British Microbiology Research Journal
4(2): 173-184, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Purification and Partial Characterization of a Non-specific Acid Phosphatase Degrading NAD from *Aspergillus oryzae* NRRL447

Thanaa H. Ali[1*], Bakry M. Haroun[2] and Amir E. Tantawy[1]

[1]Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt.
[2]Department of Microbiology, Al-Azhar Univirsity, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author THA designed the study, revised the analysis, wrote the protocol, and wrote the first draft of the manuscript. The late author BMH managed the analyses of the study. Author AET performed the analysis in the laboratories and managed the literature searches. All authors read and approved the final manuscript

Original Research Article

Received 11th July 2013
Accepted 26th September 2013
Published 23rd October 2013

ABSTRACT

A non specific acid phosphatase from *Aspergillus oryzae* NRRL447 catalyzes the phosphate hydrolysis from nicotinamide adenine dinucleotide forming nicotinamide riboside, adenosine and P_i as the final products of the reaction. The enzyme was purified to homogeneity by a sequential treatment of acetone fractionation, DEAE-cellulose chromatography and gel filtration chromatography. The enzyme was purified 400-fold. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme showed a single protein band of MW 52 kDa. The enzyme displayed maximum activity at pH 5.0 and 40 °C with NAD as substrate. The enzyme activity appeared to be stable over pH 2.0–5.0 and up to 40 °C. The enzyme activity was enhanced slightly by Mg^{2+}, Ca^{2+} whereas inhibited strongly by F^-, $Mo0_4^-$, Cu^{2+} and Fe^{2+}. The enzyme hydrolyzes several phosphate esters, suggesting a probable non-specific nature. The substrate concentration-activity relationship is the hyperbolic type and the apparent K_m for NAD^+ was 6.25×10^{-4} M.

Keywords: Aspergillus oryzae NRRL447; NAD degradation; acid phosphatase

**Corresponding author: Email: thanaa15@yahoo.com;*

ABBREVIATIONS

ACP – acid phosphatase; NAD – nicotinamide adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate; ADP –adenosine 5`-diphosphate; AMP- adenosine 5`-monophosphate; CMP- cytidine 5`-monophosphate; GMP- guanosine 5`-monophosphate; UMP- uridine 5`-monophosphate; glucose 1-phosphate; NR – nicotinamide riboside; Nm – nicotinamide; Na – nicotinate, Pi- inorganic phosphate.

1. INTRODUCTION

Acid phosphatases (ACPase, E.C.3.1.3.2) are a family of multifunctional enzymes widely distributed in both plant and animal cells [1, 2]. The enzyme can hydrolyze ester phosphate linkages of NAD, resulting ADP, nicotinamide riboside (NR) and the release of inorganic phosphates. ADP then was dephosphorylated to AMP, which later converted to adenosine [3]. NR is one of the three natural compounds containing the nicotinamide ring (nicotinamide riboside, nicotinamide, nicotinate) which used in salvage metabolic pathways as precursors of the NAD(P) biosynthetic pathway [4]. NR has shown promise for improving cardiovascular health [5], As such administration of nicotinamide riboside or a nicotinamide riboside supplemented-food product could also protect against axonal degeneration. Recently it been shown to be a promising strategy for prevention and treatment of certain diseases [6-8]. NR has been prepared by fungal acid or alkaline phosphatases such as *A. niger* [9]. While Elzainy and Ali, [10] reported, NAD was dephosphorylated by *Aspergillus terreus* extracts to nicotinamide riboside, adenine plus ribose. NAD was deaminated by *Aspergillus terreus* DSM 826 extracts to deamido NAD and complete dephosphorylation of the dinucleotide molecule by the alkaline phosphatase [11].

The second products of previous reaction, adenosine, used intravenously for treating surgical pain and nerve pain, pulmonary hypertension, and certain types of irregular heartbeat [12]. It is also given for controlling blood pressure during anesthesia and surgery and for heart tests called cardiac stress tests. Adenosine is injected into the space around the spinal cord to treat nerve pain [13]. Adenosine phosphate (AMP) is given by injection into the muscle (intramuscularly) for treating varicose veins, bursitis, pain and swollen tendons (tendonitis), multiple sclerosis (MS), neuropathy, shingles (herpes zoster infection), and poor blood circulation [14].

In the present work, the properties of the purified acid phosphatase (ACPase) from *Aspergillus oryzae* was characterized for the first time as a step towards producing NR and adenosine which are medicinally valuable. The optimum concentration of substrate NAD, pH, temperature and influence metal ions were determined.

2. MATERIALS AND METHODS

2.1 Chemicals

NAD, ADP, AMP, adenosine, inosine and adenine were purchased from Sigma Chemical Company, nicotinamide and acetamide were purchased from Merck. DEAE-cellulose and Sephadex G-100 were from Pharmacia Fine Chemicals. The protein molecular weight (MW) markers were purchased from Serva Electrophoresis GmbH D-69115 Heidelberg Carl-Benz-Str 7. All other reagents were prepared in Microbial Chemistry Department, National Research Centre.

2.2 Methods

2.2.1 Organism

Aspergillus oryzae NRRL447 was obtained from Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, Illinois, USA.

2.2.2 Medium

Aspergillus oryzae was grown and maintained on slants of solid modified Czapek Dox's medium containing g/L distilled water: glucose, 30; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and agar, 20.

2.2.3 Preparation of *A. oryzae* extracts

The 4 days old mats, grown on liquid modified Potato-Dextrose medium containing per liter: 300g of potato and 20g dextrose at 28 °C, were harvested by filtration, washed thoroughly with distilled water and blotted dry with absorbent paper. The mats were then ground with cold washed sand in a chilled mortar and extracted with cold distilled water. The slurry thus obtained was centrifuged at 1522 ×g for 10 min and the supernatant was used as the crude enzyme preparation.

2.2.4 Influence of P_i on the enzyme formation

Various concentrations of KH_2PO_4 (0.0-4.0 g L^{-1}) were used in the growth media to assess their influence on ACPase formation.

2.2.5 Enzyme assay

Acid phosphatase was determined according to the method described by Heninone and Lahti [15] and can be summarized as follows: The stock solutions consist of 10 mM $(NH_4)_6.MO_7O_{24}.4H_2O$, 1 M citric acid, and 5 N H_2SO_4, all in distilled water, they are stable at least for several weeks at 25°C. AAM solution (acetone-acid-molybdate) was prepared daily by mixing 1 vol of ammonium molybdate solution with 1 vol of 5 N H_2SO_4 and 2 vol of acetone. Inorganic phosphate determination: into test tube containing 0.5 ml of samples, 4 ml of AAM solution was added. The contents were mixed carefully with a vortex mixer and 0.4 ml of 1 M citric acid was pipetted into each tube. After mixing, the yellow color was measured at (390-420nm). A sample with no added pi was used as a blank.

Every experiment cited in this work has been repeated several times and all the results recorded were reproducible.

Specific activity was expressed as µmol Pi liberated per mg protein per min, and a unit of enzyme activity was defined as the amount of enzyme required to produce 1 mM Pi per 30 min under the assay conditions (buffer, Tris-acetate at pH 5, 80 µmol; protein, 40 µg; substrate concentrations, 5 µmol; time of reaction, 30 min; total volume, 1 mL; temp., 40 °C). Protein concentration was determined following Lowry et al. [16], 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, [17].

2.2.6 Preparation and purification of enzyme

Cold acetone (-15°C) was added to the crude extract at concentrations of 0-33%, 30-60%, 60-75% and 75-83.5% respectively. The precipitated protein was separated by centrifugation and dissolved in 6ml Tris-acetate buffer, pH 6.0 (0.02M). Dialysis of this fraction was carried against cold distilled water for three hours at about 7°C.The dialyzed solution was loaded onto a DEAE-cellulose column (1.0 x 45 cm), pre-equilibrated with 0. 1 M Tris-acetate buffer at pH 6.0. Elution was carried at room temperature by batch-wise additions of 50 ml portions of increasing molarities (0.0-0.5M) of solutions of sodium chloride in 0.1 M Tris-acetate buffer pH 6, at flow rate about 20ml/ hrs. Fractions of 5.0 ml were collected and analyzed for protein and enzyme activity. The enzyme-active fractions were concentrated by lyophilization. The concentrated solution was then loaded to Sephadex G-100 column (46 × 2.0 cm), which had been equilibrated in 0.1 M Tris-acetate buffer pH 6.0. The enzyme was eluted from the column using the same buffer at a flow rate of 30 ml/hr, at room temperature (25°C). The fractions were analyzed for protein and enzyme activity. The enzyme purity was then checked using polyacrylamide gel electrophoresis (PAGE) technique.

SDS-gel electrophoresis technique was used to detect the purity of enzymes and to determine molecular weight of the purified enzyme from *Aspergillus oryzae* NRRL447 according to the method described by Laemmli [18], by using the following proteins which were used as molecular weight standards (Fermentas) (spectra ^{TM} multicolor broad range protein ladder): 260 KDa, 135 KDa, 95 KDa, 72 KDa, 62 KDa, 42 KDa, 34 KDa, 26 KDa, 17 KDa and 10 KDa.

2.2.7 Dialysis of the extracts

Dialysis of the extracts was made against 200 fold its volume of cold distilled water for three hours at about 7°C by dialysis membrane, size 3, Inf Dia. 20/32- 15.9 mm, MEDICEI, J, International Ltd**.**

2.2.8 Determination of optimal pH, optimal temperature and stability

The optimal pH of the purified ACPase was determined by performing the standard enzyme assay in the appropriate buffers: KCl-HCl (pH 1.0–2.0); Tris-acetate (pH 3–6.0); Tris-HCl (pH 7.0–9.0). The pH stability of the purified ACPase was examined by measuring the residual activity after incubating the enzyme at each desired pH for 1 h. The optimal temperature of the ACPase was determined by performing the standard enzyme assay within the temperature range of 20–80 °C. The thermal stability of the purified ACP was examined by measuring the residual activity after incubating the enzyme at each desired temperature for 30min

2.2.9 Effect of metal ions and some modulators on the purified enzyme activity

The effects of several metal ions on ACPase activity were investigated. The purified enzyme was pre-incubated with 10 mM metal ions at 40°C for 30min in Tris- acetate buffer (50 mM, pH 5.0). The metal ions and some modulators were: Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, F^{-}, MoO_4^{2-} and N_3^{-} The ACPase activity was determined by the standard assay as described above using NAD as substrate. 100% activity corresponds to no reagents added.

2.3 Substrate Specificity

Substrate specificity was investigated by replacing NAD in the standard assay mixture with an equal concentration of the following representative phosphorylated compounds: ADP, AMP, GMP, CMP, UMP, G-1-P and Phenyl phosphate disodium salt (ph.ph).

3. RESULTS

3.1 Evidence of P_i -repression on ACPase

The effect of P_i on the enzyme formation and its specific activity was determined (Table 1). The results showed that the maximum specific activity of the acid phosphatase was achieved with absence of Pi in the growth media, and decreased gradually with the increase of Pi concentration. A complete disappearance of enzyme activity at concentration 4.0 gL^{-1}.

Table 1. Influence of Pi on the acid phosphatase formation

Concentration. Of KH_2PO_4 (gL^{-1})	Specific activity (units mg^{-1} protein)
0.00	2.90
0.20	2.30
0.25	2.00
0.50	1.60
0.75	1.25
1.00	0.75
1.50	0.60
2.00	0.25
4.00	0.00

3.2 Enzyme Purification

A summary of the different protein isolation steps carried out to purify the ACPase from *Aspergillus oryzae* and NAD was used as substrate to monitor the enzyme activity throughout the purification procedures. Acetone fractionation, anion exchange chromatography using DEAE-cellulose, gel filtration chromatography by Sephadex G-100 gave partially purified (400-fold) enzyme with a yield of 64.6 (Table 2). The homogeneity of the ACPase was confirmed by migrating as a single band around 52 kDa through SDS-PAGE run reduced (DTT) (Fig. 1).

3.3 Optimal pH and Temperature Stability

The effect of pH on the ACPase activity was examined over a pH range of 3.0 to 9.0. The ACPase displayed optimal activity at pH5.0 (Fig. 2) and showed stability within a pH range of 3.0–5.0(data not shown). At pH 8.0, the ACPase loses 80% of its activity. The optimal temperature for the ACPase was 40°C (Fig. 3). The ACPase was stable over a temperature range of 20–40 8C (see Fig. 4).

3.4 Effects by Metal Ions and Some Modulators

The effects of metal ions on the enzyme activity of the ACPase were examined under standard assay condition with addition of 10 mM metal ions. The ACPase was enhanced

slightly by Ca^{2+}, Mg^{2+}, and, inhibited strongly by addition of Fe^{3+}, Cu^{2+} $Mo0_4^-$, N_3 and F^- whereas Zn^{2+} has no effect on the enzyme activity (see Table 3).

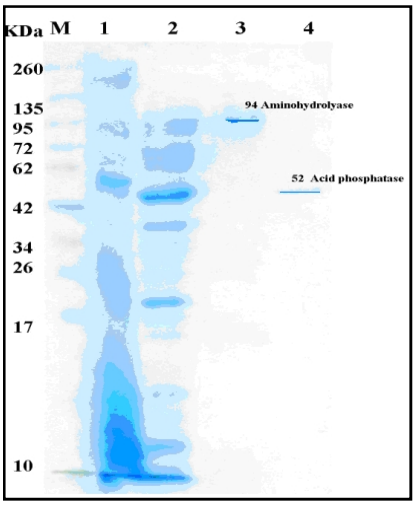

Fig. 1. Electrophoretic analysis of aminohydrolyase and acid phosphatase

Table 2. Purification of NAD dephosphorylating activity from *A. oryzae* NRRL 447 extracts

Purification steps	NAD dephosphorylating activity				
	Total activity (units)	protein (mg)	Sp. activity (units/mg protein)	Recovery (%)	Purification Fold
Crude extract	289.0	124.6	2.31	100.0	1.0
Acetone fractionation	274.8	54.0	5.1	95.0	2.23
DEAE-cellulose:	246.2	0.88	278.8	85.1	120.6
Sephadex G-100	186.7	0.202	924.2	64.6	400.0

Table 3. Effect of additions of different compounds on the purified acid phosphatase

Compounds	Relative activity (%)
	10mM
Control	100
$MgCl_2$	149
NaN_3	10
$CaCl_2.2H_2O$	129
$NaMoO_4$	13
$ZnSO_4$	100
$FeCl_3$	32
Cu SO_4	46
Na F	10

Reaction mixture contained: NAD^+, 5 µmol; protein, 25µg; buffer, Tris-acetate at pH 5, 80 µmol; vol., 1 mL; temp, 40°C; time of the reaction, 30min; concentration of compounds used, as indicated.

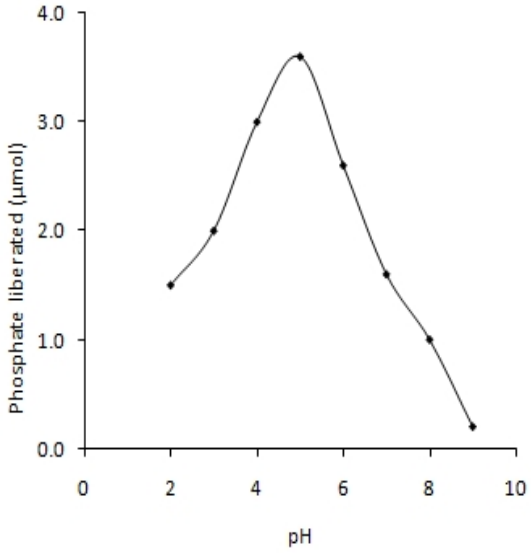

Fig. 2 Effect of pH on the purified acid phosphatase
Reaction mixture contained: NAD, 5µ mole; protein, 0.05 mg; buffer, Tris-acetate at pH5, 80µ moles; time of reaction, 30 min; temp.,40°C.

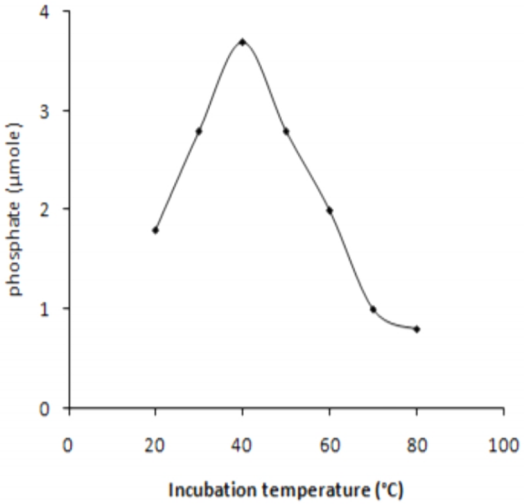

Fig. 3 Effect of different temperatures on the enzyme activity :
Reaction mixture contained: NAD, 5µ mole; protein, 0.025 mg; buffer, Tris-acetate at pH5, 80µ moles; vol., 1 mL; time of reaction, 30min; incubation temperature, as indicated

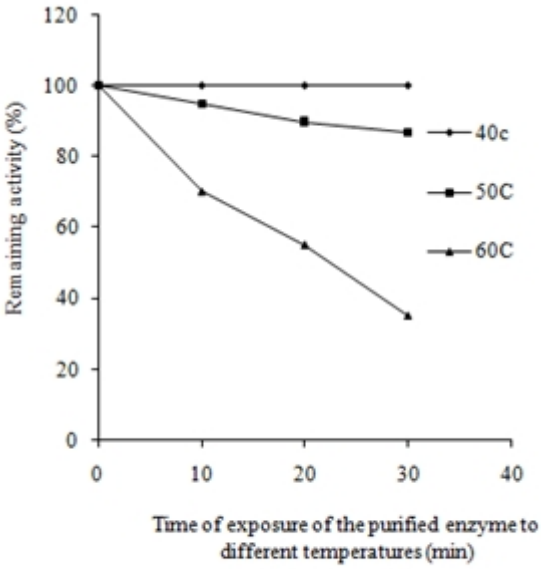

Fig. 4 Heat inactivation kinetics of the purified acid phoshatase Reaction mixture contained: NAD, 5µ mole; protein, 0.025 mg; buffer, Tris-acetate at pH5, 80µ moles; vol., 1 mL; time of reaction, 30min; incubation temperature, 40°C.

3.5 Substrate Specificity

The ACPase hydrolysed several phosphate esters at different rates; including ADP, AMP, UMP, GMP, CMP, G-1-P and ph.ph (see Table 4).

Table 4. Substrate specificity of the purified acid phosphatase activity

Substrate	Phosphatase activity (Uµg^{-1}protein)
NAD^+	6.70
ADP	6.25
AMP	3.10
UMP	2.10
GMP	2.60
CMP	1.17
G-1-P	1.38
ph.ph	2.70

3.6 Measurement of K_m and V_{max} for Acid Phosphatase

The rate of hydrolysis of the substrate NAD in the presence of *A. oryzae* acid phosphatase showed that optimum concentration of NAD was 20mM for acid phosphatase catalyzed hydrolysis into adenosine and nicotinamide riboside Fig. 5, there after stability in hydrolysis was observed. The apparent K_m value for NAD^+ was 6.25 x 10^{-4}M and the maximum reaction rate (V_{max}) was 0.3 µmole min^{-1} mg^{-1} proteins at pH5.

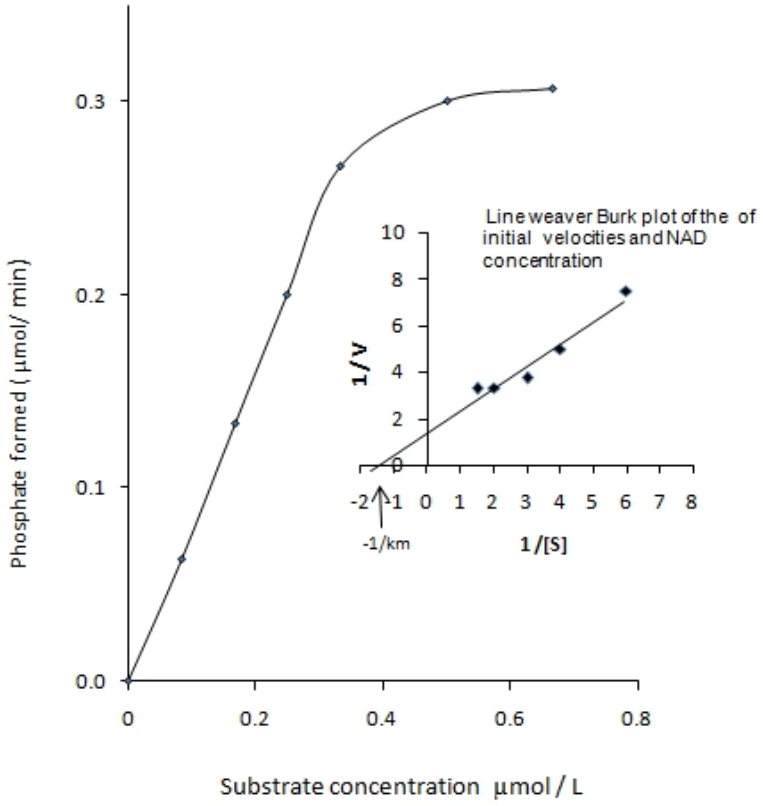

Fig. 5 Saturation kinetics of the purified acid phosphatase activity with the substrate

4. DISCUSSION

The present investigation provides data indicating involvement of non-specific phosphate-repressible acid phosphatase free-NAD deaminase from *Aspergillus oryzae* NRRL447. A complete dephosphorylation of NAD forming nicotinamide riboside, adenosine and inorganic phosphates as final products. Similar pathway behaviors were recorded previously by enzymes from an *A. niger* strain, and these enzymes were characterized as non-specific acid and alkaline phosphatases forming nicotinamide riboside, adenine plus ribose [9]. On the other hand, NAD^+ was dephosphorylated by *Aspergillus terreus* DSM 826 alkaline phosphatase to NR and adenosine [10]. NAD^+ was also converted to deamido NAD^+ and complete dephosphorylation of the dinucleotide molecule by the same extracts [11].

Aspergillus oryzae NRRL447 acid phosphatase (present work) had an acidic pH optimum at 5.0. Similarly, previous studies proved that extracts of *A. niger* contained isozymes of acid phosphatases which having their highest activities at pH4-4.5 [19]. However, the extracts of *A. niger* $NRRL_3$ strain contained two phosphatases, one of them was acidic and the other was alkaline one, each of them could catalyze sequential release of the two phosphate moieties of NAD [20].

The optimal reaction temperature for the ACPase was 40°C, which was similar to ACPases from other previous fungal species that having their optimal activities at temperatures ranging 40-50°C. *A. oryzae* ACPase was stable when incubated at 40°C for 1 h, but higher temperatures significantly deactivated the enzyme.

Aspergillus oryzae NRRL447 acid phosphatase is phosphate repressed enzyme that it is not found in mats grown on Czapek Dox's containing inorganic phosphates [3]. This enzyme is similar to acid phosphatases, have been described in *S*. *cerevisiae* [21], *A*. *niger* [22] and *P*. *chrysogenum* [23,24]. Phosphate is essential for the growth of all fungi [25], while, *A*. *oryzae* was able to grow at Czapek Dox's without Pi concentration [3].

It was showed that Cu^{2+}, significantly reduced the activity of the purified ACPase, which was consistent with the earlier reports [26,27,2]. The enzyme activity was strongly inhibited by F^{-}, N_3 and $Mo0_4$, a common inhibitor for ACPases [26,28,2].

An ACPase has been purified from *Aspergillus oryzae* with MW of 52 kDa.This molecular weight of acid phosphatase is smaller than acid phosphatase from the body wall of sea cucumber *S. japonicus* which having MW of 147.9 [2] and in agreement with acid phosphatases of mammalian tissue whose MW is 57 kDa [29].

The ACPase from *Aspergillus oryzae* had the highest activity towards both mono- and di-nucleotides such as, AMP, ADP, UMP, CMP, GMP and NAD like other species of *A. niger. A. terreus* [9,10]. Nonetheless, it also hydrolyzes glucose 1-phosphate and ph.ph effectively, suggesting that the purified ACPase has a broad hydrolytic capability. Most acid phosphatases, especially fungal types, are nonspecific and hydrolyze all phosphoric esters tested, but at different rates [9, 10, 11, 30, 2]. It is reasonable to believe that the purified *A. oryzae* ACPase is a nonspecific acid phosphatase.

4. CONCLUSION

In this study, ACPases have been purified from *Aspergillus oryzae*. The enzyme was purified to homogeneity and its molecular weight was 52 kDa. The enzyme exhibited optimal activity at pH 5.0 and 40 ^{o}C, and showed broad thermal but narrow pH stability. The enzyme appears to be a non-specific phosphatase and hydrolyses several phosphate esters. The characterization of the purified enzyme was studied.

ACKNOWLEDGEMENTS

This work was financially supported by the National Research Centre of Egypt.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Bull H, Murray PG, Thomas D, Fraser AM, Nelson PN. Acid phosphatases. Mol Pathol. 2002;55:65–72.

2. Zhu B-W, Yu J-W, Zhang Z, Zhou D-Y, Yang J-F, Li D-M, Murata Y. Purification and partial characterization of an acid phosphatase from the body wall of sea cucumber *Stichopus japonicus*, Process Biochemistry. 2009;44:875–879.
3. Ali TH, Haroun BM, Tantawy AE. Optimization of culture conditions for the production of NAD-degrading enzymes by *Aspergillus oryzae* NRRL 447. Advances in Food Sci. 2012;34:(4)195-201.
4. Belenky P, Racette FG, Bogan KL, McClure JM, Smith JS, Brenner C. Nicotinamide riboside promotes Sir2 silencing and extends lifespan via Nrk and Urh1/Pnp1/Meu1 pathways to NAD. Cell. 2007;129:473–484.
5. Tempel W, Rabeh WM, Bogan KL, Belenky P, Wojcik M, Seidle HF, Nedyalkova L, Yang T, Sauve AA, Park HW, Brenner C. Nicotinamide riboside kinase structures reveal new pathways to NAD, PLOS Biol.2007; 5: 263.
6. Bogan KL, Brenner C. Nicotinic acid, nicotinamide and nicotinamide riboside: A molecular evaluation of NAD precursor vitamins in human nutrition. Annu. Rev. Nutr., 2008; 28: 115-130.
7. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science. 2004; 305: 1010-1013.
8. Sasaki Y, Araki T, Milbrandt J. Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. J Neurosci. 2006; 26: 8484– 8491**.**
9. Elzainy TA, Ali TH. Pathways of NADP and NAD degradation by *Aspergillus niger* extracts. Annals of Microbiol., 2000; 50: 65-75.
10. Elzainy TA, Ali TH. Nature and mode of action of NAD and ATP dephosphorylating enzyme from *Aspergillus terreus* DSM 826. Antonie van Leeuwenhoek Journal of Microbiol., 2003;84: 255- 260.
11. Elzainy TA, Ali TH. NAD deamidation, a non-previously recognized reaction, by an enzyme from *Aspergillus terreus* DSM 826. Antonie van Leeuwenhoek Journal of Microbiol. 2005;87:119-129.
12. Mitchell J, Lazarenko G. Wide QRS complex tachycardia. Diagnosis: Supraventricular tachycardia with aberrant conduction; intravenous (IV) adenosine. CJEM. 2008;10 (6):572– 581.
13. Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov. 2008;**7**(9):759–770.
14. Malcolm AD, Garratt CJ, Camm AJ. The therapeutic and diagnostic cardiac electrophysiological uses of adenosine. Cardiovasc Drugs Ther. 1993;7(1):139-147.
15. Heninone JK, Lahti RJ. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. J. Bio. Chem. 1981;113**:**313-317**.**
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951;193:265-275.
17. Schleif RF, Wensink PC. Practical Methods in Molecular Biology. Springer-Verlag. New York. 1981;P. 74.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 1970; 227: 680- 685.
19. Elzainy TA, Elawamry ZA, Hassan MM, Ali TH. Nucleotide catabolism in *Aspergillus niger.* Acta Microbiol. Pol. 1989;38(1):27-38.
20. Elzainy TA, Ali TH. Extent and mode of ATP degradation by extract of *Aspergillus niger* $NRRL_{3.}$ Annali of Microbiologia ed Enzimologia. 1998;48:133-143.
21. Vogel K, Hinnen A. The yeast phosphatase system. Mol Microbiol. 1990;4:2013-2017.

22. MacRae WD, Buxton FP, Sibley S, Garven S, Gwynne D I, Davies RW, Arst HN, Jr. A phosphate-repressible acid phosphatase gene from *Aspergillus niger*: its cloning, sequencing and transcriptional analysis. Gene 1988; 71: 339-348.
23. Haas H, Redl B, Leitner E, Stoffler G. *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. Biochim Biophys Acta 1991; 1074: 392-397.
24. Haas H, Redl B, Friedlin E, Stoffler G. Isolation and analysis of the *Penicillium chrysogenum phoA* gene encoding a secreted phosphate-repressible acid phosphatase. *Gene*. 1992;113:129-133.
25. Jennings DH. Phosphorus. In The Physiology of Fungal Nutrition, 1995; 252-287. Cambridge: Cambridge University Press.
26. Jing G, Li L, Li Y, Xie L, Zhang R. Purification and partial characterization of two acid phosphatases forms from pearl oyster *(Pinctada fucata*). Comp Biochem Physiol B Biochem Mol Biol. 2006;143:229–235.
27. Terada T. Characterization of multiple acid phosphatases in bovine liver cytosol and lysosome. Inactivation of cytosolic enzymes by disulfides and its redox regulation by thiol transferase. Int J Biochem Cell Biol. 1997;29:985–992.
28. Rodrigues MC, Guimaraes LH, Liberato JL, de Lourdes Teixeira de Moraes Polizeli M, dos Santos WF. Acid and alkaline phosphatase activities of a fraction isolated from *Parawixia bistriata* spider venom. Toxicon. 2006;47:854–858.
29. Panara F, Angiolillo A, Fagotti A, Di Rosa I, Francesca S, Pascolini R. Acid phosphatases in mammalian tissues. Evidence for the existence of a 57kDa Zn^{2+} -dependent acid phosphatase form. Int J Biochem. 1992;24:1619–23.
30. Hoehamer CF, Mazur CS, Wolfe NL. Purification and partial characterization of an acid phosphatase from *Spirodela oligorrhiza* and its affinity for selected organophosphate pesticides. J Agric Food Chem. 2005;53:90–97.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=305&id=8&aid=2345