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# Effect of aluminium on the $NAD^+$ kinase activity of *Euglena gracilis* grown heterotrophically

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## Abstract

The effect of 2 mM $AlCl_3$ on $NAD^+$ kinase (E.C. 2.7.1.23) activity was studied using *Euglena gracilis* strain Z grown heterotrophically in darkness at pH 3.5 in the presence of lactate as sole carbon source. The Al-treatment slowed down the culture growth and suppressed the peak of $NAD^+$ kinase activity, which characterizes the beginning of the exponential phase of growth of the control cell cultures. There are two possible explanations of the Al effect: it *1*) either prevents the enzyme activation by the Ca-calmodulin (CaM) complex; or *2*) suppresses the CaM-dependent $NAD^+$ kinase form. In *Euglena* cells, a part of the $NAD^+$ kinase activity is enhanced by EGTA and lowered by $Ca^{2+}$: this peculiar $NAD^+$ kinase activity is unaffected by the Al treatment.

*Additional key words*: CaM-dependent $NAD^+$ kinase, cell cultures, EGTA and $Ca^{2+}$ effects.

## Introduction

Among the various aluminium species present in solution, micromolar $Al^{3+}$ concentrations can be toxic for plants (Kinraide and Parker 1989). When the soil pH decreases below 5.0, $Al^{3+}$ is released from insoluble oxides or from alumino-silicate complexes leading to an increase in $Al^{3+}$ concentration (Foy *et al.* 1978). In higher plants, the symptom of $Al^{3+}$ toxicity is an inhibition of cell elongation and root tip growth, resulting from multi-level disturbances (Aniol 1983, Haug 1984, Kochian 1995). Two types of $Al^{3+}$ resistance mechanisms have been described: *1*) external

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*Abbreviations*: CaM - calmodulin; CDC - cell division cycle; EGTA - ethylene glycol-bis(β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid; MTT - 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PVP - polyvinylpolypyrrolidone; TFP - trifluoperazine.

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resistance or exclusion mechanisms limiting $Al^{3+}$ entry into the cytosol (*e.g.* secretion of mucilage, exudation of chelator ligands, alkalization of the rhizosphere), and *2)* internal modifications, such as production of organic acids and/or acidic polypeptides playing the role of natural $Al^{3+}$-chelators, or synthesis of $Al^{3+}$-resistant isozymes (Taylor 1988, 1991, 1995). $Al^{3+}$ can induce the expression of specific genes (Snowden and Gardner 1993, Richards *et al.* 1994), and/or the synthesis of proteins (Ricon and Gonzales 1991, Basu *et al.* 1994). It also may bind to calmodulin and therefore induce conformational changes of this regulatory protein (Siegel and Haug 1983, Larkin 1987); this hypothesis is controversial (Richardt *et al.* 1985, Martin 1988, You and Nelson 1991).

$NAD^+$ kinase (EC 2.7.1.23), also called ATP:$NAD^+$2'-phosphotransferase, is an ubiquitous enzyme playing an essential role in the cellular metabolism of all organisms. $NAD^+$ kinase catalyses the only known biochemical reaction leading to the production of $NADP^+$ from ATP and $NAD^+$. The NADH (abundantly produced by glycolysis and citric acid pathways) and the NADPH (generated either by the oxidative branch of pentose phosphate pathway, by many dehydrogenases, or by the energy-linked nicotinamide nucleotide transhydrogenase (Enander and Rydström 1982) play different but complementary roles in the cell. While oxidation of NADH mainly promotes the ATP production in mitochondria, oxidation of NADPH supplies electrons and protons in most of the reduction steps necessary for the biosynthesis pathways. The $NAD^+$ kinase may therefore be a key enzyme controlling the relative importance of anabolic and catabolic pathways in the cell. In the case of $Al^{3+}$ stress, the $NAD^+$ kinase is either a direct target, because of its key role when cells start new synthesis requiring NADPH as a cofactor (and therefore primarily $NADP^+$), or an indirect target, since $NAD^+$ kinase activity uses Mg-$ATP^{2-}$ as substrate and that its activity can also be partly CaM-dependent like in plants (Muto and Miyachi 1977, Anderson *et al.* 1980, Allan and Trewavas 1985). In root tips of two wheat genotypes characterized by different degrees of tolerance to Al stress, an increase in activity due to a *de novo* synthesis of $NAD^+$ kinase has been demonstrated when Al stress was applied, which was stronger and faster in the Al-resistant than in the Al-sensitive wheat line, and which mainly implied the CaM-independent form of $NAD^+$ kinase (Slaski 1989, 1990).

*Euglena gracilis,* wild strain Z, is a fresh water protozoa which displays an unusual resistance to agents generally deleterious for ecosystems. In this study designed to test the effect of aluminium on the total $NAD^+$ kinase activity and on the relative contributions of the $Ca^{2+}$-CaM dependent and independent isoforms, this organism seemed to be an appropriate model because it can grow and divide in acidic media (Calvayrac and Douce 1970), in which the provided aluminium is under its most toxic ionic form $Al^{3+}$ (Garcidueñas Piña and Cervantes 1996).

## Materials and methods

**Cell cultures**: *Euglena gracilis* (strain Z) cells were grown in darkness at 22 °C. The culture medium (1.5 $dm^3$ placed in 5 $dm^3$ bottles for sufficient aeration) contained 33 mM lactate as the unique carbon source (Calvayrac and Douce 1970) and vitamins

B1 and B12 (pH initially 3.5). The $Al^{3+}$ concentration was 0 mM (control) and 1.52 mM (supplied as 2 mM $AlCl_3$ at the time of cell inoculation). The cells were inoculated from a control preculture grown for 7 d. From day 3 to day 8, then at day 10 or 11, 160 $cm^3$ samples of culture medium were collected; cells were pelleted by centrifugation (10 min at 10 000 *g*) and stored at -20 °C. The cell titration was estimated by three countings using a *Malassez* cell (cells immobilized in 9 % KI). The data represent the means of four independent experiments.

**Preparation of cell extracts for $NAD^+$ kinase assays:** The pelleted cells were sonicated for 4 intervals of 20 s separated by 3 min for cooling, after addition of 0.2 $cm^3$ of cold 62 mM Tris-HCl buffer (pH 7.5). The crude extracts obtained were adjusted to 1 $cm^3$ with the same buffer, then centrifuged (45 min, 39 000 *g* at 4 °C), and the supernatants kept on ice. In the text, the $NAD^+$ kinase present in the crude extracts prepared from cells grown with Al or without Al are referred, respectively, as Al-$NAD^+$ kinase, or control-$NAD^+$ kinase.

**$NAD^+$ kinase activity:** Based on previous studies (Goto 1984, Laval-Martin *et al.* 1990a,b, Slaski 1990), the final concentrations of the incubation mixture for standard assays were 50 mM Bicine-NaOH (pH 7.5), 3 mM $MgCl_2$, and 2.5 mM $NAD^+$, 2.5 mM ATP. Assays were carried out in a total volume of 0.25 $cm^3$, four test tubes were prepared identically and placed in a dry incubator at 30 °C. The reaction - which had been checked to be linear for at least 30 min - was started by the addition of 0.025 $cm^3$ of *Euglena* extract. One of the tubes was heated at 100 °C for 3 min at time zero of the reaction, thus providing the (initial) amount of endogenous $NADP^+$; the three other tubes were incubated at 30 °C for 15 min, and then the reactions were stopped by heating. The reaction media, further clarified by centrifugation, were stored at -20 °C until $NADP^+$ measurement.

The amount of $NADP^+$ formed during the $NAD^+$ kinase assay was determined using glucose 6-phosphate dehydrogenase as auxiliary enzyme. This method, using a colorimeter reader of microplates, *Ceres U.V. 900* (*Bio-Tek Instruments*, Winoosky, USA), allows the detection of pmole quantities of $NADP^+$ (Goto 1984).

Crude extracts, containing both endogenous $Ca^{2+}$ and calmodulin, were analysed in standard conditions and the corresponding activity is referred to as 100 %. Responses of the enzyme(s) to some effectors were then tested on the same extracts after addition of: *1*) 1 mM $Ca^{2+}$, to ensure that the totality of the endogenous CaM be complexed; *2*) 0.1 mM TFP, in order to inhibit CaM, or *3*) 2.5 mM EGTA, used as a specific chelator of the endogenous $Ca^{2+}$. Complementary assays combined either $Ca^{2+}$ plus TFP, or EGTA plus TFP.

**Protein, aluminium and phosphate concentrations:** Protein concentrations were assayed by the method of Bradford (1976) with bovine serum albumin as standard.

Culture samples were collected every day, and centrifuged (15 min at 20 000 *g*) to separate soluble ionic Al from insoluble Al (Koyoma *et al.* 1988) and cells. Measurements of soluble Al (by a colorimetric method, *Merck Spectroquant 14825*) of soluble inorganic phosphate (Ames 1966), and of pH were then performed.

## Results and discussion

**Effect of aluminium on cell growth:** Whereas the control cultures reached the stationary phase at day 6, the growth curve was hardly reaching the plateau value at day 11, when Al was present (Fig. 1). This effect of Al on the generation time could

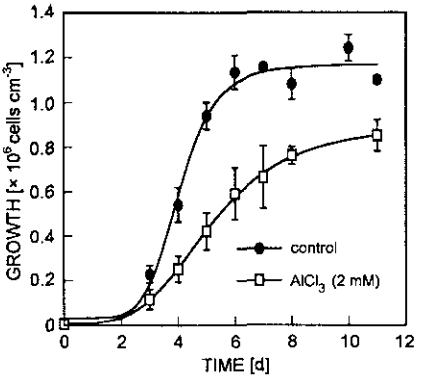


Fig. 1. Growth curves of *Euglena gracilis* (strain Z) grown heterotrophically in darkness at 22 °C, in a lactate medium with or without 2 mM $AlCl_3$. Means of four independent experiments ± SE.

be indirect, because Al could affect the pH and also the inorganic phosphate concentration in the medium. In control conditions, the pH value and inorganic phosphate concentration remained constant for 3 d; then, at day 4 of the culture, the phosphate concentration decreased to 6 mM, while the pH increased to a value of 6.5 - 7.0. The addition of 2 mM $AlCl_3$ (1.52 mM of soluble Al) in the medium, lowered the initial pH from 3.5 to 3.3, without any change in inorganic phosphate availability (7 mM); then, the Al concentration decreased from 1.52 mM at day 3 to 0.61 mM at day 4, while the phosphate concentration and pH remained constant. Such a diminution of the soluble Al is probably more due to binding with the mucopolysaccharide coat produced by the cells (Slaski 1990), than to an improbable massive intralysosomal accumulation of such a toxic ion. Finally, from days 6 to 11, the pH slowly increased to 5.5, while the inorganic phosphate concentration dropped to 4 mM and soluble Al totally disappeared, due to a precipitation of these two ions occurring at this pH (Table 1). These results indicate that, whatever the culture condition, the generation times were neither correlated to the pH changes, nor to the inorganic phosphate availability; the decrease in growth rates was, therefore, interpreted as the direct consequence of the presence of soluble Al in the medium and not as an indirect effect of Al on the pH of that medium. As a matter of fact, it was shown that the ammonium salts present in the medium are buffering the lactate medium to low pH, and the increase in pH is the direct consequence of the lactate consumption (Calvayrac 1972). In one hand, although the addition of 2 mM $AlCl_3$ to the medium induced a slight lowering of the pH from 3.5 to 3.3, this may not have any significant effect on *Euglena* growth since it has been shown that *Euglena* was able to grow heterotrophically in acidic media until pH 2 (Kempner 1982). In other

hand, soluble inorganic phosphate concentrations was not affected during the first 5 d when pH remained low and Al under its most toxic form (Garcidueñas Piña and Cervantes 1996). Thus, the decrease in growth rate and cell titer was a direct consequence of the presence of Al.

Table 1. Effect of 2 mM $AlCl_3$ on pH, soluble aluminium, inorganic phosphate, and cell generation time during culture growth (n.d. - not detected).

| Treatment | Control | | | $AlCl_3$ | | |
|---|---|---|---|---|---|---|
| Cultivation [d] | 0 - 3 | 3 - 5 | 5 - 11 | 0 - 3 | 3 - 5 | 5 - 11 |
| pH | 3.5 | 6.5 | 7 | 3.3 | 3.3 | 5.5 |
| Pi [mM] | 7 | 6 | 6 | 7 | 7 | 4 |
| Al [mM] | n.d. | n.d. | n.d. | 1.52 | 0.61 | n.d. |
| Generation time [h] | 14.4 | 17.0 | - | 18.0 | 20.0 | - |

The presence of Al during the cell growth also altered the cell titrations of the cultures reaching the stationary growth phase (Fig. 1): 1 - 1.2 × $10^6$ cells per $cm^3$ in control conditions, against about 0.8 - 0.9 × $10^6$ cells per $cm^3$ in the Al-treated cultures. Since the rate of cell division, the duration of exponential and transition phases, and the cell titration attained in the stationary phase, are all indicators of the integrated cell potential, the data are presented as a function of the cell titer rather than of the culture duration.

**Effect of aluminium on NAD$^+$ kinase activity and protein content:** NAD$^+$ kinase activity and protein concentration were measured during cell culture growth. In the control medium, and in the first half of the exponential phase of culture growth (Fig. 2), the NAD$^+$ kinase activity peaked to values 3 to 4 times that of the initial value for inoculated cells, and then decreased to a minimum at the end of the exponential phase (1 million cells per $cm^3$). When cells stopped to divide, a second increase of NAD$^+$ kinase activity led to activity similar to that of the inoculated cells (originating from a 7-d-old preculture). A curve of roughly comparable shape was observed for the evolution of the soluble protein content from the begining of the culture until the stationary phase, after which the protein content remained nearly constant (Fig. 2).

For the cells grown with Al (Fig. 2), in spite of the dispersed experimental points, an initial decrease of NAD$^+$ kinase activity occurred at the beginning of the exponential phase of culture growth. The activity then increased slightly to values characteristic of those of the inoculated cells. The content of soluble proteins progressed in a similar way (Fig. 2).

The variation in NAD$^+$ kinase activity during the growth of control cells (Fig. 2) allows to distinguish three different classes of cells: class I, from 0.2 to 0.4 million cells per $cm^3$- corresponding to the first peak of activity; class II, from 0.6 to 1.1 million cells per $cm^3$ - corresponding to the minimum of activity; and class III, 1.2 million cells per $cm^3$ - corresponding to the second peak of activity which

occurred during the stationary phase. For each class, when the total $NAD^+$ kinase activity is considered (expression per $cm^3$ of culture medium instead of per million cells), interestingly, no significant difference can be observed between classes I and II from the control, although the number of the cells in class II was nearly 3 fold greater than in class I (Table 2). These results could possibly indicate that, after an activation or a *de novo* synthesis of $NAD^+$ kinase which occurred at the beginning of the exponential phase of growth (Fig. 2), no more activation or synthesis took place during the remaining exponential phase and the total activity seemed to be split between cells, leading to a low level of activity per cell at the end of the exponential phase of growth. When cells reached the stationary phase, the activity per cell increased which reinforced the total activity.

Table 2. Specific [nmol $mg^{-1}$(protein) $s^{-1}$] and total [nmol $cm^{-3}$(medium) $h^{-1}$] $NAD^+$ kinase activities for the three classes of cells, distinguished on the basis of cell titer [$10^6$ cells $cm^{-3}$]. Means ± SE ($n$ = 3 to 11); means followed by different letters are significantly different ($P$ = 0.05), according to Mann-Whitney's test.

| Cell titer | | 0.2 to 0.6 | 0.6 to 1.1 | 1.2 |
|---|---|---|---|---|
| Specific activity | control | 23.8 ± 5.3 a | 20.2 ± 1.3 a | 42.4 ± 2.7 b |
| | Al-treated | 17.0 ± 4.6 a | 22.2 ± 3.8 a | - |
| Total activity | control | 0.92 ± 0.05 b | 1.05 ± 0.09 b | 3.66 ± 0.32 c |
| | Al-treated | 0.14 ± 0.04 a | 0.83 ± 0.19 b | - |

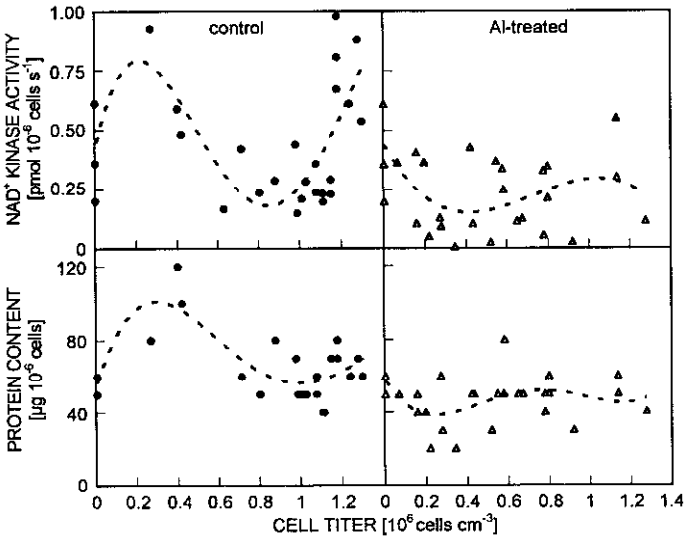


Fig. 2. Variation of the $NAD^+$ kinase activity and the soluble protein contents in control and Al-treated cells as a function of the cell titer. The data (expressed per $10^6$ cells) were plotted using a polynomial regression programme.

The total NAD$^+$ kinase activity in class I of Al-treated cells was 4-5 fold lower than that of control cells (Table 2). In class II, when the soluble Al concentration decreased in the medium, the activity corresponded to nearly 80 % of that of control. Thus, when the concentration in soluble Al decreases in the medium, the level of NAD$^+$ kinase activity seems to progressively increase to reach the level characteristic of control cells. Because no Al-treated cultures never reached cell titers over 1 million cells per cm$^3$, and because $Al^{3+}$ was no more detected in the medium after day 8; for class III, the results could not be analysed as resulting from an effect of $Al^{3+}$on NAD$^+$ kinase.

**Responses of NAD$^+$ kinase activity to different effectors:** For each cell class, three independent experiments were performed to test the effects of $Ca^{2+}$, TFP (calmodulin inhibitor) or EGTA added in the incubation mixture of the enzyme assay. Since Al slowed down growth and cell titrations never exceeded $10^6$ cells per cm$^3$, the comparative analysis only covers the first two cell classes. In both control and Al-treated culture, 2.5 mM EGTA enhanced the NAD$^+$ kinase activity (150 % in average). This result suggests that NAD$^+$ kinase may be inhibited by low $Ca^{2+}$ concentration (or other divalent cation), which is not detectable in the standard conditions of enzyme assay because of endogenous $Ca^{2+}$. This hypothesis is supported by the slight decreases (10 %) of NAD$^+$ kinase activities from both control- and Al-treated cells in the presence of 1 mM $Ca^{2+}$ (Fig. 3).

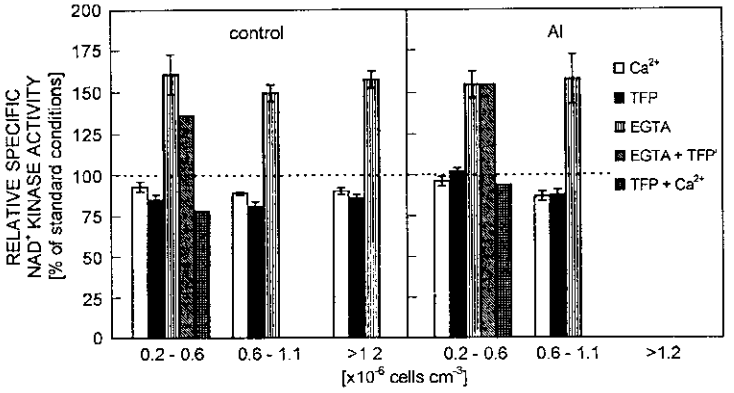


Fig. 3. Relative NAD$^+$ kinase activities in the presence of effectors (1 mM $Ca^{2+}$; 0.1 mM TFP; 2.5 mM EGTA, EGTA plus TFP or TFP plus $Ca^{2+}$). Tests were performed on extracts of the first class of cells (0 - 0.5 × $10^6$ cells cm$^{-3}$). The standard assay, described in Materials and methods, was considered as 100 % (dotted line).

TFP depressed (by 15 - 20 %) NAD$^+$ kinase activity from control cells, whatever the class considered. Therefore the enzyme activity could be partially dependent on CaM. In contrast, during the early exponential phase of growth in the presence of Al (class I cells), TFP did not modify the NAD$^+$ kinase activity. Thus, the Al treatment

either suppresses or inactivates a CaM-dependent $NAD^+$ kinase activity. Later on, in Al-class II cells, the contribution of the CaM dependent $NAD^+$ kinase activity to the total enzyme activity became comparable to that in control cells.

By comparison to the effect of TFP or EGTA alone, the effects of combined effectors (EGTA + TFP and TFP + $Ca^{2+}$), here presented only for the first class of cells, were additive (Fig. 3). Thus, $Ca^{2+}$ and TFP could act on two different forms of $NAD^+$ kinase: the CaM independent form which is possibly inhibited by an excess of free $Ca^{2+}$, and the CaM dependent form which is strongly or even totally inhibited by TFP (Allan and Trewavas 1985).

## Discussion

Previous studies have shown that when $AlCl_3$ was added to the culture medium of *Euglena*, in a concentration range 0 - 5 mM, the concentration of 2 mM (equivalent to 1.52 mM of soluble Al when colorimetrically measured) corresponded to the maximal concentration of Al for which cells were still able to undergo the cell division cycle. Therefore this concentration was used for stress experiments.

The culture growth of control cells was characterized by two peaks of $NAD^+$ kinase cellular activity, the first one at the beginning of the exponential phase and the other during the stationary phase. About 15 to 20 % of $NAD^+$ kinase activity could be CaM-dependent, since it was inhibited by TFP. The other part could be negatively regulated by $Ca^{2+}$, since it was stimulated by EGTA and inhibited by $Ca^{2+}$. When compared to control, $NAD^+$ kinase activity per cell remained very low when 2 mM $AlCl_3$ was added to the culture medium, the effect of which being to prevent lactate consumption (Calvayrac 1972). The cells, slowly growing in the presence of Al, seemed - at least for class I cells - to be devoid of CaM mediated $NAD^+$ kinase, since no inhibition was shown in the presence of TFP. Al could then supress the part of activity which is CaM dependent; nevertheless, this absence could not entirely explain the low activity of $NAD^+$ kinase observed in class I of Al- treated cells (Table 2), which suggests that Al may also act on CaM-independent activity.

In the presence of Al, the growth rate and the final cell titer were impaired. Thus, low levels of $NAD^+$ kinase activity and a slowing down of the divisions of *Euglena* cells are positively correlated. Cell growth and initiation of division have already been observed together with high concentrations of NADPH (Epel 1964, Whitaker and Steinhardt 1981) and high levels of $NAD^+$ kinase activity (Epel *et al.* 1981, Caldini *et al.* 1992).

In wheat root tips, an increase in $NAD^+$ kinase activity was observed (maximal effect for 1.5 mM Al), which was more rapid and higher in the resistant cultivar than in the sensitive one. This enhancement was mainly due to the *de novo* synthesis of the CaM-independent $NAD^+$ kinase isoform (Slaski 1989). The author concluded that the NADPH pool present before the stress could not support the adaptive biosynthesis involved in mechanisms of Al-tolerance and that an increase in $NAD^+$ kinase activity would therefore be necessary. In contrast, a decrease of the $NAD^+$ kinase activity was observed in root tips of both Al-tolerant and sensitive legumes (Slaski 1990).

Al-stress seems to induce in *Euglena* cells a decrease of $NAD^+$ kinase activity, like in monocotyledons. From macroscopic observation the Al-treated cells were always "stickier" than the control cells, as if they were producing a kind of mucilage. In any case, the low level of $NAD^+$ kinase activity observed in Al-stressed *Euglena* cells seems to be sufficient to sustain growth and division.

The results showing that a part of $NAD^+$ kinase activity in *Euglena* is stimulated by EGTA agree with a previous study (Pou de Crescenzo *et al.* 1997). Experiments are now in progress to precise the EGTA and $Ca^{2+}$ effects on crude extracts and to check if such effects still occur with purified $NAD^+$ kinase. As a matter of fact, the activities of $NAD^+$ kinase isoforms can be revealed in polyacrylamide gels after electrophoresis in native conditions (Apps 1975, Filippovich *et al.* 1990, Jalouzot *et al.* 1994, Morère-Le Paven *et al.* 1998).

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