ELSEVIER

Journal of Chromatography B, 656 (1994) 197–202

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Application of high-performance liquid chromatography to the study of the biological transformation of adiponitrile

J.L. Moreau, S. Azza, F. Bigey, A. Arnaud *, P. Galzy

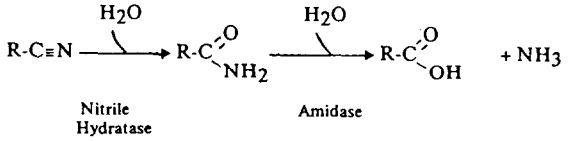
Chaire de Microbiologie Industrielle et de Génétique des Microorganismes, Ecole Nationale Supérieure Agronomique de Montpellier, Place Viala, 34060 Montpellier Cedex, France

Abstract

A procedure for the assay of nitrile hydratase and amidase activity by high-performance liquid chromatography is described. The method can be used to assay the intermediate compounds resulting from the hydrolysis of adiponitrile into adipic acid, and to determine the kinetics of the hydrolysis of these compounds using whole cells and enzyme extracts. The precision of the method makes it suitable for the determination of the enzyme parameters: K_m and V_m (nitrile hydratase and amidase). Using cyanovaleramide as substrate, K_m and V_m were respectively 370 m*M* and 2060 U/mg for nitrile hydratase and 6.6 m*M* and 33 U/mg for amidase.

1. Introduction

The biological hydrolysis of nitriles and amides involves the nitrile hydratase and amidase enzyme systems [1–8] (see Scheme 1). A gas chromatographic (GC) method for the assay of the activities of these enzymes has been described previously [3]. The usefulness of this method is, however, limited to the assay of sufficiently volatile nitriles and amides. Another simple and sensitive method was therefore required for the rapid assay of non-volatile nitriles, amides, and organic acids, particularly those involved in the transformation of adiponitrile into adipic acid (Fig. 1).



Scheme 1.

Described in this paper is the application of a high-performance liquid chromatographic (HPLC) method to the study of the biological hydrolysis of adiponitrile to adipic acid by *Brevibacterium sp.* R312 and mutant strain *Brevibacterium sp.* ACV2.

2. Experimental

2.1. Biological material

The strains used were *Brevibacterium sp.* R312 [10–12] and *Brevibacterium sp.* ACV2 [13].

2.2. Culture conditions

The strains were maintained on agar slants (25

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00091-I

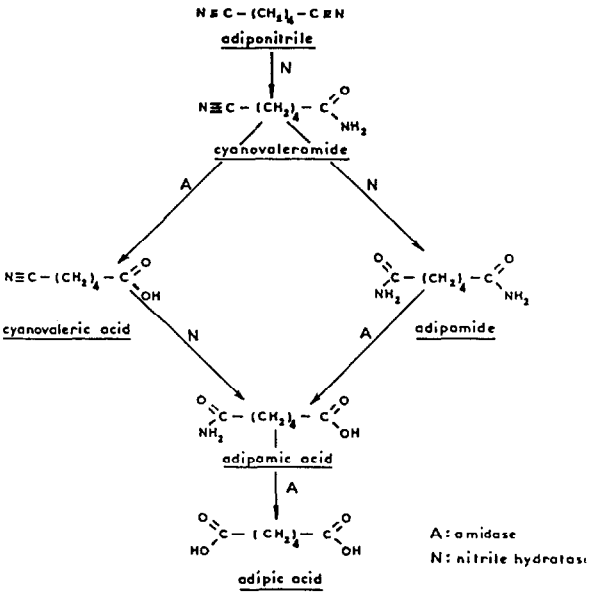


Fig. 1. Theoretical pathways for the transformation of adiponitrile into adipic acid by a nitrile hydratase (N) and an amidase (A).

$g\,l^{-1}$) containing yeast extract (3 $g\,l^{-1}$), malt extract (3 $g\,l^{-1}$), bacto peptone (5 $g\,l^{-1}$) and glucose (10 $g\,l^{-1}$), known as YMPG. A pre-culture was inoculated on MMT medium [14] into Erlenmeyer flasks filled to 10% of their volume. After *ca.* 12 h, the culture on MMT was inoculated with this pre-culture. Cells were harvested 20 h later. The culture and pre-culture were incubated at 28°C with shaking (80 oscillations min^{-1}; amplitude 8 cm).

2.3. Sonication technique

The cells were sonicated with a Branson 250 Sonifier (Branson, Danburg, CT, USA), and 0.5 g (wet mass) was suspended in 3 ml of 20 mmol/l phosphate buffer (pH 7.3). Sonication was performed for a total of 15 min by sonicating for periods of 0.3 s and then cooling for periods of 0.7 s [power, 25 W; temperature, *ca.* −15°C (maintained by using an ice–water mixture saturated with NaCl)]. The sonicated mixture was centrifuged at 15 000 *g* for 10 min and the resulting supernatant was used as the enzyme extract.

2.4. Equipment

HPLC analyses were performed on a Waters apparatus consisting of a Model 150 pump, a U6K injector, and a Model 450 UV detector. The integration–calculation of the peak areas was performed with a Model 740 integrator. The chromatographic column used was a reversed-phase 5 μm LiChrosorb RP 18 (125 × 4.0 mm I.D.; Merck, Darmstadt, Germany). The analytical conditions were as follows: mobile phase: 25 m*M* H_3PO_4–methanol (98.9:1.1, v/v), pH 2.0, flow-rate 1.0 ml min^{-1}; the detection wavelength was 200 nm. The sample volume injected was 5 μl and the loop volume was 2 ml.

3. Results and discussion

3.1. Identification of intermediary compounds and end products

The determination of the nitrile hydratase and amidase activities depends on the disappearance of substrates or the appearance of products. The five compounds resulting from the hydrolysis of adiponitrile detected by HPLC were: cyanovaleramide, adipamide, cyanovaleric acid, adipamic acid, and adipic acid (Fig. 2). The approximate limits of detection, defined as the lowest concentrations below which no peak could be integrated, were: cyanovaleramide: $5 \cdot 10^{-5}$ *M*; adipamide: 10^{-5} *M*; cyanovaleric acid: 10^{-3} *M*; adipamic acid: $5 \cdot 10^{-5}$ *M*; adipic acid: 10^{-4} *M*. Adipamic acid and cyanovaleramide had the same retention time. Fortunately, the rates of hydrolysis of cyanovaleramide into cyanovaleric acid or adipamide (respectively by amidase and nitrile hydratase) were sufficiently high so that adipamic acid appeared only when no more cyanovaleramide was left in the reaction mixture. Since cyanovaleric acid and adipamide are very slowly transformed into adipamic acid by nitrile hydratase and amidase respectively, adipamic acid and cyanovaleramide do not appear simultaneously. Thus, there was no possible confusion as to the identity of the two peaks.

The method was found not to be sensitive for

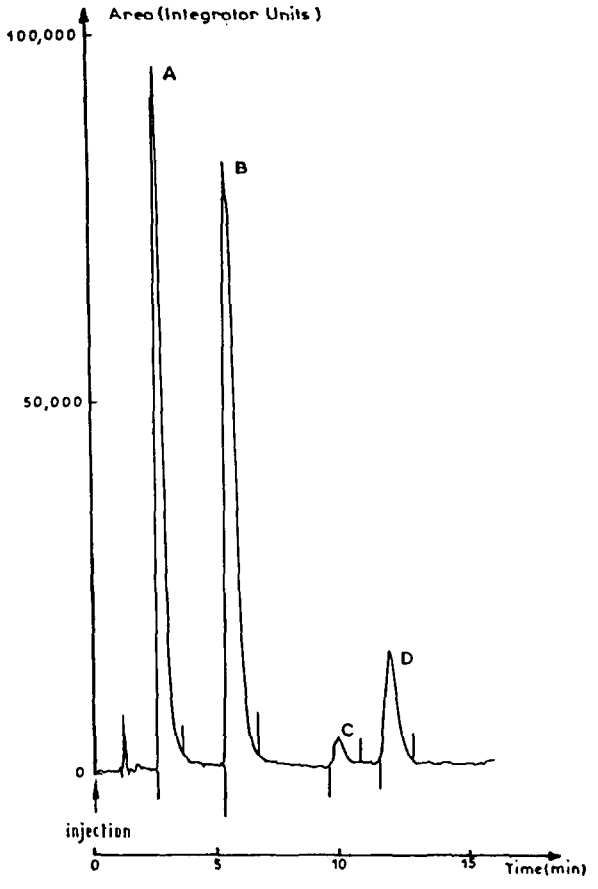


Fig. 2. Chromatogram of different hydrolysis products of adiponitrile. Peaks: A = adipamide, 20 mg l^{-1}; B = adipamic acid (50 mg l^{-1}) + cyanovaleramide (50 mg l^{-1}); C = cyanovaleric acid,250 mg l^{-1}; D = adipic acid, 200 mg l^{-1}.

the detection of cyanovaleric acid and adipic acid. During measurement of the reaction kinetics of nitrile hydratase activity with cyanovaleric acid as substrate, it was more convenient to analyse the reaction product adipamic acid. On the other hand, the kinetics of the hydrolysis of cyanovaleramide by nitrile hydratase or amidase, and that of adipamic by amidase, could be followed by monitoring the disappearance of the substrate.

3.2. Measurement of enzyme kinetics

Kinetic studies using enzyme extracts

The kinetic studies were performed in thermostated containers at 25°C for the nitrile hydratases and at 30°C for the amidases. The enzyme mixture consisted of 1 volume of enzyme extract appropriately diluted in 100 m*M* phosphate buffer (pH 7.0) and 1 volume of substrate solution in the same buffer preincubated for 2 to 3 min at the chosen temperature (usually 1 volume corresponded to 20 μl). At regular intervals, 5-μl samples of the reaction mixture were taken and injected with a chromatographic syringe. The pH of the eluent (pH 2) was sufficient to stop the enzymatic reaction. The curve for the disappearance of the substrate or the appearance of the product was calculated from the peak heights or the peak areas. The area or height at time zero was obtained by injecting substrate solution in which the added enzyme preparation was replaced by distilled water. A chromatogram showing the hydrolysis of adipamide by amidase is shown in Fig. 3. The kinetics were studied at 4 or 5 substrate concentrations and the kinetics parameters K_m and V_m were graphically determined from these data (Tables 1 and 2). Figs. 4 and 5 show the K_m and V_m determination for nitrile hydratase and amidase, respectively, using this method.

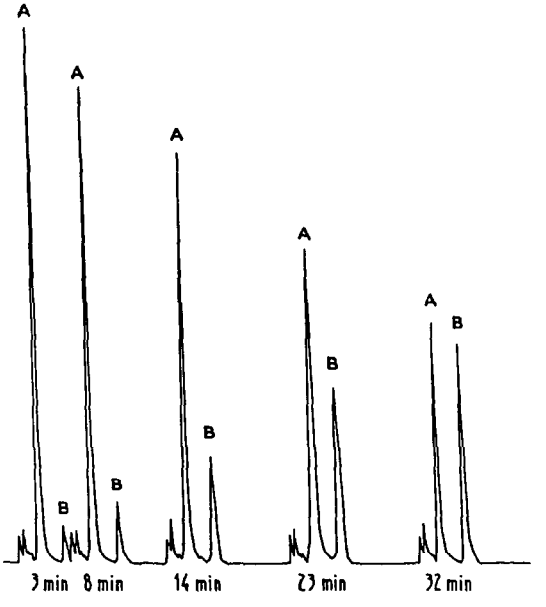


Fig. 3. Kinetics of hydrolysis monitored by HPLC. Peaks: A = adipamide; B = adipamic acid.

Table 1
K_m and V_m of the nitrile hydratase from *Brevibacterium sp.* R312 and *Brevibacterium sp.* ACV2 *versus* some compounds

Compounds	Nitrile hydratase R 312 (pH 7.0)		Nitrile hydratase ACV 2 (pH 6)	
	K_m (mmol/l)	V_m (units/mg proteins)	K_m (mmol/l)	V_m (units/mg proteins)
Propionitrile[a]	8	1800	50	2000
Adiponitrile[a]	30	1800	38	1930
Cyanovaleramide[b]	156	60	370	2060
Cyanovaleric acid[b]	28	0.25	21	5

[a]GC method [1].
[b]HPLC method.

The method was found to be sensitive, since concentrations as low as 10^{-4} *M* could be easily measured for adipamic acid, cyanovaleramide and adipamide.

Kinetics using whole cells

Sometimes it is necessary to determine the rates of hydrolysis using whole cells. The reaction temperatures were 25°C for nitrile hydratase and 30°C for amidase. Incubations were performed in 100 m*M* phosphate buffer (pH 7). The incubation mixture contained 1 volume of substrate (in buffer) and 1 volume of cells suspended in the same buffer. The amount of cells used depended on the substrate studied and the activity of the cells. The substrate concentrations were the same as those used in the experiments with enzyme extracts.

It was necessary to remove the cells before injecting the sample onto the HPLC apparatus. At regular intervals, an aliquot was removed from the reaction mixture and the same volume of 0.5 *M* orthophosphoric acid was added to the sample. The resulting pH was sufficient to stop

Table 2
Determination of K_m and V_m for the purified amidase from *Brevibacterium sp.* ACV2

Substrate	V_m (units/mg proteins)	K_m (m*M*)
Acetamide[a]	16	3
Propionamide[a]	22	2
Butyramide[a]	30	0.05
Valeramide[a]	40	0.03
Hexanoamide[b]	41	0.03
Cyanovaleramide[b]	33	6.6
Adipamide[b]	7.5	11
Adipamic acid[b]	7.5	105
Acrylamide[a]	3	0.8

[a]GC method [1].
[b]HPLC method

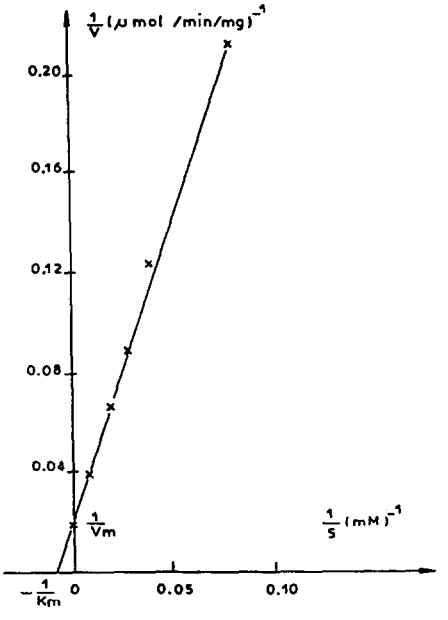


Fig. 4. Example of K_m and V_m determination of nitrile hydratase from *Brevibacterium sp.* R312 using cyanovaleramide as substrate. Lineweaver–Burk representation.

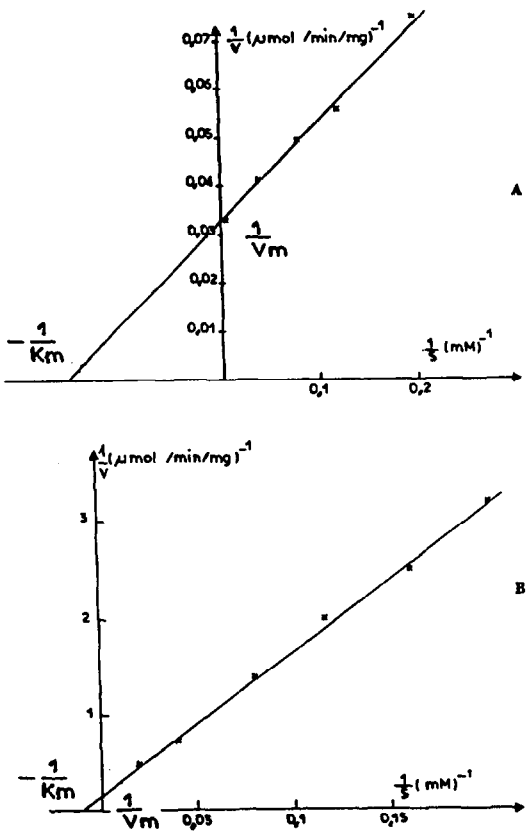


Fig. 5. Example of K_m and V_m determination of amidase from *Brevibacterium sp.* ACV2 using cyanovaleramide (A) or adipamic acid (B) as substrate. Lineweaver–Burk representation.

the reaction. Next, the cells were separated by centrifugation at 5000 *g* for 5 min and the supernatant was retained for assays. Measurement to determine the rate of hydrolysis were performed as described above.

3.3. Biological hydrolysis of adiponitrile

The hydrolysis of adiponitrile into adipic acid by strains R312 and ACV2 was monitored by HPLC. After growth of the strains on basic MMT medium (as described by Bernet *et al.* [14]) to which glucose (10 g l^{-1}) and ammonium chloride (5 g l^{-1}) were added, the cells (8 g wet weight) were dispersed in phosphate buffer (100 m*M*, pH 7) and added to an adiponitrile solution (final concentration: 50 m*M*). The results (Fig.

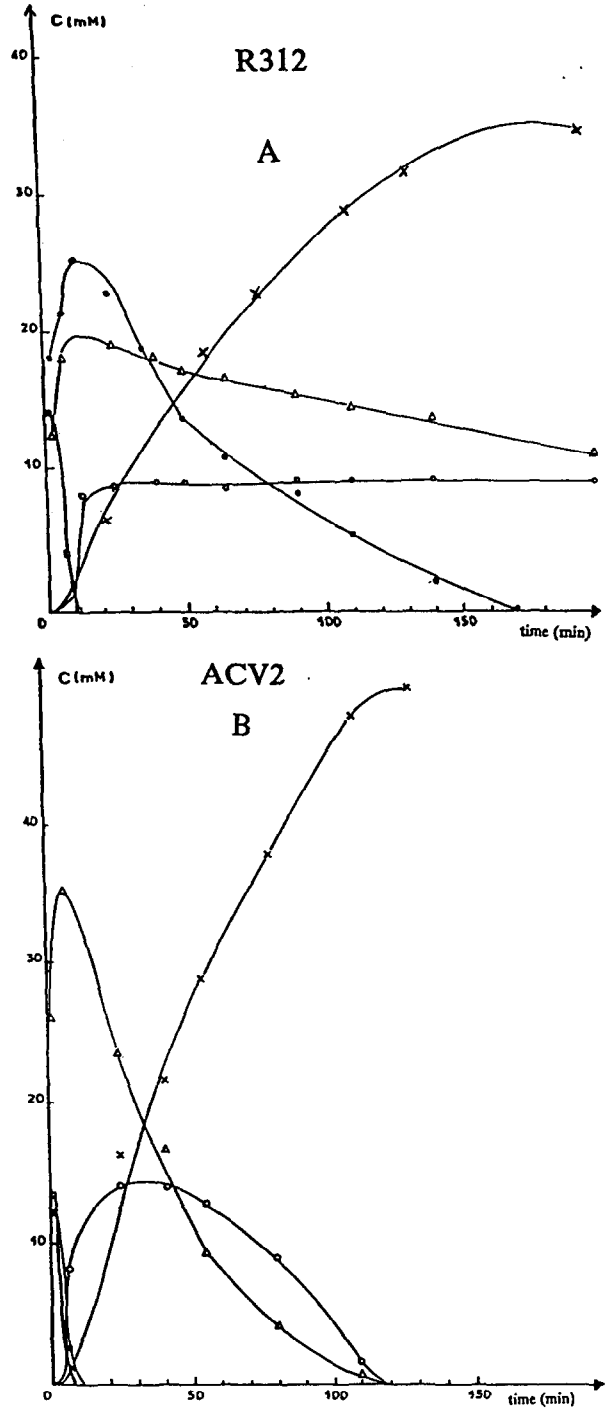


Fig. 6. Degradation of adiponitrile and assay of adiponitrile hydrolysis products by R312 strain (A) and ACV2 strain (B). (□) Cyanovaleramide; (●) cyanovaleric acid; (△) adipamide; (○) adipamic acid; (×) adipic acid.

6) showed that adiponitrile was immediately degraded by strain R312. *Brevibacterium sp.* R312 (Fig. 6A) rapidly hydrolyzed cyanovaleramide to adipamide and cyanovaleric acid. Further hydrolysis of these two compounds, especially adipamide, was slow; after 2 h, only about 25 m*M* of ammonium adipate was formed.

Like the wild strain R312, strain ACV2 immediately hydrated adiponitrile to cyanovaleramide (Fig. 6B). However, the product was then almost totally hydrolyzed to adipamide. Consequently, almost no cyanovaleric acid was produced, and any quantity produced was rapidly degraded further. The two reaction products that accumulated were adipamide and adipamic acid. These compounds were then hydrolyzed to ammonium adipate.

4. Conclusions

An HPLC method has been used for the determination of the rate of biological (whole cells and enzyme extracts) hydrolysis of nitriles, amides and acids involved in the transformation of adiponitrile into adipic acid. The precision of the method makes it suitable for the determination of the enzyme parameters [15,16]. The method was shown to be very useful for the study of the production of adipic acid by a biological pathway.

5. References

[1] A. Arnaud, P. Galzy and J.C. Jallageas, *Agric. Biol. Chem.*, 41 (1977) 2183.
[2] K. Bui, M. Maestracci, A. Thiéry, A. Arnaud and P. Galzy, *J. Appl. Bacteriol.*, 57 (1984) 183.
[3] K. Bui, H. Fradet, A. Arnaud and P. Galzy, *J. Gen. Microbiol.*, 130 (1984) 89.
[4] R. Duran, C.K.N. Chan Kwo Chion, F. Bigey, A. Arnaud and P. Galzy, *J. Basic Microbiol.*, 32 (1992) 13.
[5] J.C. Jallageas, A. Arnaud and P. Galzy, *J. Gen. Appl. Microbiol.*, 24 (1978) 103.
[6] M. Maestracci, A. Thiéry, K. Bui, A. Arnaud and P. Galzy, *Arch. Microbiol.*, 138 (1984) 315.
[7] A. Thiéry, M. Maestracci, A. Arnaud, P. Galzy and M. Nicolas, *J. Basic Microbiol.*, 26 (1986) 299.
[8] C.K.N. Chan Kwo Chion, R. Duran, *A. Arnaud and P. Galzy. Appl. Microbiol. Biotechnol.*, 36 (1991) 205.
[9] J.C. Jallageas, A. Arnaud and P. Galzy, *J. Chromatogr.*, 166 (1978) 181.
[10] A. Arnaud, P. Galzy and J.C. Jallageas, *C.R. Acad. Sci. Paris*, 287 (1976) 571.
[11] A. Arnaud, P. Galzy and J.C. Jallageas, *Rev. Ferment. Ind. Aliment.*, 31 (1976) 39.
[12] A. Arnaud, P. Galzy and J.C. Jallageas, *Folia Microbiol.*, 21 (1976) 178.
[13] J.L. Moreau, N. Bernet, A. Arnaud and P. Galzy, *Can. J. Microbiol.*, 39 (1993) 524.
[14] N. Bernet, A. Arnaud and P. Galzy, *Biocatalysis*, 3 (1990) 259.
[15] J.L. Moreau, S. Azza, A. Arnaud and P. Galzy, *Biosci. Biotech. Biochem.*, 57 (1993) 294.
[16] J.L. Moreau, S. Azza, A. Arnaud and P. Galzy, *J. Basic Microbiol.*, 33 (1993) 323.