT+SPD+SPM)	S-CAD	SH+PH-(PUT+SPD+SPM)	SH+PH-CAD	S+SH+PH-(PUT+SPD+SPM)	S+SH+PH-CAD
HMMCs from centrifuged (pH 5.3) sugarcane juice[a]						
0	2.18	2.10	3.95	3.69	6.13	5.79
24	3.46	2.82	3.67	1.69	7.13	4.51
48	5.99	2.89	2.55	0.64	8.54	3.53
72	2.36	3.07	7.71	2.61	10.07	4.93
HMMCs from clarified (pH 8.0) sugarcane juice[b]						
0	2.36	0.55	1.86	1.18	4.22	1.73
24	3.03	0.49	2.13	1.69	5.16	2.18
48	4.22	0.73	1.76	0.93	5.95	1.66
72	4.87	1.03	2.61	0.47	7.48	1.50

[a] Values are the mean of three replicates. Standard error was never higher than ±0.17.
[b] Values are the mean of three replicates. Standard error was never higher than ±0.08.

Table 3
A comparison of the time course of the amount of free PUT, SPD and SPM against conjugated (SH+PH fractions) or total PUT, SPD and SPM, and free CAD against conjugated or total CAD in the combined HMMC+MMMC fractions obtained from centrifuged (pH 5.3) and clarified (pH 8.0) sugarcane juices

Time (h)	Polyamines (μg ml^{-1})					
	S-(PUT+SPD+SPM)	S-CAD	SH+PH-(PUT+SPD+SPM)	SH+PH-CAD	S+SH+PH-(PUT+SPD+SPM)	S+SH+PH-CAD
HMMC+MMMCs from centrifuged (pH 5.3) sugarcane juice[a]						
0	6.40	5.72	5.06	4.25	11.46	9.97
24	5.76	5.19	6.67	4.17	12.43	9.36
48	9.68	5.39	5.10	2.72	14.78	8.11
72	5.14	6.01	11.12	5.29	16.26	11.30
HMMC+MMMCs from clarified (pH 8.0) sugarcane juice[b]						
0	7.54	2.53	5.62	1.97	9.79	4.50
24	5.44	1.31	5.09	2.37	10.53	3.68
48	6.46	1.61	5.25	1.87	11.71	3.48
72	8.20	2.17	6.27	1.71	14.47	3.88

[a] Values are the mean of three replicates. Standard error was never higher than ±0.17.
[b] Values are the mean of three replicates. Standard error was never higher than ±0.08.

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