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A NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE FOR SEPARATION AND DETERMINATION OF ADENYLIC AND NICOTINAMIDE NUCLEOTIDES IN *LACTOBACILLUS PLANTARUM*

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ABSTRACT

A new technique for separation and determination of ATP, ADP, AMP, NAD^+, $NADP^+$, NADH and NADPH in *Lactobacillus plantarum* has been developped. It involves acid and basic extraction of nucleotide pool, analysis by reverse-phase high performance liquid chromatography on a 5 µm Spherisorb ODS-1 column and UV detection. The method offers advantages in accurate estimations of adenylic and nicotinamide nucleotides concentrations in *Lactobacillus plantarum* during the growth phase. Such results indicate the potential of this technique as an important research tool.

INTRODUCTION

Adenine nucleotides (ATP, ADP and AMP) and oxidized and reduced pyridine coenzymes ($NADP^+$, NADPH, NAD^+, NADH) play a well-known role in intermediairy metabolism through the maintenance of the energetic and redox states. The determination of nucleotide pools by HPLC is a well-established procedure for animal and plant cell cultures (Zakaria and Brown, 1981; Stocchi et al., 1985 and 1987; Nieman and Clark, 1984; Meyer and Wagner, 1985a; 1985b). Nevertheless, nucleotide extraction remains somewhat difficult with plant material (low concentration, instability of nucleotides, post-extraction hydrolysis of reduced nucleotides, etc, ..). In despite of all these difficulties, we adapted this method for bacteria, especially for *Lactobacillus plantarum,* to test its availability for such a material which contains high concentration of nucleotides.

With the improvement of HPLC equipment and extraction techniques, it may be possible to increase nucleotide separation and thus determine cellular nucleotide concentrations for this bacteria. The present work describes the separation of 20 nucleotides by HPLC and the determination of adenylic and nicotinamide nucleotides using the procedure developed for plant material adapted for the extraction, purification and HPLC separation of these compounds from *Lactobacillus plantarum.*

During our studies on physiology of *Lactobacillus plantarum,* we applied this method to relate the energy and redox charge to lactic acid production during growth. Better knowledge of these mechanisms would help to optimize the growth of *Lactobacillus plantarum* in order to obtain better lactic acid production.

MATERIAL AND METHODS

Strain and culture medium : The microorganism used was *Lactobacillus plantarum* (Lacto-labo Company , France). The strain was maintained at 4°C and subcultured monthly on the agar slants prepared by adding 1.4 % agar to MRS medium (Difco). Experiments were carried out with MRS medium in which glucose was added to the medium to a final concentration of 50 g/l.

Fermentation conditions : fermentation was carried out in a 10 l bioreactor (LSL Biolafitte, France)) at 31°C. The pH was set at 6.0 with 10 N NaOH and was maintained throughout. The medium was inoculated at 10 % (v/v) level and was agitated at 200 rpm and without aeration. During the exponential growth phase (five hours after inoculation), 500 ml of culture were removed and centrifuged at 3,000 g for 25 min, three grammes of cell were used for nucleotide pool extraction and were frozen with liquid nitrogen before being stored at -80°C.

Nucleotide extraction : we used acid extraction for adenylate nucleotides and oxidized nicotinamide nucleotides, and alkaline extraction for reduced forms.

Frozen cells were resuspended in ice cold medium with 0.5 M $HClO_4$ diluted with 10 % methanol (v/v) for acid extraction,

or with 0.5 M NaOH diluted with 10 % methanol (v/v) for basic extraction. The samples were homogenised with an Ultra-Turrax (Ika-Werk) at 20,000 rpm for 15 sec, transferred to a Potter-Elvehjem glass homogenizer and further homogenised (10 times at 250 rpm).

The suspension was centrifuged (5,000 g) for 15 min at 4°C. The pellet was extracted with 0.2 M $HClO_4$ or 0.2 M NaOH diluted with 10 % methanol (v/v), respectively and centrifuged in the same conditions. The supernatant were combined and adjusted to pH 5.0 with saturated KOH solution in the acid extract, or to pH 8.0 with 1 M HCl in the alkaline extract. After centrifugation (5,000 g) for 10 min at 4°C, the supernatants were frozen with liquid nitrogen and immediately lyophilized. Dry extract was dissolved in 2 ml of 0.1 M KH_2PO_4, pH 6.5 and the suspension centrifuged (5,000 g) for 10 min at 4°C.

Twenty microliter of supernatant was used for HPLC analysis.

HPLC procedure : nucleotide analyses were carried out throughout this work on the liquid chromatograph PU system (Philips), using a 5 µm Spherisorb ODS 1 column (C-18, 25 cm x 4.6 mm), and a Brownlee RP-18 guard column (5 cm x 4.6 mm). A PU 4021 multi-channel detector provided a continuous series of instantaneous spectra as the eluent passed through its flowcell. Samples were injected by a PU 4700 autosampler with a 20 µl injection loop. Spectra, retention times and peak areas were obtained by a PU 6030 electronic integrator.

The mobile phase consisted of two eluents : 0.1 M KH_2PO_4 with 8 mM TBAHS (tetra-n-butylammonium hydrogen sulfate), pH 6.0, adjusted with Tris-base (buffer A) and 0.1 M KH_2PO_4 with 8 mM TBAHS, pH 6.0, containing 20 % (v/v) methanol (buffer B). Buffers were filtered through a 0.22 µm Millipore membrane before use. Chromatographic analysis was performed at 10°C.

The conditions used to obtain the chromatograms shown in Figures 1, 3 and 4 were as follows : 12 min at 100 % of buffer A, 4 min at up to 25 % of buffer B, 4 min at up to 50 % of buffer B, 2 min at up to 100 % of buffer B, and finally hold for 8 min .at 100 % of buffer B. The initial conditions were restored in 5 min. The flow rate was 1.3 ml min^{-1} and detection was performed at 254 nm. The chromatogram in Fig. 2 was obtained in the same conditions but with buffer B at 25 % (v/v) methanol.

Standard nucleotides and nucleosides were prepared as 10 mM stock solutions in buffer A.

Calibration was carried out by injection of a standard mixture of nucleotides or nucleosides used at different concentrations. Therafter, the equipment permit us to assume automatically the quantitative measurement of nucleotides extracted from such a biological material.

RESULTS AND DISCUSSION

Using the chromatographic procedure developped in the present studies, at least 14 different nucleotides and nucleosides could be separated in the standard mixture of 20 compounds within 35 min as shown in Fig. 1. Retention times and absorbance wavelengths for 20 nucleotides or nucleosides are summarized in Fig. 2.

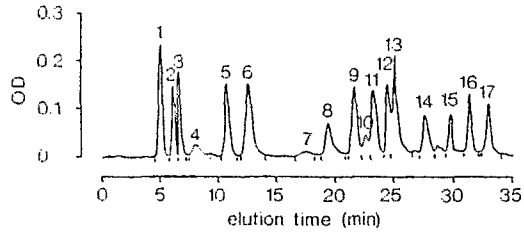
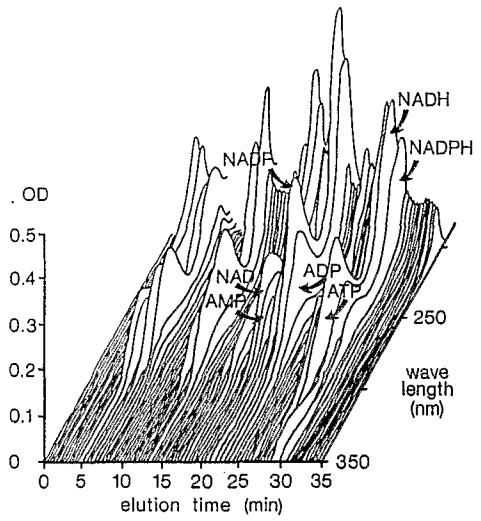


Figure 1. Separation of nucleotides and nucleosides by HPLC.
A standard mixture, prepared with 10 mmol of each compound, was analyzed as described in Material in Methods. Each compound was detected at 254 nm. Each peak was identified as follows : 1, cytidine, CMP; 2, uridine; 3, UMP; 4, CDP; 5, GMP; 6, UDP, guanosine; 7, CTP; 8, GDP; 9, NAD^+; 10, UTP; 11, AMP; 12, adenosine; 13, $NADP^+$, GTP; 14, ADP; 15, ATP; 16, NADH; 17, NADPH.

The data indicate that separation of different nucleotides or nucleosides depends on the elution temperature, the eluent (% of methanol) and the pH gradient (results not shown). The conditions used correspond to the best obtained for the separation of adenylic and nicotinamide nucleotides. Thus, as excepted, $NADP^+$, adenosine, AMP, ATP, NAD^+, NADH and NADPH were well separated.

The spectrum presented in Fig. 2 showed that every nucleotide or nucleoside had a high absorbance around 264 nm. The sensitivity of detection of NADH and NADPH at 343 nm was lower than at 254 nm. The detection limit for reduced nicotinamide nucleotides was different : 5 pmol at 254-264 nm and 100 pmol at 343 nM. Nevertheless, the absorbance peak at 343 nm allowed us to conclude the presence of NADH and NADPH in any biologi cal samples analyzed by such a method.



Nucleotide or nucleoside	Retention time (min)	Absorbance wavelength (nm)
Cytidine	5.0	275
CMP	5.0	275
uridine	6.0	266
UMP	6.6	266
CDP	8.0	266
GMP	10.7	266
UDP	12.5	266
Guanosine	12.5	258/277
CTP	17.2	277
GDP	19.2	258/277
NAD	21.4	264
UTP	22.4	264
AMP	23.1	264
GTP	24.2	264
NADP	24.7	264
Adenosine	24.7	264
ADP	27.2	264
ATP	29.4	264
NADH	31.1	264/343
NADPH	32.6	264/343

Figure 2. Chromascan (3D-analysis) of a standard mixture of nucleotides and nucleosides prepared at 10 mM. The axes were defined as follows : the X- and the Y-axes corresponded to the elution chromatogram; the Z-axis, to the absorption spectrum of each molecule eluted. In the table, we precised the retention time and the absorption optimum for each molecule.

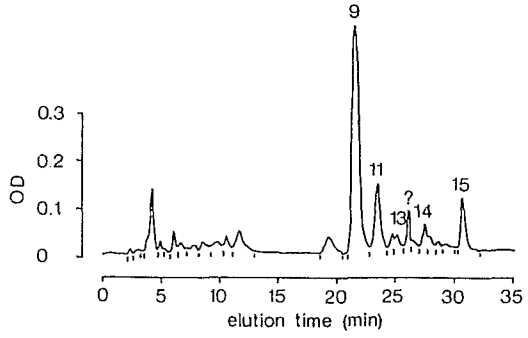


Figure 3. Separation of nucleotides and nucleosides after their acid extraction from *Lactobacillus plantarum* cells. Each peak was referred as in Fig. 1.

Acid extraction from *Lactobacillus plantarum* gave a chromatogram (Fig. 3) where adenylic and nicotinamide nucleotides were well separated. The majority of nucleotides extracted could be identified. Nevertheless, as observed by Quebedeaux (1981), acid extraction did not allow us to obtain reduced nicotinamide nucleotides.

With the basic extraction (Fig. 4), NADH and NADPH were extracted. The amounts calculated seemed to be low , 7 and 28 nmol respectively (Table 1).

When we compared the two modes of extraction, the amount stayed unchanged for ADP and NADP. For the other nucleotides, this amount decreased. Furthermore, from basic extraction, we obtained two unknown peaks with retention times of 25.7 and 28.4 minutes. These peaks could be regarded as a degradation product of reduced nicotinamide nucleotide. Indeed, the low contents of reduced nicotinamides and the presence of these unknown peaks could be interpreted as a higher degradation of nicotinamide reduced nucleotides during the basic extraction.

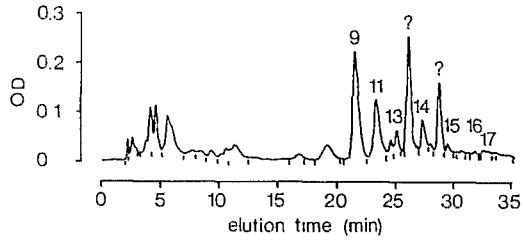


Figure 4. Separation of nucleotides and nucleosides after their basic extraction from *Lactobacillus plantarum* cells. Each peak was referred as in Fig. 1.

Table 1. Estimation of the adenylic and nicotinamide nucleotides concentration in *Lactobacillus plantarum* cells during the exponential phase. g.f.w. : gram fresh weight; g.d.w. : gram dry weight.

Nature	Acid extraction		Basic extraction		Nucleotide amount
of molecule	nmol/g.f.w.	mg/g.d.w.	nmol/g.f.w.	mg/g.d.w.	nmol/g.f.w.
AMP	408	0.9	35	0.80	408
ADP	141	0.4	141	0.40	141
ATP	333	1.0	54	0.20	333
NAD+	1260	6.0	498	2.30	1260
NADP+	37	0.2	46	0.25	46
NADH	-	-	7	0.03	7
NADPH	-	-	28	0.10	28

During the growth phase of *Lactobacillus plantarum*, we have defined the amount of adenylic and nicotinamide compounds (Table 1). The range of values described in this paper is in good agreement with those found in other bacteria with the other classic methods more tedious to use. After optimization ot the extraction conditions of reduced nicotinamide we will able to measure variations of the nucleotide pool during fermentation of *Lactobacillus plantarum*.

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