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Purification and some kinetic parameters of NAD glycohydrolase from *Penicillium brevicompactum* NRC 829

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ABSTRACT

The current work reports the purification and characterization of some kinetic properties of nicotinamide adenine dinucleotide glycohydrolase (NADase) from Penicillium brevicompactum NRC 829. The enzyme was purified about 286 fold with a specific activity of 400 U/mg protein through a 3-step purification procedure. The purified NADase was shown to have a relative low molecular mass of 48 kDa by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). Nicotinamide and adenosine diphosphoribose (ADP-ribose) were the sole products formed when the purified NADase was incubated with NAD. This result was confirmed by Thin Layer Chromatography (TLC) analysis. The enzyme exhibited a broad pH profile with optimum pH for hydrolysis at 6.0. In addition to NAD and NADP, a number of NAD analogs were shown to serve as substrates for that enzyme. The Kinetic studies using NAD as a substrate followed Michaelis-menten type with 5.1 x 10^{-4} M of K_m indicates the high substrate affinity of NADase. Product inhibition studies demonstrated nicotinamide to be a noncompetitive inhibitor which has a K_i of 2 x 10^{-2} M, while ADP-ribose was found to be a competitive inhibitor with a K_i of 3.6 x 10^{-2} M.

Key words: NAD degradation, NAD^+ glycohydrolase, *Penicillium brevicompactum* NRC 829, purification, characterization.

INTRODUCTION

Nicotinic acid adenine dinucleotide (NAD^+) is a molecule that has central roles in cellular metabolic process and energy production. NAD^+ acts as a coenzyme in numerous redox reactions in cells. The abundance of the NAD^+ pools inside the cells is dependent upon the enzymes that catalyze the synthesis of NAD^+ and site inside the cells. Furthermore, the abundance of NAD^+ is also controlled by the enzymes that break down it. There are several classes of enzymes that cleave NAD^+ to create nicotinamide and ADP-ribosyl product [1]. NAD^+ glycohydrolases (NADase; E.C 3.2.2.5), belong to the class of mono (ADP-ribose) transferase, are distinguished from the other classes by their ability to use water rather than simple amino acid as the acceptor of ADP-ribose and resulting in free ADP-ribose [2]. NADase catalyze the hydrolysis of the nicotinamide–ribose bond of NAD^+ to yield nicotinamide (also known as Vitamin B3) and adenosine diphosphoribose (ADP-ribose). The reaction equation is as follow:

$$\mathbf{NAD^+ + H_2O \xrightarrow{NADase} Nicotinamide + ADP\text{-}ribose + H^+}$$

The function of nicotinamide remains uncertain, while ADP-ribose works as a substrate for ADP-ribosylation. Mono ADP-ribosylation of various proteins has been documented to result in significant modifications in function such as inactivation of the protein [3]. In this concern Honjo et al. [4] reported that in the existence of diphtheria toxin,

ADP-ribose from NAD^+ was transferred to aminoacyl transferase II led to the inactivation of the enzyme. While, poly ADP-ribosylation is involved in several fundamental processes targeted at maintenance of the functional integrity of the genome [5]. NAD^+ glycohydrolases have been demonstrated in different types of organisms, ranging from microorganisms (including bacteria, yeast and fungi) to mammals [6-8]. The properties of NADase vary widely among species with respect to molecular weight, subunit composition, specific activity, K_m value and transglycosidase activity [9]. Microbial NADases are different from mammalian NADases in such a way that the mammalian enzymes are non-soluble membrane bound enzymes associated with microsomal particles and can only be purified after solubilization by treatment with enzymes [10], while microbial NADases are soluble and can be isolated from the culture supernatant [11-12]. Besides, the mammalian NADases are more sensitive to inhibition by nicotinamide than are the microbial enzymes. However, the most striking contrast between microbial and mammalian NADases is the high thermal stability of some of the microbial enzymes. For example, the NADase of *Mycobacterium tuberculosis* retains 70% of its activity after 1 min at 100°C [13] while that of *Mycobacterium butyricum* retains 60% after 20 min at 100°C [14]. On the other hand, the NADases of *Streptococcus* sp. [15] and *Neurospora crassa* [16] are not heat-stable. Therefore, the developing importance of the participation of NAD in nonoxidation-reduction reactions such as ADP-ribosylation as well as the synthesis of poly ADP-ribose has promoted a renewed interest in the properties of NAD glycohydrolases (NADases), especially with respect to the catalysis of transglycosidation reactions. In the present study, the NADase from a potent filamentous fungus *Penicillium brevicompactum* NRC 829 was purified 286-fold to electrophoretic homogeneity as well as properties of the enzyme were characterized. To the best of our knowledge, this is the first report on the purification of NADase from *P. brevicompactum* NRC 829.

EXPERIMENTAL SECTION

Chemicals

Adenosine, inosine, cytidine, AMP, GMP, NAD, CMP and UMP were purchased from Sigma Chemical Company. Nicotinamide and nicotinic acid were purchased from Merck. DEAE-Sephadex A-25 and Sephadex G-100 were purchased from Pharmacia Fine Chemical. All other reagents were prepared in Microbial Chemistry Dept., National Research Centre.

Microorganism

Penicillium brevicompactum NRC 829 was obtained from culture collection of Microbial Chemistry Dept., National Research Centre, Egypt.

Maintenance and growth of *Penicillium brevicompactum* NRC 829

The culture was maintained by sub-culture every 4 weeks on slopes of solid modified Czapek Dox's medium containing g/L: glucose, 30; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; KCl, 0.5 and agar, 20. Seven days old slants were used for the preparation of the inoculum; conidia of the fungus were scraped using an inoculation needle under aseptic conditions and 5.0 ml of sterile distilled water was added to each slant. Two ml aliquots (v/v) of inoculum size (2.1×10^6 spores/ml) was adopted to inoculate 250 ml Erlenmeyer flask containing 50 ml of liquid potato dextrose medium adjusted at pH 5.5 before autoclaving at 121°C (15 min). The inoculated flasks were incubated for 4 days at 28°C in a static condition.

Preparation of cell-free extracts

At the end of incubation time, the 4 days old mats were harvested by filtration, washed thoroughly with distilled water and finally blotted to dry with absorbent paper. The mats were ground with cold washed sterilized sand in a chilled mortar and extracted with cold distilled water. The slurry obtained was centrifuged at 6000 rpm for 10 min and the clear supernatant obtained was used as a source of crude enzyme preparation.

Enzyme assay

NAD^+ glycohydrolase was assayed by a modified method described by Yuan and Anderson [17]. The assay mixture contained, in a volume of 3.0 ml: 1 mM β-NAD, 50 mM Tris-acetate buffer (pH 6.0) and NADase. After incubation for 30 min at 50ºC, the reaction was terminated with 5 µL of 2 M HCl and finally centrifuged at 10,000 rpm for 10 min. The initial velocity was measured by transferring 0.2 ml aliquots from the clear supernatant obtained to a test tube containing 2.8 ml of 1 M potassium cyanide. The absorbance was measured at 327 nm against a blank (0.2 ml of 50 mM Tris-acetate buffer (pH 6.0) and 2.8 ml of 1 M potassium cyanide). The unit of enzyme activity referred to is the international unit (1 µmol of substrate transformed/min).

All results are expressed as the mean of three independent experiments.

Protein determination

Protein concentration was determined according to Lowry et al. [18], using bovine serum albumin (BSA) as a standard. The protein content of the purified enzyme fractions was determined by the UV absorbance according to the method of Schleif and Wensink [19].

Purification of NADase from *P. brevicompactum* NRC 829

All purification steps were performed at room temperature unless otherwise stated.

Acetone fractionation

Fractional precipitation with organic solvent was done according to the procedure of Kaufman [20]. The cold acetone at -20°C was added to the cell free extract until the required concentration was reached (0-30%) with continuous stirring for 20 min at 4°C. The observed precipitate was collected by cooling centrifugation at 12.000 rpm for 15 min and dissolved in a minimal volume of 20 mM Tris-acetate buffer (pH 6.0), then dialyzed against the same buffer for 3.0 h at 5°C. Further cold acetone was added to the supernatant fluid and the process repeated until the final concentration reached (30-60 and 60-90%).

DEAE-Sephadex A-25 columnn chromatography

The partially purified NADase was loaded onto a DEAE-Sephadex A-25 columnn (1.0 × 45 cm) pre-equilibrated with 50 mM Tris-acetate (pH 6.0). A linear gradient of sodium chloride solutions (0.0-0.5 M) in 100 mM Tris-acetate buffer (pH 6.0) was used for elution at a flow rate of 3 ml/10 min. Fractions containing NADase activity were pooled, dialyzed against the same buffer and concentrated by lyophilization (Fraction 2).

Sephadex G-100 gel filtration

A column was packed with Sephadex G-100 column (2.0 × 100 cm) and equilibrated with 50 mM Tris-acetate buffer, pH 6.0. Fraction 2 was applied to the column and eluted with the same buffer at a flow rate of 6.0 ml/10 min. Fractions of 5 ml were collected and examined for NADase activity and protein content. Fractions containing NADase activity were pooled, concentrated and stored at -20ºC for further studies.

Molecular mass determination

For molecular mass determination and enzyme purity detection, the purified enzyme preparations and molecular mass markers were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [21]. The following proteins were used as molecular mass standards (Fermentas, spectra ^{TM} broad range protein ladder): 250, 150, 100, 70, 50, 40, 30, 20 and 10 KDa.

Thin layer chromatography (TLC) analysis

Aliquots of the enzymatic reaction products and authentic samples were chromatographed on TLC Silica gel 60254 (Aluminum sheet; 20 × 20 cm) [22-23], developed in a mixture of: *n*-butanol: acetone: acetic acid (glacial): ammonia (5%): water (45:15:10:10:20) [24] and finally visualized under UV at 254 nm. The retention values (R_f) of the authentic samples and the products of the reaction mixtures were then calculated according to the following equation:

$$R_f = \frac{\text{Distance from baseline travelled by sample}}{\text{Distance from baseline travelled by solvent}}$$

Effect of pH on the purified NADase activity

The activity of the purified NADase was evaluated at different pH values. The enzyme was incubated using 50 mM of four buffers system ranging from pH 1.0 to pH 10, under the standard assay conditions. Buffers used were KCl-HCl (pH 1.0 - 2.0); Tris-acetate (pH 3.0 - 6.0); Tris-HCl (pH 6.0 - 9.0) and Carbonate - bicarbonate (pH 9.0 -10.0).

Temperature dependence of NADase activity

Optimum temperature for enzyme activity was determined by performing the standard enzyme assays at temperatures ranging from 20 - 80ºC. The enzyme activity was determined under the standard assay conditions.

Determination of K_m and V_{max} of NADase activity

Rate of reaction of enzyme activity (μmol of NAD^+ consumed/min) was determined using various concentrations of β-NAD^+ (1.0 - 20 μM) in 50 mM Tris-acetate buffer (pH 6.0). Kinetic parameters K_m and V_{max} were calculated by linear regression from Lineweaver-Burk plot [25].

Product inhibition of NADase activity

In the present research, product inhibition by nicotinamide and ADP-ribose was studied to determine the minimum kinetic mechanism for the hydrolysis of NAD. Inhibition constants for each product were determined at a constant inhibitor concentration while varying substrate concentration. Two different inhibitor concentrations were used in each experiment. Kinetic parameter for each inhibitor concentration was plotted according to Lineweaver - Burk plot.

Substrate specificity of the purified NADase

The substrate specificity of the purified enzyme was determined by replacing NAD in the assay mixture with an equimolar amount of the representative phosphorylated, aminated riboside compounds. The enzyme activity obtained with NAD was used as the control (100%).

RESULTS AND DISCUSSION

Purification of NAD^+ glycohydrolase

NADase was purified from *P. brevicompactum* NRC 829 as described in Materials and methods section and the sequential multi-steps purification procedure was summarized in Table 1. The crude enzyme was precipitated at 30 - 60% acetone saturation with a recovery yield of 83% and 1.8 purification fold. The partial purified enzyme from the above step was loaded on DEAE-Sephadex A-25 column chromatography and a typical elution profile is shown in Fig. 1, which revealed that the enzyme was purified to 120 fold with a yield of 51% and specific activity of 166.6 U/mg protein. Fractions containing NADase activity were pooled and concentrated by lyophilization (Fraction 2).

The elution profile of Fraction 2 loaded on Sephadex G-100 column is illustrated in Fig. 2. A sharp distinctive peak of NADase activity with an increase in the specific activity was noticed. This increase corresponds to about 286-fold purification and 28% recovery yield. The most active fractions with specific activity of 400 U/mg protein were pooled together, concentrated with lyophilizer and stored at -20ºC for further studies.

Table 1. Purification of NAD^+ glycohydrolase from *P. brevicompactum* NRC 829

Purification step	Total activity (U)	Total protein (mg)	Sp. activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	289.0	200	1.41	100.0	1.0
Acetone fractionation	240.8	90	2.6	83	1.8
DEAE-Sephadex A-25	150.2	0.9	166.6	51	120
Sephadex G-100	80	0.2	400	28	286

Table 2. Substrate specificity of NADase purified from *P. brevicompactum* NRC 829

Substrate	Product (μM)
NAD	0.16
Nicotinamide Riboside	0.18
Thymidine	0.17
Uridine	0.17
Cytidine	0.15
CMP	0.14
UMP	0.15
AMP	0.00
ADP	0.00
Adenosine	0.00

Molecular mass determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of *P. brevicompactum* NRC 829 NADase revealed a single distinctive protein band with an apparent molecular weight of 48 kDa (Fig. 3), which clearly indicates that the enzyme is monomer. Likewise, this result has been reported earlier by Menegus and Pace [12] for NADase isolated from 'zinc-deficient' mycelium of *N. crassa*. While Yost and Anderson [26] reported that the molecular mass of *Bungarus fasciatus* NADase is 130 kDa.

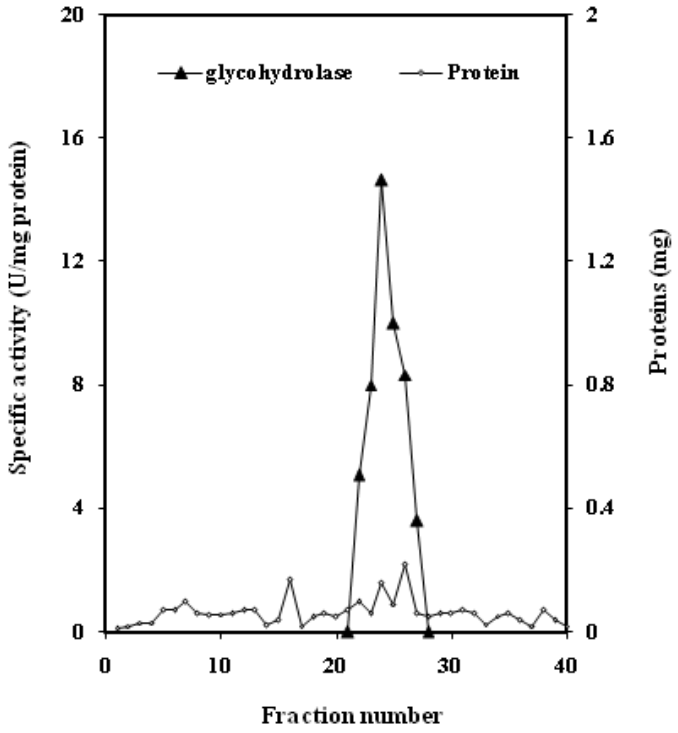

Fig. 1 Elution diagram of NAD^+ glycohydrolase from DEAE Sephadex A-25

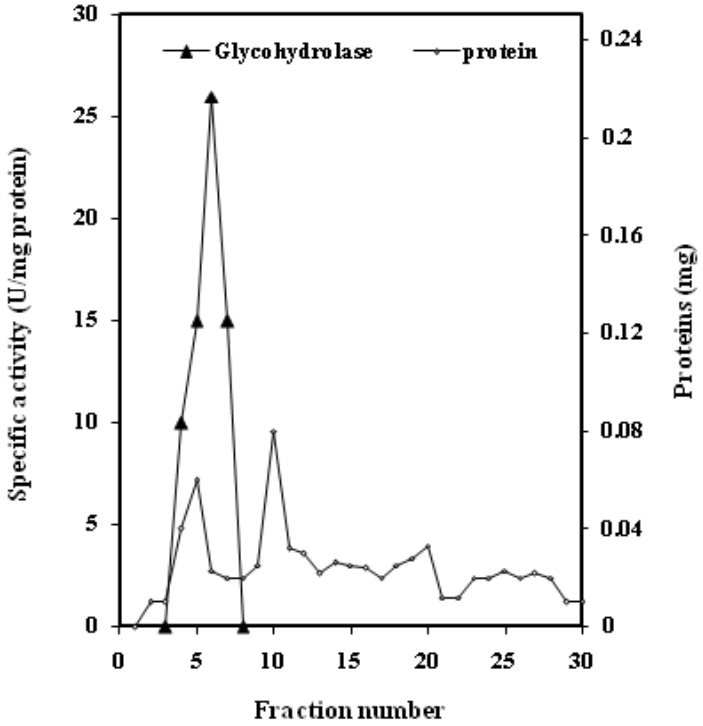

Fig. 2 Elution diagram of NAD^+ glycohydrolase by Sephadex G-100

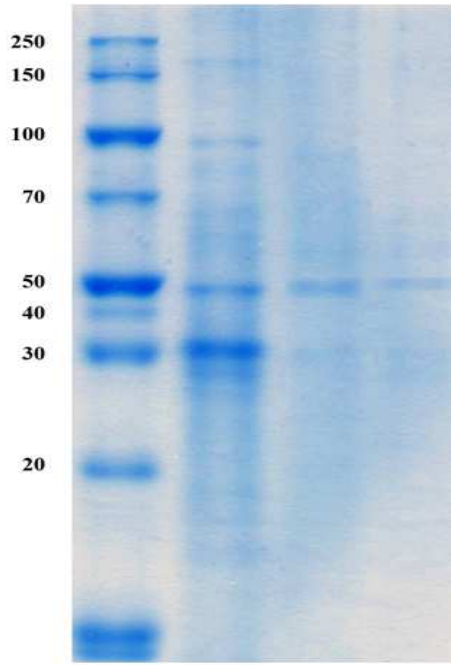

Fig. 3 Electrophoretic analysis of *Penicillium brevicompactum* NRC 829 NADase. Separation was performed on a 12% (w/v) SDS Polyacrylamide gel and stained with Coomassie brilliant blue. From left to right: *lane 1*, molecular mass markers; *lane 2*, fractional precipitation by chilled acetone; *lane 3*, Partial purified NADase on DEAE-Sephadex A-25, *lane 4*, purified NADase on Sephadex G-100

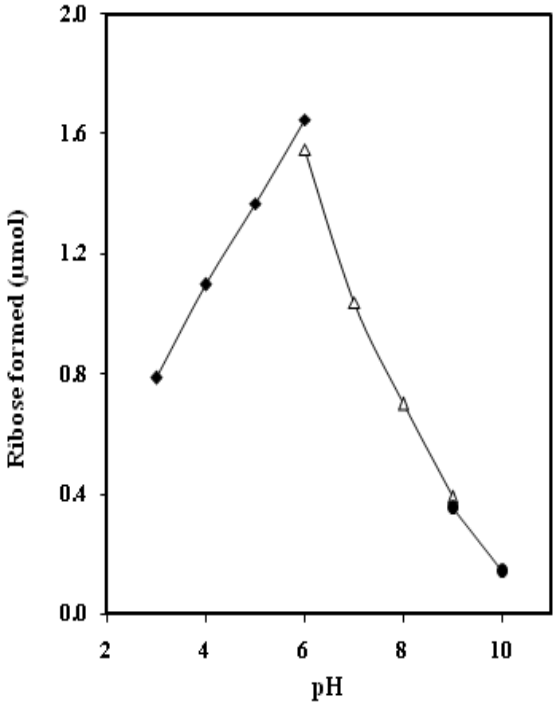

Fig. 4 pH dependance of the purified NAD^+ glycohydrolase activity

Thin layer chromatography analysis

TLC clearly indicated that NAD^+ was partially consumed. In addition no accumulation of either ADP or AMP or NR was detected. Instead, the presence of nicotinamide and ADP-ribose were as evident for those lanes (data not shown).

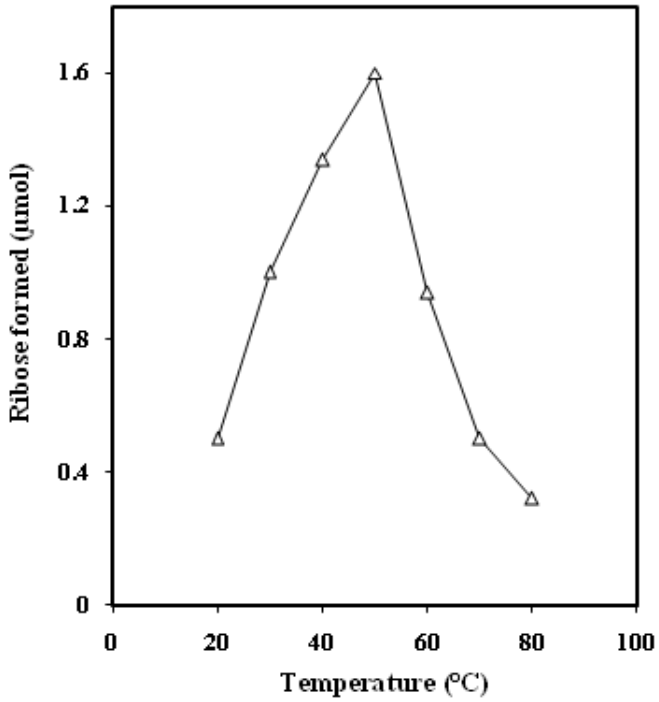

Fig. 5 Effect of temperature on purified NAD$^+$ glycohydrolase

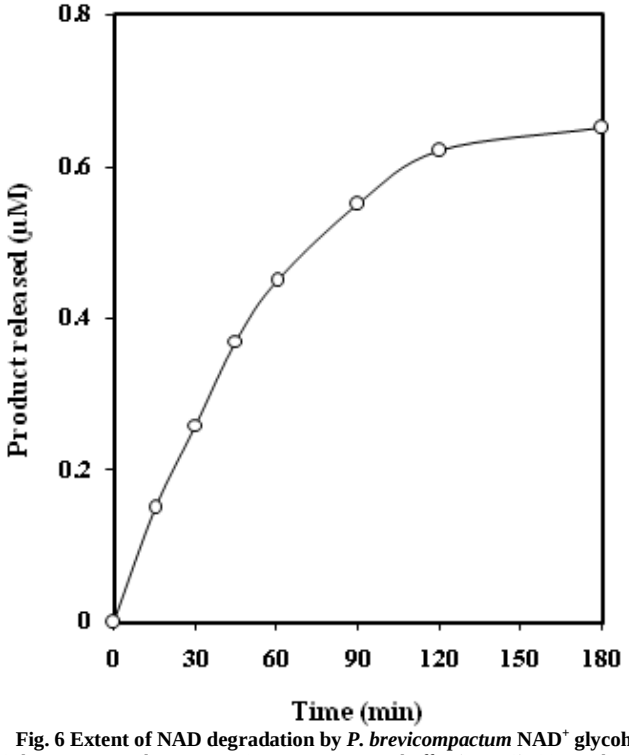

Fig. 6 Extent of NAD degradation by *P. brevicompactum* NAD$^+$ glycohydrolase

Reaction mixture contained: Substrate, 2 µmol; Protein, 50 µg; Tris-acetate buffer (pH 6.0), 80 µmol; Total vol., 1.0 ml; Time of the reaction, as indicated; Temp., 40ºC.

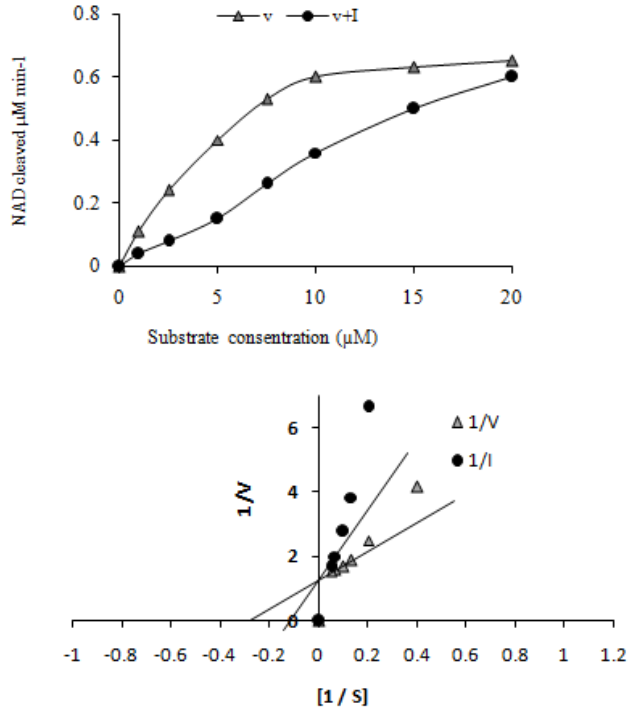

Fig. 7 Competitive inhibition of NADase by ADP-ribose

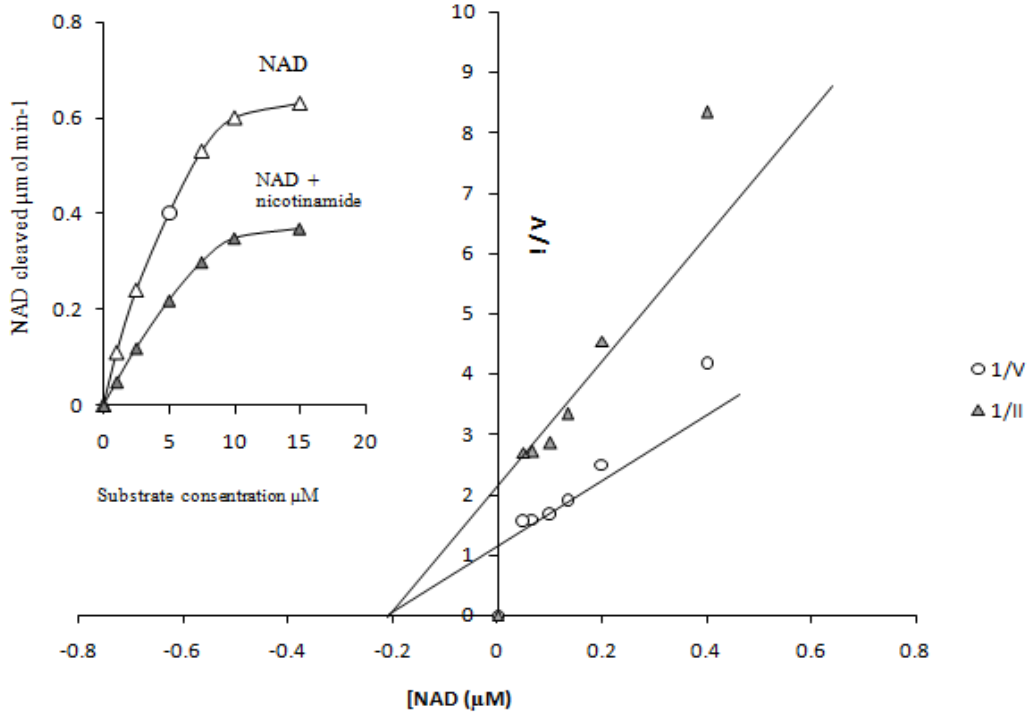

Fig. 8 Noncompetitive inhibition of NADase by nicotinamide

Effect of pH on NAD^+ glcohydrolase activity

Broad pH optima are characteristic of number of NADases [27-29]. In the present study, the purified NADase was found to be active in a wide pH range of 4.0 to 7.0, with an optimum pH at 6.0 (Fig. 4). In addition, it was reported

that as pH value diverged from the optimum level, the efficient functioning of the enzyme was decreased, most probably due to the changes occurred in the active site conformation. On the other hand, no enzyme activity was detected at pH 2.0, while 10% of the initial enzyme activity was observed at pH 10, which may be attributed to the better binding of the enzyme to the substrate at the alkaline pH. This result is closely related to that reported for the optimal pH (7.0) of NADase purified from *B. fasciatus* venom [26].

Temperature dependence of NADase activity

In the present study, optimal temperature for NADase activity purified from *P. brevicompactum* NRC 829 was reported in the range of 40 - 60°C (Fig. 5). In addition, the results obtained revealed that the enzyme activity was gradually declined at temperatures beyond 60^{o}C, which may be due to the denaturation of enzyme results from breaking of the weak hydrogen bonds within the enzyme structure. On the other hand, lower optimal temperatures of 13 - 45^{o}C have been reported for NADase activity purified from *B. fasciatus* [26].

Inhibition of NAD^+ glycohydrolase activity by the products of the reaction

The disappearance of NAD in the presence of purified fungal NADase was attributed solely to the hydrolysis of the nicotinamide ribosidic bond, yielding only ADP-ribose and nicotinamide as products, which verified previously by TLC analysis. When NAD was incubated with purified enzyme under the optimum assay conditions reached above, only about 30% of NAD was converted into products (Fig. 6). Incompletion of the glycosylation reaction observed in Fig. 6 leds to the suggestion that it could probably be due to product inhibition. Substantiation of this assumption can be observed in addition of nicotinamide or ADP ribose to the reaction mixture containing NAD as a substrate at the beginning of the reaction resulted in partial inhibition of the product.

Determination of the type of inhibition exerted

The plot of partially purified enzyme activity versus NAD concentration in the absence and presence of nicotinamide or ADP-ribose clearly shows the significant inhibition in the enzyme activity. It was found that the enzyme was inhibited by nicotinamide and ADP-ribose; these inhibitions are shown in Figs. 7 and 8, respectively. Replots of slopes and apparent K_i values (or intercept values) are shown in the insets of these figures. ADP-ribose was a linear competitive inhibitor with a K_i of 2.0×10^{-2} M. On the other hand, nicotinamide was a purely non competitive inhibitor with a K_i of 3.6×10^{-2} M. The same patterns were observed in product inhibition studies of bovine seminal Plasma NADase [27], calf spleen NADase [28] and *B. fasciatus* venom NADase [26].

Determination of K_m and V_{max} of NAD^+ glycohydrolase

The K_m and V_{max} of NADase for β-NAD was determined under the optimal assay conditions by measuring the enzyme activity of NADase using different concentrations of the substrate as described in the Materials and Methods. The concentration of NAD^+ was varied from 1.0 to 20 µmol and the initial velocities were determined at the definite enzyme concentration. K_m and V_{max} were determined and plotted according to Lineweaver - Burk plot in Fig. 7, from which K_m **a**nd V_{max} for NAD of 5 µM and 600 µM, respectively, were reported. NAD hydrolysis was directly proportional to the amount of NADase added at saturating substrate concentrations. *Penicillium brevicompactum* exhibited lower K_m value might possibly be explained to the high substrate affinity of NADase to the substrate. In comparison, the membrane-associated bovine erythrocyte 16 µM while other membrane-bound erythrocyte NADases exhibited K_m values from 10 to 80 µM [30]. The purified calf spleen NADase, a membrane-bound enzyme, exhibited a K_m value of 56 µM for NAD [28]. While Yuan and Anderson in 1973 [27] determined that the soluble bovine seminal plasma NADase exhibited K_m of 100 µM.

Substrate specificity of the purified NAD^+ glycohydrolase

Studies of the substrate specificity of the *P. brevicompactum* NADase indicated that number of pyridine nucleotides or their corresponding nucleosides served as substrates for the enzyme (Table 2). The enzyme was observed to be more sensitive to the pyridine moiety of the substrate such as NAD, CMP, UMP, uridine, thymidine, cytidine, and nicotinamide riboside. Although the adenyl portion of the dinucleotide was essential for substrate functioning, substitution of other purines for adenine did not greatly alter the functioning of the resulting dinucleotides as substrates. The substrate specificity of *P. brevicompactum* NADase is more closely related to that reported for *B. fasciatus* venom NADase and that reported for calf spleen NADase [31] than that observed for bovine seminal plasma NADase [27].

CONCLUSION

NAD^+ glycohydrolase was purified and characterized from *P. brevicompactum* NRC 829 crude extracts that demonstrates several important implications. Whereas, the removing of other NAD-hydrolyzing enzymes will yield a great analog formation besides the analysis of the products will be less complicated.

Acknowledgments
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