Journal of Chromatography, 539 (1991) 517–523
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 900

Purification of NAD glycohydrolase from *Neurospora crassa* conidia by a polyclonal immunoadsorbent

MARIO PACE*, DARIO AGNELLINI, GUIDO LIPPOLI, PIER GIORGIO PIETTA and PIER LUIGI MAURI

Dipartimento di Scienze e Tecnologie Biomediche, Sez. Chimica Organica, University of Milan, Via G. Celoria 2, 20133 Milan (Italy)

and

SERGIO CINQUANTA

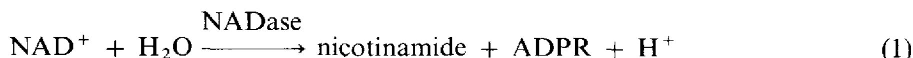
CNR, c/o Ist. Patologia Vegetale, University of Milan, Via G. Celoria 2, 20133 Milan (Italy)

ABSTRACT

NAD glycohydrolase from *Neurospora crassa* conidia was purified by affinity chromatography on a column of polyclonal antibodies bound to an agarose matrix. The procedure was easy, non-denaturating and suitable for repetitive use of the gel. The enzyme obtained appeared homogeneous by sodiumdodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

NAD glycohydrolase (NADase, E.C. 3.2.2.5) from *Neurospora crassa* conidia is a hydrolytic enzyme which uses NAD^+ as a substrate, catalysing the reaction



$$NAD^+ + H_2O \xrightarrow{\text{NADase}} \text{nicotinamide} + \text{ADPR} + H^+ \quad (1)$$

NADase is a glycoprotein of 33 000 dalton and was purified by affinity chromatography [1] using a competitive inhibitor, 4-methyl-NAD, bound to a Sepharose-4B gel through a hydrophilic spacer arm. The enzyme possesses hydrophobic characteristics which were used for its immobilization onto propyl–Sepharose [2] and for the isolation of the protein by hydrophobic chromatography [3]. However, both purification methods used show some disadvantages:

(a) 4-methyl-NAD is not a commercial nucleotide and its preparation is a long and cumbersome process;

(b) the crude extract of conidia contains pyrophosphatases and nucleotidases which hydrolyse the ligand and shorten the life of the affinity column;

(c) hydrophobic chromatography can be performed only under well established conditions, *i.e.*, with a propylic residue as a ligand and a gel capacity in the range 0.09–0.17 mmol/g; and

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V.

(d) the elution of the protein from propyl–Sepharose gels can be accomplished only under the conditions that lead to a high percentage of denaturation (6 *M* urea or 1 *M* *n*-propylamine).

For these reasons, we prepared a new column with a polyclonal antibody to NADase bound to an agarose gel and in this paper we report the results obtained for the purification of the enzyme.

EXPERIMENTAL

NAD^+ was purchased from Boehringer (Mannheim, Germany) and adenosine diphosphoribose (ADPR) from Sigma (St. Louis, MO, U.S.A.). Acrylamide, N,N-bis-acrylamide and sodium dodecyl sulphate of electrophoresis grade and Affi-Gel 10 were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). All other reagents were of analytical-reagent or high-performance liquid chromatographic (HPLC) grade. A crude extract of NADase was obtained by washing the *Neurospora crassa* conidia with water and centrifuging the suspension at 5000 *g* for 15 min at 2°C.

NADase was previously purified by affinity chromatography [1] in order to obtain polyclonal antibodies.

NAD glycohydrolase activity was assayed by HPLC [4] when the enzyme was in phosphate buffer, or by titrating the hydrogen ions produced in the reaction with 5 m*M* sodium hydroxide through a pH-Stat apparatus (Radiometer) when a dialysed solution was used.

HPLC was carried out on a C_{18} Resolve column (Waters Assoc., Milford, MA, U.SA.) (150 × 4.5 mm I.D.; 5 μm average particle size) with a Corasil precolumn (35 × 3.9 mm I.D.), eluted with 4% (v/v) methanol in 0.1 *M* phosphate buffer (pH 6.2) using a Waters Assoc. apparatus. The instrument consisted of a Model 590 pump equipped with a Model U6K universal injector, a Lambda-Max Model 480 UV detector and a Model 730 data module. The chromatographic assay was accomplished at a flow-rate of 2.0 ml/min and the eluent was monitored by UV absorption at 254 nm (0.05 a.u.f.s.).

One NAD glycohydrolase unit is defined as the amount of enzyme which catalyses the hydrolysis of 1 μmol of NAD^+ per minute at 37°C and pH 7.0.

Preparation of the polyclonal antibodies of NADase

Antisera were obtained in rabbits by a series of 4-weekly injections of NADase, previously purified by affinity chromatography, emulsified with complete Freund's adjuvant and bleeding 1 week after the last injection. Antibodies against NADase from *Neurospora crassa* conidia were purified according to the rivanol method [5], and linked to alkaline phosphatase by means of glutaraldehyde [6] in order to assay their specificity. For extended storage the derivatives were kept frozen in working aliquots and each sample was assayed by enzyme-linked immunosorbent assay (ELISA) using *p*-nitrophenyl phosphate as substrate. The working dilution of each stock solution of enzyme–antibody conjugate in the ELISA assay was 1:100 and the reaction lasted 30 min.

ELISA assay

Each well of a polystyrene plate was filled with 200 μl of antibody solution [1

μg/ml in phosphate buffer (pH 7.5)] and allowed to stand at 4°C for 18 h. After washing three times with phosphate buffer (pH 7.5) containing 0.9% NaCl [phosphate-buffered saline (PBS)] and 0.05% (v/v) Tween-20, 200 μl of NADase (1 μg/ml) in PBS was added to each well and the plate incubated for 1 h at 37°C. The wells were then washed three times with PBS–Tween-20 and 200 μl (5 μg/ml) of antibody, linked to alkaline phosphatase by means of glutaraldehyde, were added to some of the wells. The wells containing no antigen were filled with conjugate as a control and the plate was incubated for 1 h at 37°C. After addition of 200 μl of *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine (pH 9.8), containing 1 m*M* $MgCl_2$, the plate was incubated at 37°C for 30 min. For each determination, triplicate wells were pooled and the absorbance at 405 nm was measured.

Immobilization of antibodies onto Affi-Gel 10

A 7.5-ml aliquot of the N-hydroxysuccinimide ester of derivatized cross-linked agarose gel beads (Affi-Gel 10) was washed with 23 ml of isopropanol and 23 ml of chilled water, according to the procedure of Ikura *et al.* [7]. To the gel was added 1 ml of 0.1 *M* phosphate buffer (pH 8.0), containing 48 mg of antibodies; after reaction for 4 h with gentle shaking at 4°C, the gel was washed with 23 ml of 0.1 *M* phosphate buffer (pH 8) and 1 ml of 0.1 *M* lysine (pH 8.0) was added. After 1 h at room temperature with shaking, the gel was washed with 10 m*M* Tris–HCl buffer (pH 7.5) containing 0.15 *M* NaCl, 0.1 m*M* dithiothreitol and 1 m*M* EDTA.

Chromatography of NADase on the polyclonal antibody–Affi-Gel 10 column

Five small columns were filled with 1.5 ml of polyclonal antibody conjugated with Affi-Gel 10, and each column was loaded with 3 ml of the extract of *Neurospora crassa* conidia in water (4 mg/ml) according to the following procedure: 1 ml of extract was adsorbed into each gel at a flow-rate of 0.5 ml/min and allowed to rest in the column for 10 min, then the gels were washed with a second portion of 1 ml of extract and after 10 min replaced with the remaining enzyme solution. The eluate from each gel was applied again to the column according to the same procedure.

The gels were then washed with 7 ml of 0.1 *M* phosphate buffer and eluted with the following solutions:

1st gel: 9 ml of 0.1 *M* citrate buffer (pH 2.2) containing 1 *M* NaCl;
2nd gel: 9 ml of 0.1 *M* phosphate buffer containing 0.5% Triton X-100;
3rd gel: 9 ml of 10% dioxane in water (pH 7.0);
4th gel: 9 ml of 10% dioxane in 20 m*M* HCl (pH 2.5);
5th gel: 9 ml of 50% ethylene glycol in 20 m*M* HCl (pH 2.5).

The entire process was carried out at 4°C.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Weber and Osborn [8] after concentration of the eluate from the immunoadsorbent gel by means of Centricon (Amicon, Danvers, MA, U.S.A.).

RESULTS AND DISCUSSION

Polyclonal antibodies obtained from active and denatured NADase cross-reacted with both types of enzyme and were mixed before their immobilization onto Affi-Gel 10 in order to recognize all the forms of the enzyme.

TABLE I

EFFECT OF VARIOUS SOLUTIONS ON THE CATALYTIC ACTIVITY OF NAD GLYCOHYDROLASE AFTER STORAGE FOR 1 h AT 4°C AND 25°C

The samples in citrate and in Triton X-100–phosphate buffer were assayed by HPLC and the other samples were tested by automatic titration.

Solution	Activity after storage at 4°C	Activity after storage at 25°C
Water	3.25 (100%)	2.5 (100%)
Citrate–NaCl (pH 2.2)	2.0 (61%)	2.75 (110%)
Triton–phosphate (pH 7.0)	3.9 (120%)	2.7 (108%)
Ethylene glycol (pH 2.2)	3.45 (106%)	2.45 (98%)
10% dioxane in water	3.1 (95%)	2.25 (90%)
10% dioxane (pH 2.2)	2.6 (80%)	1.85 (74%)

The total amount of polyclonal antibody obtained was 62.5 mg, as calculated spectrophotometrically by the extinction coefficient 1.4 for 1 mg protein/ml at 280 nm, whereas the amount of antibody bound to Affi-Gel 10 was 5.9 mg/ml of gel (92% yield).

The eluents for the immunoaffinity column were chosen from recommendations in the literature [9–11]. However, before the chromatography of the enzyme, the solutions chosen were tested in order to assess their effect on the NADase activity, after exposure for 1 h at 4 and 25°C. The behaviours of the five solutions described above at both temperatures tested were similar, whereas at alkaline pH (pH 10.5) (data not shown here) the enzyme was completely inactivated. The results are reported in Table I.

After elution of the gels with the solutions described above, NADase activity was assayed in the eluates and in the gels. Table II shows the recovery of the activity for a typical experiment.

The data in Table II show that no enzyme activity was found in the 0.1 *M* citrate–1 *M* NaCl eluate and virtually all NADase remained immobilized in the gel.

TABLE II

NADase ACTIVITY IN THE GELS DETERMINED BY AUTOMATIC TITRATION AFTER EXHAUSTIVE WASHING WITH DOUBLY DISTILLED WATER

The enzyme in the column washing and in the eluate was assayed by HPLC and then checked with the pH-Stat method after dialysis against water.

Eluent	Activity in the gel (units)	Activity in the washings	Activity in the eluate
Citrate–NaCl (pH 2.2)	6.62	0.3	0.0
Triton–phosphate (pH 7.0)	0.28	3.1	2.8
Ethylene glycol (pH 2.2)	1.93	3.5	0.75
10% dioxane in water	2.33	3.75	0.1
10% dioxane (pH 2.2)	2.59	3.82	0.05

TABLE III

NADase ACTIVITY DETERMINED BY AUTOMATIC TITRATION AFTER DIALYSIS OF THE SAMPLES AGAINST WATER

Run No.	NADase activity in the washings (%)	NADase activity in the eluate (%)
1	51	45
2	54	47
3	40	51
4	54	48
5	34	66
6	55	40
7	51	45
8	50	44

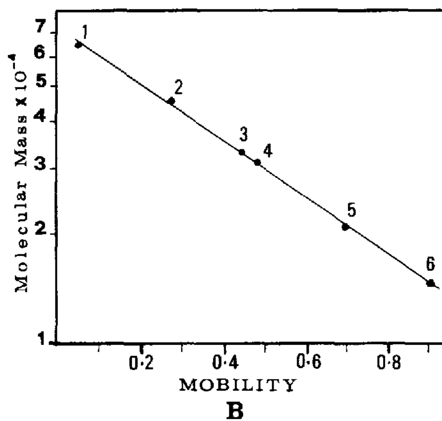


Fig. 1. (A) SDS-PAGE of the eluate with Triton X-100–phosphate buffer (pH 7.0). (B) Mobility of NADase (3) compared with (1) bovine serum albumin (M_r 66 200), (2) ovalbumin (M_r 45 000), (4) carbonic anhydrase (M_r 31 000), (5) soybean trypsin inhibitor (M_r 21 000) and (6) lysozyme (M_r 14 500).

Most of the NADase activity was similarly found in the gels eluted with 10% dioxane both in water and in 20 m*M* HCl and with ethylene glycol in 20 m*M* HCl, whereas their eluates contained only traces of activity. In contrast, the gel eluted with 0.5% Triton X-100 in 0.1 *M* phosphate buffer (pH 7.0) showed a minimum of NADase activity adsorbed, and most of the enzyme was found in the eluate and in the washings with 0.1 *M* phosphate buffer (pH 7.0).

The last column was repeatedly used for a total of eight runs and the results obtained were similar in each instance, as shown in Table III.

The purity of the samples eluted by Triton X-100–phosphate buffer was assessed by SDS-PAGE and by the spectrum of the eluate in the ultraviolet region.

SDS-PAGE showed only one band, corresponding to a protein of about 33 000 dalton (Fig. 1), as determined by comparison with standard proteins.

The UV spectrum showed the characteristic maximum of NADase at 234 nm [12], with a ratio A_{234}/A_{280} of 5.1, while the enzyme purified by affinity chromatography had a ratio of 5.6. The difference may be due to the presence of Triton X-100 in the eluate.

CONCLUSIONS

The purification method for NADase reported here seems to have some advantages over previous procedures. First, the immunoadsorbent can be used many times without a loss of efficiency, as showed in Table III. Moreover, a unique extraction with water of *Neurospora crassa* conidia is sufficient for loading the column, whereas affinity chromatography on 4-methyl-NAD–Sepharose needs at least two kinds of extraction (water and 0.17 *M* KCl) in order to prolong the life of the column [1]. The entire procedure causes no denaturation, in contrast to elution from the hydrophobic gel [3], because it is done under milder conditions. The capacity of the immunoadsorbent gel is not very high, as shown by the ratio of enzyme held by the column to enzyme washed with buffer, when only a few units were loaded onto 1.5 ml of gel (see Table II). On the other hand, the possibility of reusing the gel many times makes it suitable to the purification of larger amounts of NADase. The enzyme eluted with Triton X-100 in phosphate buffer (pH 7.5) is apparently homogeneous by SDS-PAGE and seems to be analogous to that purified by affinity chromatography.

ACKNOWLEDGEMENTS

This work was supported by the CNR Target Project on Biotechnology and Bioinstrumentation.

REFERENCES

1 F. Menegus and M. Pace, *Eur. J. Biochem.*, 113 (1981) 485.
2 M. Pace, D Agnellini, P. G. Pietta, A. Cocilovo and L. Bonizzi, *Prep. Biochem.*, 14 (1984) 349.
3 M. Pace, P. G. Pietta, D. Agnellini and P. L. Mauri, in O. M. Nejissel, R. R. van der Meer and K. Ch. A. M. Luyben (Editor), *Proceedings of the 4th European Congress of Biotechnology*, Vol. 2, Elsevier, Amsterdam, 1987, pp. 142–145.
4 P. G. Pietta, M. Pace and F. Menegus, *Anal. Biochem.*, 131 (1983) 533.
5 J. Horejsi and R. Smetana, *Acta Med. Scand.*, 155 (1956) 65.

6 S. Avrameas, *Immunochemistry*, 6 (1969) 43.
7 K. Ikura, K. Okumura, M. Yoshikawa, R. Sasaki and H. Chiba, *J. Appl. Biochem.*, 6 (1984) 222.
8 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
9 K. K. Andersson, Y. Benyamin, P. Dozou and C. Balny, *J. Immunol. Methods*, 25 (1979) 375.
10 H. D. Caldwell and C.-C. Kuo, *J. Immunol.*, 118 (1977) 437.
11 Y. Ikeda and M. Steiner, *J. Biol. Chem.*, 251 (1976) 6135.
12 M. Pace, D. Agnellini, P. G. Pietta, P. L. Mauri, F. Menegus and R. L. Berger, *Plant Physiol. (Life Sci. Adv.)*, 7 (1988) 115.