ISOLATION, CLONING AND EXPRESSION OF NAD^+ GLYCOHYDROLASE FROM *NEUROSPORA CRASSA*

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Summary

NAD^+ glycohydrolase (NADase) from *Neurospora crassa* is a glycoprotein that catalyzes the hydrolysis of NAD^+ to ADP-ribose and nicotinamide. It is used as one of the reagents in the cycling assay which functions to remove endogenous NAD^+. Conidia were found to have higher NADase activities than mycelia. Conidial NADase is different from mycelial NADase in terms of their optimum pH, K_m and carbohydrate moiety. Conidial NADase has a K_m of 280 μM while the K_m of mycelial NADase is 500 μM. Optimum pH for conidial NADase is pH 7. The mycelia NADase is active over a wide range of pH. N-linked deglycosylation reduced the size of the protein from 42 kDa to 32 kDa which suggested that the carbohydrate contributes 20% of the molecular mass. The native form of the protein is predominantly a dimer of 75 kDa without interdisulfide bond. Conidial NADase was purified using affinity columns, either cibacron blue 3GA agarose or blue sepharose CL-6B. The sequence of NADase was revealed and identified by mass spectrometry analysis. The DNA sequence was cloned into intracellularly expression vector, pPICZB and secretion expression vector, pPICZαA. The recombinant protein was expressed in the methylotropic yeast, *Pichia pastoris.* The extracellularly expressed protein has higher molecular weight than intracellularly expressed protein due to glycosylation. The native recombinant protein is a dimer or trimer bonded together by interdisulfide bond. The enzyme activity was confirmed by in-gel substrate staining and fluorimetric NADase assay. The recombinant proteins were applied in the cycling assay for NAD^+. It has been shown that the recombinant proteins are effective in removing NAD^+.

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ABBREVIATIONS

ADPR	Adenosine dinucleotide phosphate ribose
AOX1	Alcohol oxidase 1
ATP	Adenosine triphosphate
bp	Base pair
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
cADPR	Cyclic ADPR
cDNA	Complementary deoxyribonucleic acid
ºC	Degree celsius
dATP	Deoxyadenylate triphosphate
dCTP	Deoxycytidylate triphosphae
dGTP	Deoxyguanylate triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidylate triphosphate
ddH_2O	Double-distilled water
e-NAD	$1,N^6$-etheno-NAD
g	Gram
hr	Hour
LB	Luria Bertani
LDAO	Lauryl dimethylamine N-oxide
µg	Microgram
µl	Microliter
µM	Micromolar

ml	Milliliter
min	Minute(s)
M	Molar
MD	Minimal dextrose
MM	Minimal methanol
NGD^+	Nicotinamide-guanine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAD^+	Nicotinic acid adenine dinucleotide
PMSF	Phenylmethylsulfonyl fluoride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT	Reverse transcription
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophosis
Tris	Tris(hydroxymethyl) methylamine diamine tetra-acetate
V	Volt
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

1. INTRODUCTION

1.1 Overview of NAD^+ Metabolism and NAD^+ Glycohydrolase

NAD^+ is a molecule that has central roles in cellular metabolism and energy production. It acts as a coenzyme in many redox reactions in cells, including those in glycolysis and in citric acid cycle of cellular respiration. Besides, it also participates in non-oxidation reduction reaction which involves enzymatic transfer of ADP-ribose of NAD^+. Tryptophan is the *de novo* precursor of NAD^+ in almost all eukaryotes. The *de novo* synthesis and salvage pathway of NAD^+ involves several enzymes as shown in Figure 1.1.

The synthesis of NAD^+ has been associated with diseases. For example, it has been shown that axonopathy or Wallerian degeneration (nerve fiber damage) is always accompanied by ATP and NAD^+ depletion. Mice which are resistance to Wallerian degeneration (*wld*^{s}), protect neuronal NAD^+ levels (Wang *et. al.*, 2005). Increasing NAD^+ levels in nucleus, cytoplasm or mitochondria protects against the neurodegeneration (Belenky *et. al.*, 2007). Besides, NAD^+ also shows a protective role against *Candida glabrata* during urinary tract infection. *C. glabrata* is nicotinic acid auxotroph (Domergue *et. al.*, 2005). Under the condition of NAD^+ depletion, genes that encode adhesins (EPA1, EPA6 and EPA7) are transcribed as a result of derepression activity by NAD^+-dependent Sir2 (Gallo *et. al.*, 2004). In addition, NAD^+ synthesis is also associated with aging and regulation of cholesterol levels (Belenky *et. al.*, 2007).

The abundance of the NAD^+ pools in the cells depends on the enzymes that catalyze the synthesis of NAD^+ and location inside the cells. In addition, the

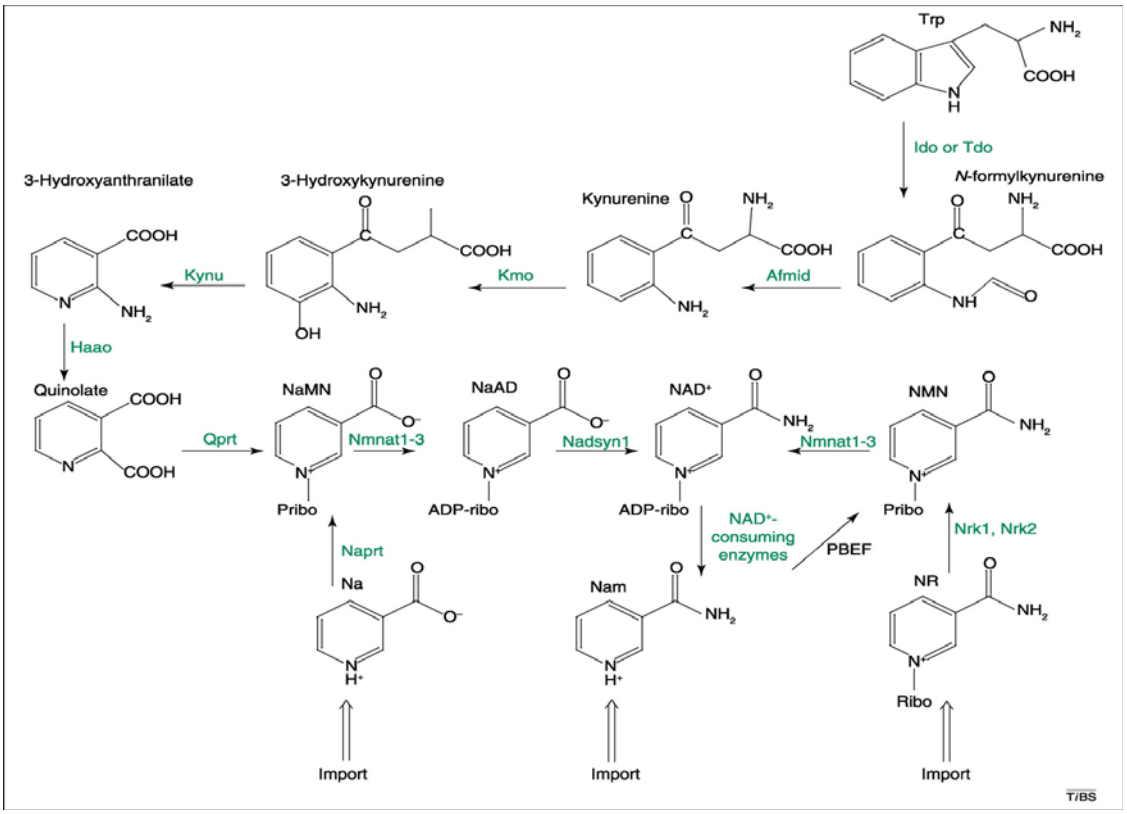


Figure 1.1. *De novo* synthesis and salvage pathway of NAD^+. *De novo* synthesis begins with the conversion of tryptophan to N-formylkynurenine by either indoleamine dioxygenase (Ido) or tryptophan dioxygenase (Tdo). Kynurenine is then formed by afmid. Kynurenine monooxygenase (kmo) converts kynurenine to 3-hydroxykynurenine which is the substrate of kynureninase (kynu) to form 3-hydroxyanthranilate. This is followed by the formation of quinolate and then NaMN. NaMN is adenylylated by Nmnat1, Nmant2 and Nmnat3 to form nicotinic acid adenine dinucleotide (NaAD). It is then converted into NAD^+ by glutamine-dependent NAD^+ synthetase (Nadsyn1). NAD^+ consuming enzymes like NADase break down the substrate into nicotinamide (Nam) and ADP-ribose. Nam is salvaged by Nam phosphoribosyltransferase to form nicotinamide mononucleotide (NMN) which can also be adenylylated to form NAD^+. Nicotinic acid (NA) and nicotinamide riboside (NR) ingested from diet are salvaged by Na phosphoribosyltransferase (Naprt) and nicotinamide riboside kinases (Nrk1 and Nrk2) respectively. (Pictured adapted from Belenky *et. al.*, 2007)

abundance of NAD^+ is also controlled by the enzymes that break down the NAD^+.

There are many classes of enzymes that cleave NAD^+ to generate nicotinamide and ADP-ribosyl product such as mono(ADP-ribose) transferases, poly(ADP-ribose) transferases, sirtuins and ADP-ribose cyclases. Collectively, these enzymes are called NAD^+-dependent ADP-ribosyl transferase (Sauve, 2006). NAD^+ glycohydrolase or NADase (E.C 3.2.2.5) belongs to the class of mono(ADP-ribose) transferase. It is distinguished from the other classes of ADP-ribosyltransferases by their ability to use water rather than simple amino acid as the acceptor of ADP-ribose and resulting in free ADP-ribose (Jacobson *et. al.*, 1995). It hydrolyzes the bond between nicotinamide and ribose moieties in NAD^+. The reaction equation is as follow:

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

NADase can be found on various types of organisms, ranging from microorganisms to mammals (Cho *et. al*., 1998). The properties of NADase may vary widely among species and from tissue to tissue. Differences in the purified NADase from several organisms are found with respect to molecular weight, subunit composition, specific activity, and Km value and transglycosidase activity as shown in Table 1.1 (Kim *et. al*., 1993). The microorganism NADase is different from mammalian NADase in such a way that microorganism NADases are readily soluble (Everse *et. al.*, 1980; Gopinathan *et. al.*, 1964; Menegus and Pace, 1980; Stine, 1969) while mammalian NADases are non-soluble membrane bound enzymes (Alivisatos *et. al.*, 1956; De wolf *et. al.*, 1985; Kim *et. al*., 1988; Muller *et. al*., 1983; Pekala and Anderson, 1978). The functional significance of NADase remains a puzzle even

though the enzyme from different sources has been studied for many decades. Consequently, NADase has received little attention.

Nicotinamide and ADP-ribose are the products generated from NADase. Nicotinamide is also known as Vitamin B3. Its role remains uncertain. ADP-ribose serves as a substrate for ADP-ribosylation. MonoADP-ribosylation was first found on diphtheria toxin (Honjo *et. al.*, 1968). MonoADP-ribosylation of several proteins has been documented to cause significant alterations in function such as inactivation of the protein (Ziegler *et. al*., 1997). For example in the presence of diphtheria toxin, ADP-ribose from NAD^+ was transferred to aminoacyl transferase II resulting in the inactivation of this enzyme (Honjo *et. al.*, 1968). Poly(ADP-ribosylation) is involved in a variety of fundamental processes aimed at maintenance of the functional integrity of the genome. The function of poly(ADP-ribose) was first reported by Shall and coworkers showing the involvement of poly(ADP-ribose) in DNA excision repair especially in the ligation step (Durkacz *et. al.*, 1980). The poly-ADP-ribose levels have been shown to increase 500 folds after DNA damage (D'Amours *et. al.*, 1999). In addition to DNA damage response, it influences processes such as recombination, mitosis, gene expression, differentiation and caspase-independent cell death (Ziegler, 2000; Gagné *et. al.*, 2006).

Table 1.1. Comparison of purified NADase from several sources. (Adapted from Kim *et. al*., 1993)

Source	Molecular weight (kDa)	Subunit M_r (kDa)	Specific activity (μmol/min/ mg)	K_m for NAD (μM)	Optimal pH	Transglycosidase activity
Rabbit erythrocytes	65	65	23	43	7.0	No
Snake Venom	124	62	1380	14	7.5	Yes
Bovine thyroid	120	ND*	1.3	400	7.2	Yes
Calf spleen	24	ND	9.6	56	7.4	Yes
Bull semen	36	ND	33	320	7.5	No
Bacillus subtilis	26.2	26.2	0.82	550	7.5	No

* ND, not described

1.1.1 Mammalian NADases

NADases from various mammalian sources, such as pig brain (Swislocki *et. al*., 1976), calf spleen (Schuber *et. al*., 1976), bull semen (Anderson *et. al*., 1980), snake venom (Yost *et. al*., 1981), bovine thyroid (De Wolf *et. al*. 1985), rabbit erythrocytes (Kim *et. al*., 1993) and bovine liver (Ziegler *et. al*., 1997) have been isolated and purified. The richest sources of NADases are generally the spleen, brain and liver (Price and Pekala, 1987). The catalytic properties of these enzymes appear closely related; however, significant differences were found among their physical properties (Everse *et. al*., 1975). Molecular weights of these NADases range from 24,000 to 124,000 and some of the enzymes show multiple forms that are enzymatically active. Several mammalian NADases have been shown to catalyze a transglycosidation reaction, frequently referred to as the pyridine base-exchange reaction (Yost *et. al*., 1981, Augustin *et. al.*, 2000) as shown in Table 1.1. The property of transglycosidase has been used for the preparation of pyridinium analogs of $NAD(P)^+$ (Price and Pekala, 1987; Anderson, 1982)

Mammalian NADases are generally found in association with the plasma membranes, therefore insoluble, and inhibited by nicotinamide (Pekala and Anderson, 1978; Yost and Anderson, 1981; Kim *et. al.*, 1993). Most mammalian NADase activity is associated with the membrane and directed toward the extracellular environment. The reason for NADase localization on the exterior of the membrane can be speculated on the basis of the findings that extracellular NAD^+, which is entirely impermeable to the membrane, can be converted to ADPR and nicotinamide, which then may be transported or diffused (Kim *et. al*., 1993).

In addition to NADase activity, many mammalian NADases also have ADP-ribosyl cyclase activity which is able to convert NAD^+ to cyclic ADP-ribose (cADPR) and nicotinamide in a 1:1 stoichiometry and cADPR hydrolase activity (Figure 1.2). ADP-ribosyl cyclase has been first purified from the ovotestis of *Aplysia californa* (Hellmich and Strumwasser, 1991). However, the purified *Aplysia* cyclase generates only cADPR rather than ADP-ribose (Lee and Aarhus, 1991; Hellmich and Strumwasser, 1991). In invertebrates, cADPR is exclusively generated by ADP-ribosyl cyclase from NAD^+ and the cyclase is a soluble enzyme. cADPR formation also has been found out in various mammalian tissues. In mammalian tissues, no equivalent enzyme could be detected. But, CD38, a mammalian NADase, shares the sequence homology with the cyclase from *A. californa* (States *et. al.*, 1992).

CD38, a 45-kDa type II transmembrane glycoprotein (Zilber *et. al*., 2000), is predominantly a NADase. It functions as a surface antigen (Ziegler, 2000) found on the plasma membranes of thymocytes, resting macrophages, activated B- and T-cells, and on many tumours. Like most of the any other NADases, CD38 is a multifunctional ecto-enzyme that has NADase activity, cyclase activity and cADPR hydrolase activity. Like any other mammalian NADases, it produces mainly ADPR with little amounts of cADPR. The cyclase and NADase activity of CD38 share a common mechanism involving the transient formation of covalent ADP-ribosyl cyclase complex. Dissociation of this intermediate to yield either cADPR or ADP may depend on the availability of water molecule at the active site (De Flora *et. al*., 1997). Mammalian NADases are able to consume NAD^+ analogues as substrate such as NGD^+. NGD^+ is converted into cGDPR in high yield while cADPR conversion is less than 2% of the

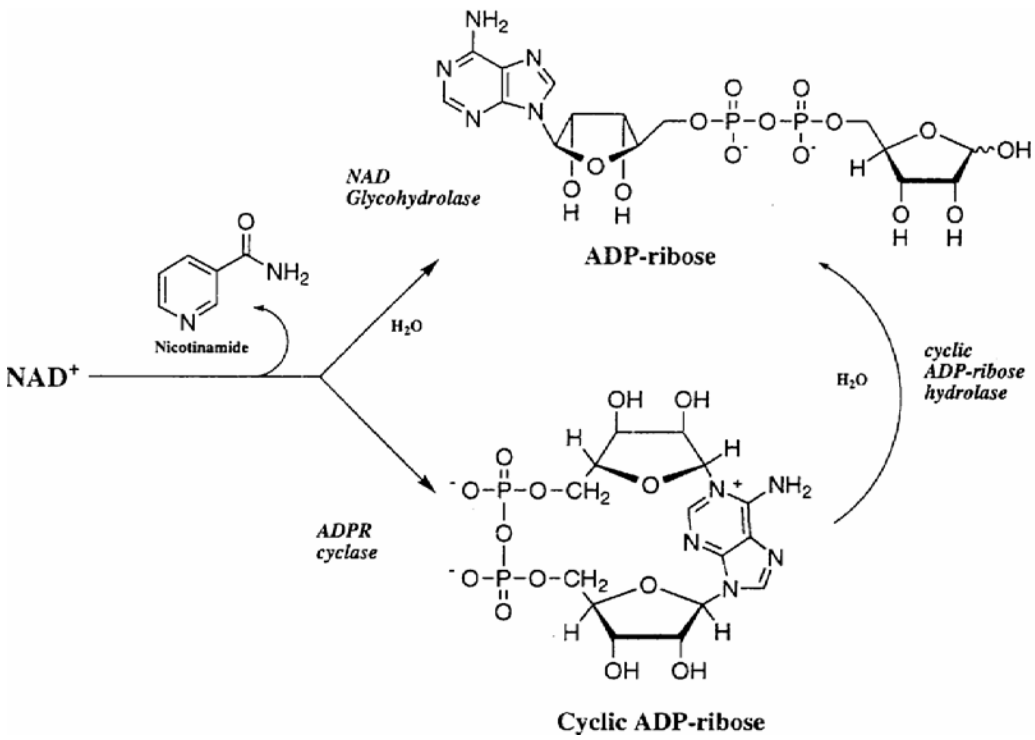


Figure 1.2. Reaction mechanism of NADase and ADP-ribosyl cyclase. (Picture adapted from Wall *et al*., 1998)

reaction products as the N7-position in guanine is more favorable than N1-position in adenine for cyclization formation (Muller-Steffner *et. al.*, 1997).

1.1.2 *Neurospora crassa* NADase

NADase was first detected in Neurospora crassa by Kaplan *et. al*. (1951) and has been associated with the process of macroconidiation. It is an ectoenzyme (Zalokar and Cochrane, 1956). NADase appears in high concentrations in *Neurospora crassa* grown on zinc-deficient medium (Nason *et. al*., 1950; Kaplan *et. al*., 1951). This increase is from 10- to 20-fold when compared to *N. crassa* grown on medium containing zinc (Nason *et. al*., 1950). NADase has long been isolated extensively from mycelium mats of *N. crassa* for its enzymatic reaction studies.

N. crassa NADase has a relative molecular weight of 38 kDa on SDS-PAGE under reducing conditions. The native form NADase migrates at a relative molecular weight of 70 kDa in gel filtration. This indicates that this enzyme can be associated as a dimer under non-reducing conditions (Cho *et. al.*, 1998). In addition, it is active in a wide range of pH. The activity begins to fall off only below pH 3 and above pH 9 (Kaplan, 1951).

In additional to mycelial NADase, NADase from conidia has been purified and characterized by Menegus and Pace (1981). They are the first and only one group who has ever looked into the conidial NADase. Conidial NADase has a molecular weight of 33 kDa, similar to that of mycelial NADase (38 kDa). But, they have shown that conidial NADase is different from mycelial NADase grown in "zinc-deficient" medium. The turnover number of conidial NADase (1.6×10^6) is higher than the

turnover number of mycelial NADase (5 x 10^3). Besides, Conidial NADase has lower hexose content compared to mycelial NADase. Hyrophobic character of conidial NAdase is not noticed in mycelial NADase.

So far, only one enzyme of several studied, NADase (EC 3.2.2.5) from *N. crassa*, has failed to produce cADPR. In addition, they do not catalyze the transfer of nicotinamide or some other pyridine analog to the ADPR moiety of NAD^+. This enzyme is designated as classical NAD^+ glycohydrolase (Reviewed by Mathias, 2000).

In contrast to mammalian NADases, NADases from this fungus is readily soluble and not sensitive to nicotinamide. NADase from *N. crassa* is only inhibited by nicotinamide at high concentrations which is about 0.1 M. The inhibition of nicotinamide is competitive in contrast to the noncompetitive inhibition observed in the bovine spleen system. The enzyme operated by a different mechanism from that of the animal tissue NADase in such a way that it does not form NAD^+ analogs.

Even though the protein has long been studied and characterized, the sequence of *N. crassa* NADase is not known yet. Neson *et. al*. (1975) has revealed that the NADase gene, *nada*, is localized on linkage group IV by developing a screening method for rapid identification of *N. crassa* mutants that are deficient in NADase and NADPase activities. They have shown that mutations at *nada* locus did not affect the expression of NADase during cell differentiation and general effect on NAD catabolism.

1.2 cADP-ribose and Ca^{2+} Signalling

The NAD^+ metabolite, cADPR was first discovered in 1987 as a Ca^{2+} mobilizing molecule in sea urchin eggs (Clapper *et. al.*, 1987). The cADPR Ca^{2+} signalling pathway is different from inositol 1,4,5-triphosphate (IP_3) Ca^{2+} signalling pathway. cADPR appears not to activate IP_3 receptors. In addition, cADPR-dependent pathway is insensitive to inhibition by heparin, a competitive inhibitor of IP_3 receptor and not inhibited by desensitization of IP_3 pathway by increasing IP_3 concentrations (Lee *et. al*., 1995). cADPR-mediated Ca^{2+} release is an important intracellular signalling system in living organisms such as protozoa, plants, invertebrates and vertebrates. Changes in intracellular calcium homeostasis occur in response to extracellular stimuli such as hormones, mediators, cell-cell contacts or physical stimuli.

Up to date, there are 4 models have been proposed to explain how the cADPR is synthesized from ADP-ribosyl cyclase. The first model, proposed by Lee, suggests that ADP-ribosyl cyclase *A. california* binds NAD^+ in a folded conformation, releases nicotinamide by forming an ADP-ribosyl intermediate and cyclizes the molecule by forming the intramolecular bond between the nitrogen atom 1 of the adenine and the anomeric carbon atom 1 of the second molecule (Lee, 1999). Second model explains that the sea urchin eggs ADP-ribosyl cyclase and neurosecretary PC12 cells are activated by nitric oxide which in turn activates guanylyl cyclase (Graeff *et. al.*, 1998; Galione *et. al.*, 1993; Willmott *et. al.*, 1996; Clementi *et. al.*, 1996). cGMP produced activates the cGMP-dependent protein kinase which then phosphorylates ADP-ribosyl cyclase. Third model suggest that the ADP-ribosyl cyclase from human Jurkat T cells is activated in response to Tyr-phosphorylation as a result of activation of T cell

receptor/CD3 complex (Guse *et. al.*, 1999). Fourth model involves the synthesis of cADPR by human CD 38 (Guse, 2000). CD 38 is located on the plasma membrane and surface of immune cells. Upon the binding of NAD^+ to CD 38, nicotinamide is released and an enzyme-bound ADP-ribose intermediate is formed. The anomeric carbon of the intermediate is in an activated state. N1 of adenine ring would react with the activated anomeric carbon to form cADPR (Metha and Malavasi, 2000)

There is pharmacological evidence showing that Ca^{2+} is released from intracellular Ca^{2+} pool via ryanodine receptor (RyR), mainly type 2 and 3 RyR (Guse, 2000). For example, the calcium-induced and caffeine-induced Ca^{2+} release are potentiated by cADPR (Lee, 1993) while ruthenium red and high Mg^{2+} concentrations inhibit cADPR-mediated Ca^{2+}-release (Galione *et. al.*, 1993; Guse *et. al.*, 1996). However, the exact mechanism of how cADPR exerts its Ca^{2+}-release effect inside the cells is not fully understood. Possibilities are direct binding of cADPR to RyR or via a separate cADPR binding protein (Noguchi *et. al.*, 1997; Tang *et. al.*, 2002). RyR is sensitized by cADPR to Ca^{2+} activation and hence promoting calcium-induced calcium-release.

In pancreatic cells, glucose induces an increase cADPR as a result of an increase in ATP. ATP is shown to exhibit inhibitory effect on cADPR hydrolase activity of CD38 (Kato *et. al.*, 1995). Extracellular NAD^+ is converted into cADPR and transported into cells by CD38 (Guida *et. al.*, 2002). Thus, CD38 acts as an enzyme and transporter. Subsequently, cADPR stimulates the Ca^{2+} release and insulin secretion (Takasawa *et. al.*, 1998). Autoantibodies against CD38 found in patients

with non-insulin-dependent diabetes mellitus implies that diseases may be related to the cADPR Ca^{2+} signalling system.

1.3 *N. crassa* NADase in Cycling Assay

To investigate roles and functions of cADPR in various tissues, it is important to monitor cellular levels of cADPR under various physiological conditions. Six strategies have been developed in order to measure cADPR in tissue levels. They include thin-layer chromatography (Galione *et. al.*,1993; Higashida *et. al.*, 1997), bioassays that exploit the ability of cADPR in cell extracts to mobilize calcium (Wu *et. al.*, 1997), radioimmunoassay (RIA) (Takahashi *et. al.*, 1995), high-performance liquid chromatography methods (Da Silva *et. al.*, 1998), radioreceptor assay (Reyes-Harde *et. al.*, 1999) and cycling assay(Graeff and Lee, 2002).

Among the strategies developed, cycling assay (Figure 1.3) is the most sensitive assay. It can detect the cellular cADPR with nanomolar sensitivity, as low as 50 fmol. It has advantages over other assays as all components of the assay are commercially available and the sensitivity of cycling assay can be further increased to sub-nanomolar range by prolonging the cycling reaction. In addition, unlike RIA, the assay does not require the synthesis of radioactive and purification of radioactive cADPR nor the antibodies against cADPR.

In this assay, NAD^+ is produced as a result of conversion from nicotinamide and cADPR by ADP-ribosyl cyclase under high concentrations of nicotinamide. NAD^+ is then coupled to cycling reaction under enzymatic reaction of alcohol dehydrogenase and diaphorase. One molecule of fluorescent resozurin is generated

when NAD^+ goes through one cycle of cycling reaction. Hence, endogenous concentrations of cADPR in nanomolar range can be measured. To remove endogenous NAD^+, the sample is pre-treated with *N. crassa* NADase. (Graeff and Lee, 2002).

N. crassa NADase is used instead of NADases from other organisms because most mammalian NADases are multifunctional enzymes which also catalyze ADP-ribosyl cyclase activity as described earlier. This will interfere with the cADPR level determinations in cycling assay. There are other pure mammalian NADases but they are not readily soluble which causes a problem in removing endogenous NAD^+ in solution assay.

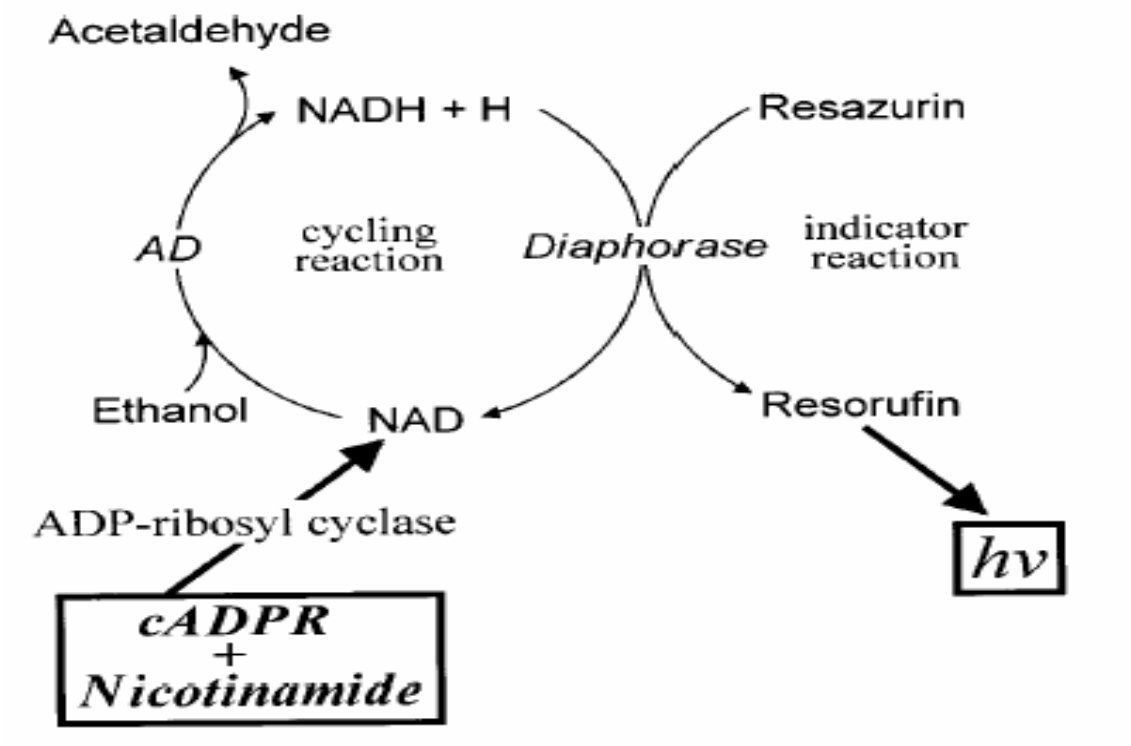


Figure 1.3. Cycling assay for cADPR
Abbreviations used: AD, alcohol dehydrogenase, *hv*, fluorescence light (Adapted from Graeff and Lee, 2002)

1.4 *Neurospora crassa*

N. crassa is a multicellular filamentous fungus of phylum *Ascomycota*. The genus name means "nerve spores". This is because the characteristic striations on the spores resemble axons (http://en.wikipedia.org/wiki/Neurospora_crassa). Its asexual life cycle is very simple which consists of three different cell types, vegetative hyphae, aerial hyphae and asexual spores called conidia as shown in Figure 1.4 (Menegus and Pace, 1981). Mycelium mats give rise to aerial hyphae which later produce conidia.

N. crassa can be grown under conditions that either promote vegetative growth or induce conidiation. When the culture is grown in liquid medium with continuous agitation, only vegetative growth occurs. However, growing the culture on solid surface or when the mycelia are harvested onto filter paper and incubated under aerobic conditions, conidiation is promoted possibly in response to aerobiosis and dessication or nutrient limitation (Berlin and Yanofsky, 1985). Conidiation can be completed within 12-14 hr (Springer and Yanofsky, 1989). Upon induction, aerial hyphae growth occurs and this is followed by apical budding formation to form minor constriction chains. Continual apical budding forms major constrictions chains which have constrictions between adjacent cells. Nuclei migrate into proconidial chains and cell walls are formed between adjacent proconidia. This is the pre-mature conidia. After each of these cells become mature, they are free conidium (Springer, 1993).

N. crassa has been first documented in 1843 as a contaminant in bakeries in Paris and been developed as an experimental organism in 1920s (Shear and Dodge, 1927; Lindergren, 1936). One of the well-known examples is done by Beadle and Tatum for their experiments leading to "one gene one enzyme" hypothesis (Beadle

and Tatum, 1941). They mutated *Neurospora* by exposing the fungus to X-rays. The experiment showed that mutation of a particular gene causes the defect of a particular enzyme in metabolic pathways. They were awarded the Nobel Prize in 1958 for their "one gene one enzyme" proposal. In the later part of 20th century, it has been widely used as a eukaryotic model organism in providing the fundamental understanding of genome defence systems, DNA methylation, mitochondrial protein import, circadian rhythms, post-transcriptional gene silencing and DNA repair (Davis, 2000). It is also used to study cellular differentiation and development in addition to other aspects of eukaryotic biology (Davis and Perkins, 2002).

N. crassa has a genome of some 40 Mb in seven chromosomes (linkage group LG I to LG VII) (Mannhaupt ***et. al***., 2003). Genome sequencing started on cosmid and BAC clones ordered along the individual chromosomes (Aign ***et. al***., 2001). At a later stage, a whole genome shotgun approach was initiated by whitehead Genome Center, Cambrigde, MA and it has been completely sequenced in 2003 (Galagan ***et. al.,*** 2003). This is the first complete genome sequence of developmentally complex filamentous fungus.

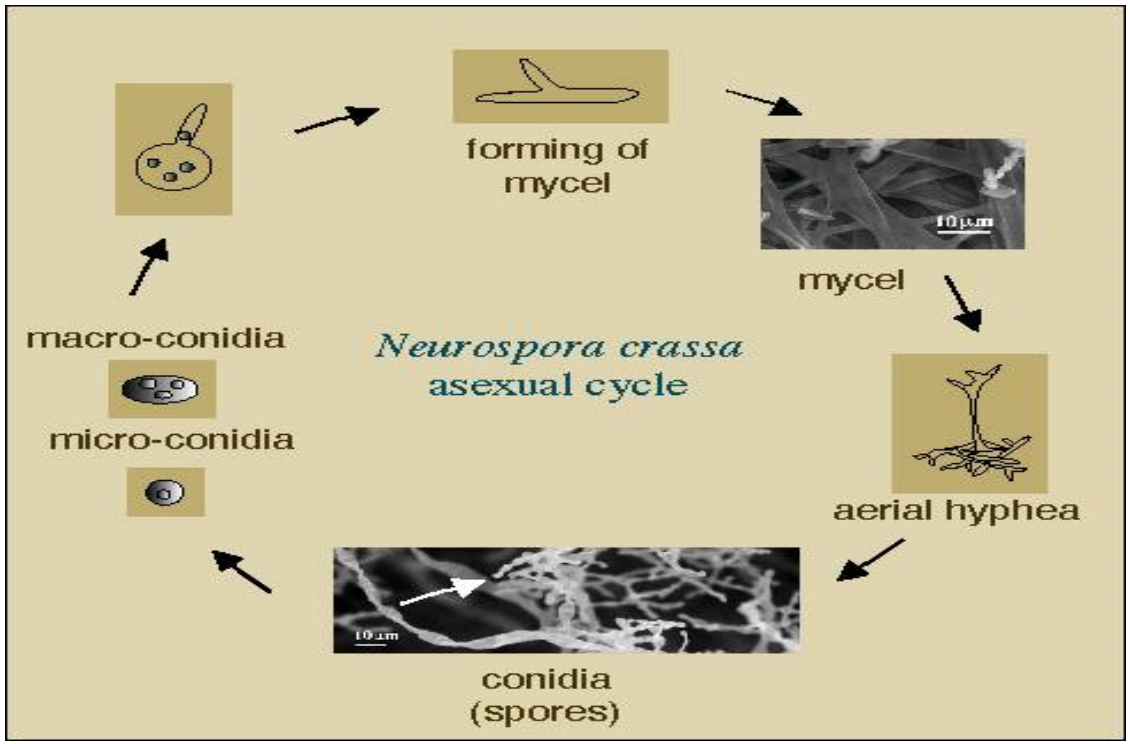


Figure 1.4. Asexual cycle of *Neurospora crassa.* The asexual cycle of *N. crassa* basically consists of 3 different cell structures, mycelium, aerial hyphae and conidia. (Picture adapted from http://www.ux.his.no/~ruoff/Neurospora_Rhythm.html)

1.5 *Pichia pastoris*

1.5.1 Background

Pichia pastoris is methylotropic yeast. It can be grown in methanol using it as the sole carbon source in the absence of glucose. Methylotrophic bacteria have been known since the beginning of 20th century and have been used extensively in 1960s to produce single-cell-protein from methanol. It was relatively easy to culture and grew well on methanol. During the time, several companies like ICI and Hoechst developed single-cell-protein processes based on methylotrophic bacteria. *P. pastoris* was not known until 1969. It was reported by Ogata *et. al.* (1969) and was initially used by Phillips Petroleum Company to produce single-cell-protein production in the early of 1970s.

When the yeast is grown on methanol, alcohol oxidase (AO) is the most abundant protein in the cell and it can be expressed up to 35% of total cellular proteins (Cauderc and Baratti, 1980). AO is the first enzyme involved in methanol metabolism pathway. It converts methanol into formaldehyde and hydrogen peroxide in the presence of oxygen as shown in Figure 1.5. The expression of AO is tightly regulated by the carbon source. When the yeast cells are cultured in media containing glycerol or ethanol, AO level in the cell is not detectable. AO is tightly regulated at transcriptional level (Roa and Blobel, 1983; Roggenkamp *et. al.* 1984). The promoter of AO was identified and isolated by Ellis and co-worker (1985). Following this, Cregg and co-worker (1985) has successfully developed a transformation system using *Pichia pastoris* as a host.

The yeast is now widely used as a host to produce a variety of recombinant proteins in both research and industrial use (Table 1.2). The advantages of using *Pichia* expression system include: (1) molecular genetic manipulations are relatively simple and easy, (2) recombinants proteins can be expressed at high level either intracellularly or extracellularly, (3) it can carry out protein modifications such as glycosylation, disulfide-bond formation and proteolysis (Cregg *et. al*., 2000), (4) it has strong inducible promoter known as alcohol oxidase 1 (AOX1) promoter which can regulate the expression of foreign protein, (5) it is insensitive to oxygen limitation, (6) it can be grown in simple culture media with very few endogenous proteins secreted out, thus simplifying the purification and recovery of proteins, (7) the media for growing cultures (containing glycerol, methanol, biotin, salts and trace elements) are economical and well-defined which is ideal for large scale production.

1.5.2 The *Pichia* Expression System

The primary features that are unique to *P. pastoris* expression system are a direct consequence of the inherent transcriptional properties of the promoter commonly used to control foreign gene expression. The most frequently used promoter to control foreign gene expression is AOX1 gene promoter. Under the control of AOX1 gene promoter, foreign gene expression can be "switched-off" by growing the cells on a non-methanolic carbon source and "switched-on" by shifting to methanol.

P. pastoris strains can be divided into several groups: 1) wild type strains (X-33, Y-1134), 2) auxotrophic mutants that are defective in histidinol dehydrogenase

Table 1.2. Heterologous proteins expressed by *P. pastoris*.

Intracellular proteins	Secreted proteins
Tumor necrosis factor (Sreekrishna *et. al.*, 1989)	Invertase (Tschopp *et. al.*, 1987)
β-galactosidase (Cregg and Madden, 1988)	Bovine lysozyme (Digan *et. al.*, 1989)
Hepatitis B surface antigen (Cregg *et. al.*, 1987)	Mouse epidermal growth factor (Clare *et. al.*, 1991)
Tetanus toxin fragment C (Clare *et. al.*, 1991)	Aprotinin analog (Vedvick *et. al.*, 1991)
Pertactin (Romanos *et. al.*, 1991)	*Aplysia* ADP-ribosyl cyclase (Munshi and Lee, 1997)
Human serum albumin (Wegner, 1990)	Human Insulin (Wang *et. al.*, 2001)

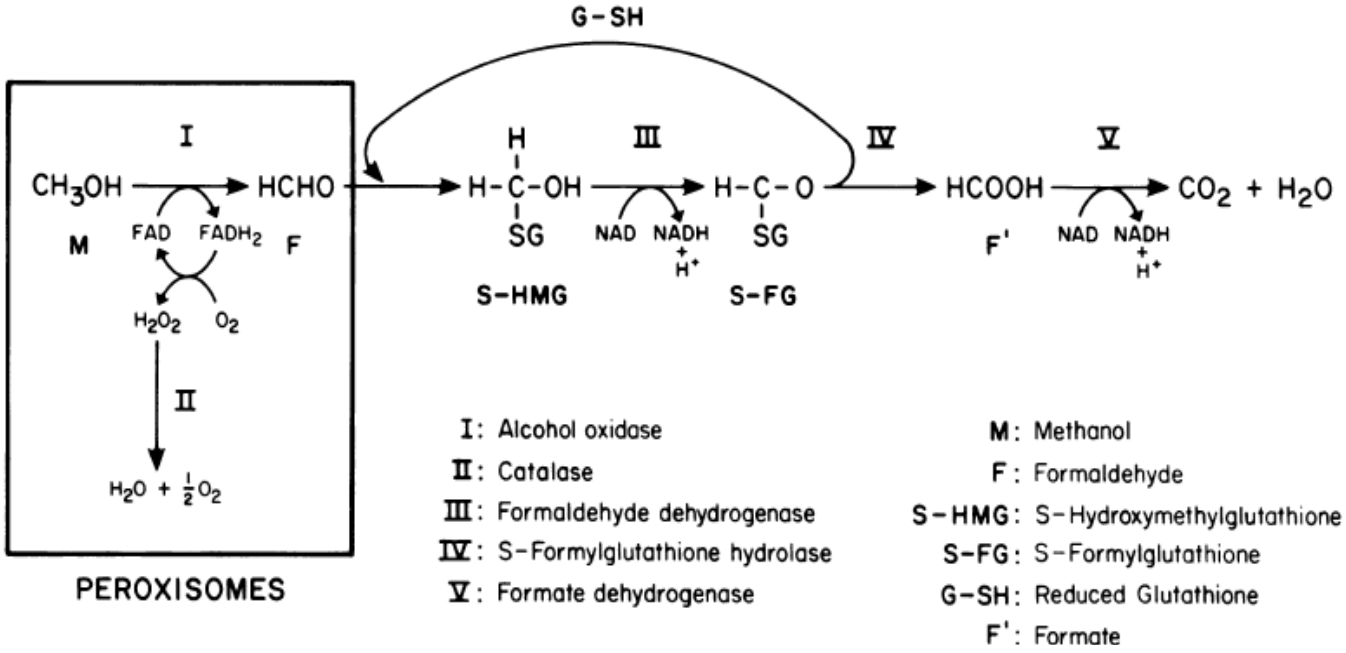


Figure 1.5. Methanol oxidation pathway in *Pichia pastoris.* (Picture adapted from Ellis *et. al.*, 1985)

(GS115), 3) mutants that are defective in genes involved in methanol utilization (KM71, MC 100-3), 4) protease-deficient strains (SMD1163, SMD1165, SMD1168) (Higgins and Cregg, 1998). The wild type yeast is wild-type in regard to the AOX1 and AOX2 genes. They are able to grow on methanol and metabolize methanol at normal rate. These are methanol utilization plus (Mut^+) strains. The AOX1 gene of KM71 strain is mostly deleted and replaced by ARG4 gene of *Saccromyces cerevisiae*. Therefore, this strain relies on weaker AOX2 gene to metabolize methanol at slow rate. This strain is referred to as Mut^s strain.

Several common features of plasmids vectors designed for foreign protein expression in *P. pastoris* are listed out in Table 1.3. The expression vectors contain AOX1 promoter followed by one or more restriction enzyme sites for the insertion of foreign genes. The 3' end of the multiple coding site contains the transcriptional termination sequence from AOX1 gene that directs efficient 3' processing and polyadenylation of mRNAs. Most of the vectors contain HIS4 gene as a selectable marker for transformation into his4 mutant host of *P. pastoris.* Other selectable markers include bleomycin and kanamycin resistance gene. Most of the vectors also contain the sequences required for plasmid replication and maintenance in bacteria, such as ColE1 replication origin and ampicillin resistance gene. Other features include AOX1 3' flanking sequences from 3' of the AOX1 gene. It can be used to direct the cassette containing foreign gene to integrate at the site of AOX1 locus by gene replacement. To secrete the foreign gene expressed, the construct contains a secretion signal right after the promoter but before the multiple coding sequences. The most frequently used secretion signal is α-factor prepro signal sequence from *S. cerevisiae.*

Table 1.3. Common features of *P. pastoris* expression vectors. (Adapted from Higgins and Cregg, 1998)

Vector name	Selectable marker	Features
Intracellular		
pHIL-D2	HIS4	NotI sites for AOX1 gene replacement
pAO815	HIS4	Expression cassette bounded by BamHI and BglII sites for generation of multicopy expression vector
pPIC3K	HIS4 and *kan*^{r}	Multiple cloning sites for insertion of foreign genes; G418 selection for multicopy strains
pPICZ	*ble*^{r}	Multiple cloning sites for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
pHWO10	HIS4	Expression controlled by constitutive GAP_P
pGAPZ	*ble*^{r}	Expression controlled by constitutive GAP_P; multiple cloning sites or insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
Secretion		
pHIL-S1	HIS4	AOX1p fused to PHO1 secretion signal; *Xho*I, *Eco*RI, and *Bam*HI sites available for insertion of foreign genes
pPIC9K	HIS4 and *kan*^{r}	AOX1 fused to α-MF prepro signal sequence; *Xho*I (not unique), *Eco*RI, NotI, SnaBI and AvrII sites available for insertion of foreign genes; G418 selection for multicopy strains
pPICZαA	*ble*^{r}	AOX1 fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
pGAPZα	*ble*^{r}	Expression controlled by constitutive GAP_P; GAP_P fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags

The vectors of *Pichia* expression systems contain at least one *P. pastoris* DNA segment (AOX1 or GAP promoter segment). Linearized vectors can generate stable transformants of *P. pastoris* via homologous recombination. The vectors can be integrated into yeast genome by single crossover type (Figure 1.6A) or gene replacement (Figure 1.6B) with the single crossover has higher possibility than gene replacement. There is only 10% to 20% of gene replacement event occur which the AOX1 gene is deleted and replaced the expression cassette and marker gene (Cereghino and Cregg, 2000). Transfomants resulting from AOX1 single crossover events are phenotypically Mut^+ while Mut^s strains are generated from gene replacement integration.

The frequency of multiple gene insertion is 1% to 10% of all Zeocin resistant (Zeo^R) transformants (Higgins and Cregg, 1998; Cereghino and Cregg, 2000). The event can occur at AOX1 or his4 locus. The multicopy strains which contain more than one copy of integrated expression cassettes sometimes have higher protein yield than single-copy strains (Thill *et. al.,* 1990; Clare *et. al.,* 1991). The multicopy events can be detected by DNA analysis methods or by examining the levels of the foreign protein directly. There is a need to screen hundreds to thousands Zeo^R transformants in order to locate strains with 20 or more copies.

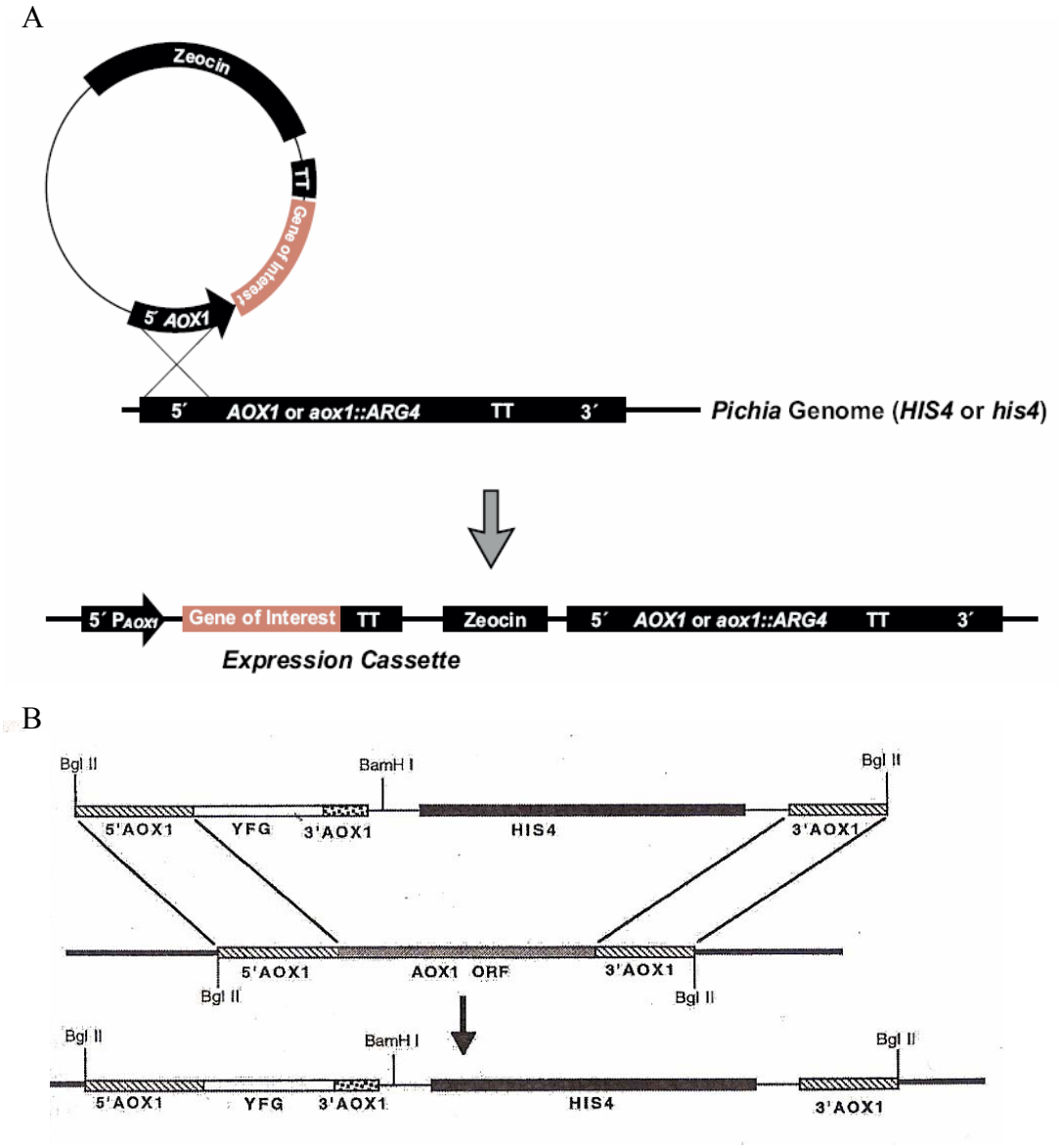


Figure 1.6. Integration of expression vectors into *P. pastoris* genome. (A) Integration of vectors into *Pichia* genome at AOX1 locus by single cross over event. (Picture adapted from EasySelect *Pichia* Expression Kit manual, Invitrogen) (B) Gene replacement event at AOX1 gene. (Piture adapted from Higgins and Cregg, 1998)

1.5.3 Expression of Foreign Proteins

Expression of foreign proteins can be done in shake-flask cultures but protein levels are much higher in fermentation cultures. This is because only under the fermentative condition, the environment of the cultures such as pH, aeration and carbon source feed rate, are well controlled. This enables the cultures to grow to ultra-high cell densities (>100 g/L dry cell weight) (Higgins and Cregg, 1998; Cereghino *et. al.*, 2002). In addition, the transcription level resulted from AOX1 promoter can be 3 to 5 times higher in fermentative cultures than cells grown in excess methanol (Higgins and Cregg, 1998). Besides for the 2 reasons stated above, oxygen consumption rate is higher in shake-flask culture, expression of foreign proteins is negatively affected by oxygen limitation (Higgins and Cregg, 1998). Under controlled environment of a fermenter, the oxygen levels of the cultures can be monitored and adjusted accordingly. Thus, the fermentation cultures can express higher levels of foreign proteins than shake-flask cultures.

1.5.4 Posttranslational Modifications

The advantage of *Pichia* expression system is the ability of *P. pastoris* to perform posttranslational modifications resemble to higher eukaryotes. The posttranslational modifications include processing of signal sequences, folding, disulfide bond formation and O-/N-linked glycosylation.

Even though there are many secretion signal sequences can be used, the most common and successfully used secretion signal is *S. cerevisiae* α-factor prepro peptide. The signal sequence consists of a 19-residue (pre) signal sequence followed by a 66-

residue (pro) signal sequence containing 3 consensus N-linked glycosylation sites and 1 dibasic Kex2 endopeptidase processing site (Kurjan and Herskowitz, 1982). The cleavage of signal peptide involves 3 basic steps. First, the pre signal is removed by endopeptidase in endoplasmic recticulum followed by cleavage of pro leader sequence by Kex2 endopeptidase at Arg-Lys site. The last step involves the cleavage of Glu-Ala repeats by Ste13 protein (Brake *et. al.,* 1984).

Pichia pastoris system has the ability to produce disulfide-bonded heterologous proteins which is not achievable by prokaryotic system due to the reducing environment (White *et. al.*, 1994). The disulfide-bonded proteins produced by the *Pichia* expression system include thrombomodulin containing 2 epidermal growth factor-like domains and coagulation protease (Macauley-Patrick *et. al.,* 2005). The activity of proteins might be affected by the disulfide bond present (Debski *et. al.*, 2004).

Pichia pastoris is able to perform O-linked and N-linked glycosylation on heterologous protein. O-oligosaccharides which compose of only mannose (Man) residues are added to hydroxyl groups of Ser and Thr residues of secreted proteins. In contrast to higher eukaryotes such as mammals, varieties of O-oligosaccharides including N-acetylgalactosamine, galactose and sialic acid, are added to Ser and Thr residues. One cannot assume that a heterologous protein is not O-linked glycosylated even if the protein is not glycosylated by its native host. Moreover, the specific Ser and Thr residues for glycosylation in original host might not be the same as the Ser and Thr residues for glycosylation in *Pichia* expression system (Cereghino and Cregg, 2000; Macauley-Patrick *et. al.,* 2005).

In all eukaryotes, N-linked glycosylation begins on the cytoplasmic site of endoplasmic reticulum with the transfer of a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$ to asparagine at the consensus recognition sequence, Asn-X-Ser/Thr. The oligosaccride core is then trimmed to $Man_8GlcNAc_2$. In mammalian Golgi apparatus, the oligosaccharide cores undergo a series of trimming and addition reaction with 3 final products generated. They are $Man_{5-6}GlcNAc_2$ (high mannose), a mixture of several different sugars (complex), or a combination of both (hybrid) (Figure 1.7).

However, in *Pichia,* the oligosaccharide chains of secreted proteins remain unaltered and consist of $Man_{8-9}GlcNAc_2$ (Montesino *et. al.*, 1998). In *S. cerevisiae*, heterologous secreted proteins produced are always hyperglycosylated where the mannose residues are typically 50-100 in length. The high mannose oligosaccharide structures in the recombinant proteins represent a significant problem in pharmaceutical industry. The proteins are antigenic and rapidly cleared from the blood when introduced intravenously. Besides, the high mannose structures may interfere with the folding and biological activity of the foreign proteins. The *Pichia* oligosaccharides are also different from *Saccharomyces* oligosaccharides by not having any terminal α-1,3-linked mannose structures (Montesino *et. al.*, 1998; Verostek and Trimble, 1995). In addition, α-1,6-linked mannose are found in *P. pastoris*-secreted invertase (Trimble *et. al.*, 1991) and kringle-2 domain of tissue-type plasminogen activator (Miele *et. al.*, 1997). This structure is not found on oligosaccharide structures of *Saccharomyces* secreted proteins.

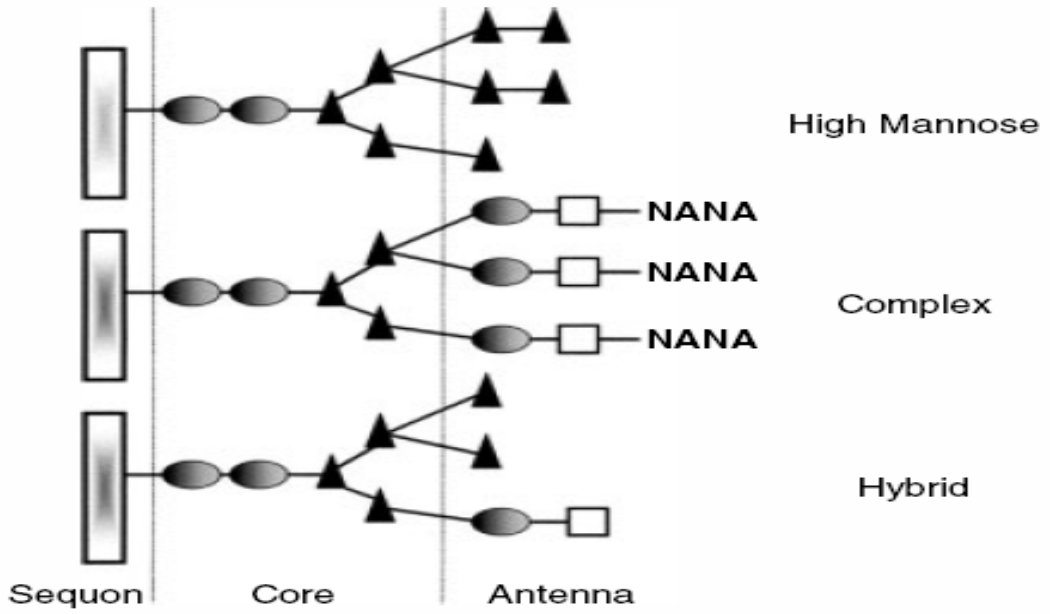


Figure 1.7. Three types of oligosaccharide chains in mammalian Golgi apparatus. The core is attached to the asparagine residue of protein sequence. The core is attached to high mannose, complex or hybrid. ● , GlcNAc; ▲ , mannose; □ , galactose. (Picture adapted from Macauley-Patrick *et. al.*, 2005)

1.6 The objectives

In this project, NADase from conidia of *N. crassa* was purified using affinity chromatography namely cibacron blue 3GA agarose and blue sepharose CL-6B. Purified NADase was then analyzed by SDS-PAGE or native PAGE, followed by in-gel substrate staining. The band which showed activity on gel was subjected to mass spectrometry analysis to identify peptide sequence of NADase from protein databases. The sequence was then cloned into yeast expression vectors, pPICZαA and pPICZB vector to express the protein extracellularly and intracellularly, respectively. This recombinant NADase was applied in the cycling assay for NAD^+ to test its efficacy in removing NAD^+.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

Standard analytical grade laboratory chemicals for the preparation of general reagents were obtained from BDH, Poole, England; Merck, Darmstadt, Germany; Sigma Chemicals Co., St. Louis, MO, USA and J.T. Baker, Phillipsburg, NJ, USA.

2.1.2 Genetic Strain

Bd strain (FSGC #1859) of *N. crassa* fungal culture was obtained from Fungal Genetic Stock Center (Kansas City, USA). Wild type (ATCC 10815) *N. crassa* was a gift from Dr. Kim Uh-Hyun (Cho *et al*., 1998). *Pichia* expression vectors (pPICZαA and pPICZB), *Escherichia coli* strain (TOP10F') and wild type *Pichia pastoris* strain (X33) were generous gifts from Prof. Chua Kaw Yan from Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore.

2.2 *Neurospora crassa* Fungal Culture

2.2.1 Mycelia Culture

Starter cultures of wild type (ATCC 10815) *N. crassa* were initiated with inoculations from frozen stocks into 2 to 3 ml of Zinc-deficient medium in green cap tubes. Starter cultures were grown at 30^{o}C in the incubator (Boekel Incubator Model 133000) until the fungus grew. Starter cultures were transferred into 1 l zinc-deficient medium prepared in 2-l conical flasks for big scale fungus growth. After six to seven days' growth, mycelium mats were collected by filtering through filter paper and

washed with Mili-Q water. Weights of mycelium mats were recorded. Mycelium mats were frozen at -80^{o}C for at least 1 hour before homogenized to facilitate enzyme extraction. From 10 l of medium, 17-20 g of mats were obtained.

2.2.2 Conidia Culture

Bd strain was grown in 250 ml-, 500 ml- or 1 l- conical flasks on Vogel's minimal medium (Davis *et al.,* 1970) with 1.5% sucrose as carbon source and solidified with 2% agar (Biomed Diagnostics, Sparks, USA). The fungal cultures were incubated at 30^{o}C for 3 to 4 days in the incubator (MRC, London, UK). Inocula were prepared in 96 mm x 16 mm petri dish (Greiner Bio-one, Frankfurt, Germany) starting from conidia frozen stock.

2.3 Isolation of *N. crassa* Proteins

2.3.1 Isolation of Mycelia Proteins

Crude extract of mycelia was prepared as described by Cho *et. al.* (1998) with slight modifications. Frozen mycelium mats were homogenized in three times their weight of ice-cold lysis buffer using pestle and mortar and 55-ml Dounce-type glass homogenizer with ten times tight passes. This was followed by centrifugation at 12,000 x g (Beckman Coulter, Fullerton, CA) for 30 min at 4^{o}C.

2.3.2 Isolation of Conidia Proteins

Conidia proteins were prepared as described by Menegus and Pace (1981) with modifications. Three milliliters, 5 ml and 10 ml of Mili-Q H_2O (Millipore, Bedford,

MA) were dispensed into 250 ml-, 500 ml- and 1 l-conical flasks respectively. Conidia on the surface of agar were resuspended and collected. The suspension was centrifuged at 20,000 x g for 30 min at 4^{o}C. Water was then discarded and the wet weight of conidia was recorded. Three volume of ice-cold 0.17 M of KCl was used to resuspend and wash 1 g of conidia. This is followed by centrifugation at 20, 000 x g for 30 min at 4^{o}C. This step is repeated for 3 times. All the extracts from 3 times of repeating resuspension and washing were pooled together. Pooled extracts were then concentrated into small volume and desalted with Buffer A by ultrafitration using Centripep 30 (Millipore, Billerica, MA).

2.4 Column Chromatography

All operations were performed at 4^{o}C unless otherwise stated.

2.4.1 Cibacron Blue Agarose

Five milliliter of cibacron blue agarose (Sigma Chemicals Co., St. Louis, MO, USA) was equilibrated with 25 ml of Buffer A. Concentrated sample of crude extract was mixed with cibacron blue agarose for 1 hour and 30 min at 4^{o}C with Intelli-mixer (Elmi, Riga, Latvia). After batch-wise mixing, beads were packed into 0.8 cm x 0.9 cm glass column. The column was washed with 50 ml of buffer A. Proteins were eluted out using 1 M potassium chloride in buffer A in gravity flow. Fractions containing the enzyme activity were pooled together. Sample was topped up with buffer A to lower the concentration of potassium chloride and concentrated using centripep 30 (Millipore, Billerica, MA).

2.4.2 Blue Sepharose CL-6B

Every 5 mg of crude extract proteins were loaded into 1 ml of Buffer A-equilibrated blue sepharose CL-6B beads (Amersham Biosciences, New Jersey, USA). Five column volume of washing buffer containing 0.5 M of sodium chloride in 50 mM of sodium phosphate, pH 7.0 was used to wash the column. Bounds proteins were eluted with 1 M of sodium chloride in 50 mM of sodium phosphate, pH 7.0. After the first CV of elution buffer was dispensed into the column and collected, the column was left for 30 min for complete disruption of interaction between the protein and the beads. The remaining elution buffer was then applied until no significant enzyme activity was found in the collected fractions. Enzyme activity was checked for collected fractions. Fractions contained enzyme activity were pooled together and concentrated using Centripep 30 (Millipore, Billerica, MA).

2.4.3 Superdex 75

Purification using an ÄKTA-FPLC system (Amersham Biosciences, New Jersey, USA) was accomplished using a 24 ml superdex 75 10/300 GL (Amersham Biosciences, New Jersey, USA) gel filtration column following the protocol recommended by the manufacturer. In brief, the column was equilibrated with 2 CV of 50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0 at recommended flow rate, 0.5ml/min. Blue sepharose sample was centrifuged at 10, 000 x g for 10 min at 4^{o}C to remove any insoluble particulate. A maximum volume of 500 μl sample was then injected into the column. The protein was eluted using 2 CV of equilibration buffer mentioned above. The fractions of 0.6 ml each were collected using fraction collector

Frac-950 (Amersham Biosciences, New Jersey, USA). Enzyme activity was checked for each fraction.

2.4.4 His-Tag Column

Intracellularly expressed recombinant proteins were solubilized in binding buffer. The His-tag columns used were His SpinTrap mini columns (GE Healthcare, Uppsala, Sweden). The purification steps were done according to manufacturer's recommendation. The column was repeatedly inverted and shaken to resuspend the medium. Cap was loosened and bottom closure was broken off. The column was spun for 30 s at 100 × g in a 2-ml microcentrifuge tube to remove the storage liquid. After that, the column was equilibrated with 600 µl binding buffer followed by centrifugation for 30 s at 100 × g. A maximum volume of 600 µl sample was applied into the column and centrifuged for 30 s at 100 × g. The column was then washed with 600 µl binding buffer and centrifuged for 30 s at 100 × g. Target protein was eluted twice with 200 µl elution buffer and centrifuged for 30 s 100 × g.

2.5 Bio-Rad Protein Assay

The Bio-Rad Protein Assay, based on the method of Bradford (1976), is a simple and accurate procedure for determining concentration of solubilized protein. Protein concentration was determined by Bio-Rad protein Assay Kit (Bio-Rad, Hercules, CA) using BSA (Promega, Madison, WI) as the standard. A standard curve was plotted using a range of concentrations of 2, 4, 6, 8, 10 and 12 µg/ml. The BSA stock was diluted to 0.1 mg/ml and mixed in each tube as follows:

Final concentration (µg/ml)	Volume of BSA (µl)	Volume of ddH_2O (µl)
Blank	0	800
2	20	780
4	40	760
6	60	740
8	80	720
10	100	700
12	120	680

200 µl of concentrated Bradford reagent was added to each tube, vortexed and spun down before incubating at room temperature for 5 min (total volume of 1 ml). The absorbance was measured immediately at 595 nm in DU® 640B spectrophotometer (Beckman Coulter, Fullerton, CA). A linear plot ($r^2 > 0.95$) of BSA standard was used to determine the concentration of protein samples.

2.6 NAD Glycohydrolase Enzyme Assay

2.6.1 Potassium Cyanide Method

This method was a modification of the assay described by Colowick *et al*. (1951). The microcentrifuge tube containing 52 µl of 0.1 M potassium phosphate, pH 7.0 and 17 µl β-NAD^+ was incubated at 37°C. The reaction was initiated by adding in 17 µl of properly diluted enzyme. At 0 min and 7.5 min, the reaction was terminated by pippetting in 514 µl of 1 M potassium cyanide. Potassium cyanide will react with β-NAD^+ to form a NAD-cyanide complex which has absorbance maximum at 327 nm. The reaction mixtures were cooled to room temperature and absorbance was read

against a blank of 52 μl 0.1 M potassium phosphate, pH 7.5, 17 μl H_2O, 17 μl diluted enzyme and 514 μl 1 M potassium cyanide.

A β-NAD^+ standard with a range of concentratrations of 45 μM, 90 μM, 135 μM and 180 μM was prepared as follows:

β-NAD^+ concentrations (μM)	**5.4 mM β-NAD^+ stock (μl)**	**H_2O (μl)**	**1 M potassium cyanide (μl)**
0	0	86	514
45	5	81	514
90	10	76	514
135	15	71	514
180	20	66	514

Enzyme activities of samples were calculated based on the standard curves constructed above. Total Activity and Specific Activity are defined as:

Total Activity = [μmol β-NAD^+ consumed/min/ml] x [total volume of sample]

Specific Activity = Total Activity/total protein (μmol/min/mg protein)

2.6.2 Fluorimetric Assay

NADase activity was determined as described by Ziegler *et. al.* (1996) using substrate analog, etheno-NAD^+ (Sigma, St. Louis, MO). The assay was carried out in a final volume of 180 μl reaction mixtures consisting of 50 mM potassium phosphate, pH 7.0 and 10 μM etheno-NAD^+ at 37^{o}C. The reaction was initiated by an addition of a properly diluted enzyme. The fluorescence intensity was monitored continuously at an excitation wavelength of 310 nm and emission wavelength at 410 nm. The

fluorescence measurement was done using LS 50B luminescence spectrophotometer (PerkinElmer, Wellesley, MA). The specific enzymatic activity was calculated from the initial linear slope of the fluorescence change (Δ) and expressed as Δ fluorescence intensity/min/mg protein.

2.7 ADP-Ribosyl Cyclase Activity Assay

The enzymatic activity of ADP-ribosyl cyclase was determined as described in Graeff *et. al*. (1994). In brief, proteins were incubated at 37°C for 15 min with 100 μM NGD^+ (Sigma, St. Louis, MO) in 20 mM Tris-HCl pH 7.2. The fluorescence intensity was monitored continuously at an excitation wavelength of 300 nm and an emission wavelength of 410 nm using LS 50B luminescence spectrophotometer (PerkinElmer, Wellesley, MA). The specific enzymatic activity was calculated from the initial linear slope and change in fluorescence (Δ) was calibrated from standard curves constructed with known concentrations of cGDPR. Results are expressed as a mean ± SD cGDPr formed in nmoles mg^{-1} min^{-1} protein.

2.8 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Native PAGE

SDS-PAGE and Native PAGE was run according to the method of Laemmli (Laemmli, 1970) by using a mini-PROTEAN® II electrophoresis system (Bio-Rad, Hercules, CA).

2.8.1 Non-Reducing SDS-PAGE

A 8% SDS-PAGE gel (see Appendix) was used to separate proteins of interest. Proteins were mixed with 5X non-reducing sample buffer (see Appendix) and placed on ice. The protein samples were loaded into each well and electrophoresis was carried out in cold room. Initially a voltage of 100V was applied until the sample had fully entered the stacking gel, after which the voltage was increased to 150 V. When the dye reached the bottom of the gel, the electrophoresis was stopped. For the determination of molecular mass, prestained SDS-PAGE standard protein marker, Precision Plus Protein™ Standards, (Bio-Rad, Hercules, CA, USA) was used.

2.8.2 Reducing SDS-PAGE

Proteins were mixed with 5X reducing sample buffer (see Appendix) and denatured by boiling water for 5 min before cooling in ice. After the samples had been loaded into wells, the electrophoresis was run at the room temperature with an initial voltage of 100V followed by 150V when the samples had fully entered stacking gel. Prestained SDS-PAGE standard protein marker, Precision Plus Protein™ Standards, (Bio-Rad, Hercules, CA, USA) was used.

2.8.3 Native PAGE

An 8% native PAGE gel (see Appendix) was used to separate proteins of interest. Proteins were mixed with 5X sample buffer for native PAGE (see Appendix) and placed on ice. The protein samples were loaded into each well and electrophoresis was carried out in cold room. Initially a voltage of 100V was applied until the sample

had fully entered the stacking gel, after which the voltage was increased to 150 V. When the dye reached the bottom of the gel, the electrophoresis was stopped.

2.9 Identification of NADase Activities in Gels after SDS-PAGE

In-gel substrate staining was done as described by Ziegler *et al.* (1997). After SDS-PAGE separation, the gel was washed 3 times in a solution containing 50 mM Tris/HCl, pH 7.5, and 0.5% LDAO with each 15 min. To visualize NADase activity, gels were then incubated for 15 min in a buffer consisting of 50 mM Tris/HCl, pH 7.5, 0.03% LDAO and 150 μM e-NAD^+. For native PAGE, the gel was subjected to buffer containing substrate without going through washing step. Gels were imaged using a gel-doc system running the GeneSnap program (Syngene, Cambridge, UK) and photographs were taken.

2.10 Silver Staining

Gels were fixed overnight with 25 ml/gel of fixer (see Appendix) with continuous shaking at room temperature. Gels were then rinsed twice with milli-Q H_2O for 5 min each. After the gels were incubated with sensitizer solution (see Appendix) for 30 min, gels were rinsed thrice with milli-Q H_2O again for 5 min each. Gels were incubated with cooled staining solution (see Appendix) for 20 min at 4^oC and rinsed twice with milli-Q H_2O for 1 min each before soaking in developing solution. Development was stopped when bands were clearly seen by displacing the developing solution (see Appendix) with stopping solution (see Appendix) for 10 min. Gels were rinsed with milli-Q H_2O thrice for 5 min each. Gels were imaged using a

gel-doc system running the GeneSnap program (Syngene, Cambridge, UK) and photographs were taken.

2.11 Immunoblotting

Immunoblotting was performed according to the method of Towbin *et al.* (1979). Briefly, the proteins resolved in the gel were electrophoretically transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA) using tank transfer system mini trans-blot® cell (Bio-Rad, Hercules, CA) at 100 V for 1.5 hours at room temperature. The transfer buffer (see Appendix) was prepared according to the method of Bjerum and Schafer-Nielsen (1986). After the proteins were transferred, the membrane was placed in blocking solution (see Appendix) and incubated at room temperature for 1 hour. The blot was then incubated with 1:1000 dilution of mouse-monoclonal anti-c-MYC (Sigma, St. Louis, MO) diluted with 1X TBST (see Appendix) containing 5% skim milk for overnight at 4^{o}C with continuous shaking. The blot was washed thrice with ample amounts of 1X TBST, 15 minutes for each wash at room temperature. After washing in 1X TBST, the blot was incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) diluted 5000X with 1X TBST containing 5% skim milk for 1 hr with continuous shaking. The blot was then washed with 1X TBST as before and developed using the ECL™ kit (GE Healthcare, Buckinghamshire, England) as instructed by the manufacturer. Briefly, an equal volume of ECL™ detection solution 1 was mixed with detection solution 2 (both solutions are provided in the ECL™ Western blotting kit). This mixture was directly added to the blot, which was subsequently incubated for 1

min at room temperature and immediately wrapped in plastic cling wrap. The signals on the blot were visualized by exposing to CL-X Posure^{TM} film (Pierce, Rockford, IL) for 2 to 5 min. A standard protein marker (Bio-Rad, Hercules, CA) was electrophoresed simultaneously for comparing the molecular weights of the visualized proteins in the membrane.

To probe the same membrane with mouse monoclonal anti-His (C-term) antibody (Invitrogen, Carlsbad, CA), the membrane was stripped with 10 ml of stripping buffer by incubating it at 50^{o}C for 30 min with occasional shaking. The membrane was then washed twice with TBST, 10 min for each. The membrane was blocked with 5 % skim milk dissolved in 1X PBST for 1 hour at room temperature with continuous shaking. The membrane was washed in 1X PBST 2X for 5 min each. Membrane was then incubated in 1:5000 dilution of anti-His (C-term) antibody in blocking buffer. The membrane was incubated at room temperature for 1 hour or at 4^{o}C for overnight with shaking. Membrane was transferred to a tray containing 1X PBST and washed twice for 5 min each. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) diluted 5000X with 1X PBST containing 5% skim milk for 1 hr with continuous shaking. The membrane was washed with 1 X PBST for 2X, 5 min each. This is followed by ECL detection as described earlier with exposure time of 2 to 10 min.

2.12 K_m Determination

Rate of reaction of enzyme activity (μmol of NAD^+ consumed/min) is determined using different concentration of β-NAD^+ (5 μM, 8 μM, 10 μM, 20 μM, 30

μM, 40 μM, 50 μM). A lineweaver-burke plot was then plotted. K_m was determined from the X-axis intercept.

2.13 Effect of pH on the Enzyme Activity

Enzyme assay was performed at a wide range of pH, pH 2 to 11. Enzyme acitivity was determined and compared to enzyme activity performed at pH 7.0.

2.14 Effect of Temperature on the Enzyme Activity

NADase was incubated at 4^{o}C for overnight, 55^{o}C for 1 hour and 80^{o}C for 10 min. Enzyme activity was then measured at different time points.

2.15 N-Linked Deglycosylation

Deglycosylation was performed according to manufacturer's instruction with modification. Basically, NADase was deglycosylated in native conditions. Twenty micrograms of proteins were brought up to 38 μl with deionized water. Ten microliters of 5X reaction buffer was added and followed by 2 μl of enzyme (5 Units/ml), Endoglycosidase H (Sigma, St. Louis, MO). The mixture was incubated at 37^{o}C for 1 hour and 3 hour. Enzyme activity was checked after each incubation time point and in-gel substrate staining was performed to check the size of the protein after deglycoslation.

2.16 Total RNA Preparation

Conidia were cultured in Vogel's minimal media and grown at 30^{o}C with shaking at 200 rpm for 18 hours. Culture was then harvested and washed with 0.1 % DEPC-treated water. Total RNA was extracted according to the procedure described by Sokolovsky *et al*. (1990). All manipulations were performed at room temperature if not stated otherwise. Culture was pulverized in a mortar with liquid nitrogen and transferred into a 2-ml Eppendorf tube containing 0.75 ml RNA extraction lysis buffer and 0.75 ml phenol (saturated with 0.1 M Tris-HCl, pH 8.0). The tube was mixed for 15-20 min using Intelli-mixer (Elmi, Riga, Latvia). Mixture was then centrifuged at 10,000 rpm for 10 min. Upper aqueous phase was transferred into an equal volume of Tris-HCl saturated phenol and vortexed. The mixture was then spun down again at 10,000 rpm for 10 min. Upper aqueous phase was once again transferred into a tube containing 0.75 volumes of 8 M LiCl and stored at 4^{o}C for overnight. The mixture was vortexed briefly before it was centrifuged at 10,000 rpm for 10 min. Pellet was resuspended with 0.3 ml 0.1 % DEPC-treated water, mixed with 3 M sodium acetate (pH 5.2) and 0.75 ml ethanol. The mixture was stored at -20^{o}C for 2 hours or at -70^{o}C for 30 min. After that, it was centrifuged at 10,000 rpm for 10 min. Precipitate was washed with 70 % ethanol. RNA pellet was then dried and dissolved in appropriate amount of 0.1 % DEPC-treated water. Total RNA was quantitated at 260 nm wavelength using DU® 640B spectrophotometer (Beckman Coulter, Fullerton, CA). The integrity of the extracted RNA was confirmed by electrophoresis in a 1.2% denaturing agarose gel (see Appendix).

2.17 First Strand cDNA Synthesis

First-strand cDNA was synthesized by mixing 5 µg total RNA with 300 ng oligo dT primer (Promega, Madison, WI) and incubating at 65˚C for 5 min, followed by cooling it at 42˚C for 2 min before adding 8 µl 5X reverse transcription buffer, 4 µl 0.1 M dithiothreitol (DTT), 20 U RNasin® ribonuclease inhibitor (Promega, Madison, WI), 20 nmol dNTP, 200 U Superscript™ II reverse transcriptase RNase H– (Invitrogen, Carlsbad, CA) and topped up with nuclease-free water to 20 µl. The reaction was incubated at 42˚C for 90 min, 70˚C for 10 min and held at 4˚C in a Mastercycler® ep Gradient S thermal cycler (Eppendorf, Hamburg, Germany). The cDNA was stored at –20˚C before analysis.

2.18 Polymerase Chain Reaction

PCR reaction was done using 100 ng of cDNA, 25 pmol of each gene-specific primer pairs, 5X Phusion HF buffer, 10 nmol dNTP, 1.0 U High Fidelity DNA polymerase (Finnzymes, Espoo, Finland) and topped up with ddH2O to 50 µl. Gene specific primers used were as follows: 1) forward primer for secretion expression 5'-gc**ctcgag***aaaaga*cttcccactagctcctcct-3' (with built in restriction site *Xho*I in bold, Kex2 cleavage signal is in italics); 2) forward primer for intracellular expression 5'-cg**gaattc**atgaagttcaccctcctctc-3' (with built in restriction site *EcoR*I in bold); 3) reverse primer 5'-aa**gcggccgc**gtagtttctaggaaccagcc-3' (with built in restriction site *Not*I in bold). PCR was performed in a Mastercycler® ep Gradient S thermal cycler (Eppendorf, Hamburg, Germany) under the amplification profile of 98ºC for 10 s, 57°C for 30 s, 72ºC for 30 s, for 35 cycles at the exponential phase of amplification. Samples

replaced with distilled water were used as water controls. The amplified PCR products were analyzed on an ethidium bromide-stained 1.2% TAE agarose gel and electrophoresed at 100 V for 20 min. The product size was determined by concurrently separating a 1 kb DNA ladder (Promega, Madison, WI) on the gel and visualized under UV transillumination. Gels were imaged using a gel-doc system running the GeneSnap program (Syngene, Cambridge, UK). Densitometric analyses were performed using Gel-Pro Analyser version 3.1 software (Media Cybernetics, Silver Spring, MD).

2.19 Restriction Enzyme Digestion

PCR products were purified using gel extraction kit (GeneAll, Seoul, Korea) and sequenced using BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) to confirm the sequence. Around 1.5 μg-1.8 μg of PCR products and 2 μg of plasmids vectors (pPICZB and pPICZalpha A, Figure 2.1) were added with 4 μl of 10X RE buffer, 2 μg acetylated BSA, 5 U of *Xho*I enzyme (secretion expression) or 5 U of *EcoR*I (intracellular expression) and 5 U of *Not*I enzyme (Promega, Madison, WI). The reaction mixture was topped up to 40 μl with ddH_2O and incubated at 37^oC for 1.5 hour. The RE digested products were gel-extracted and ready to be used for ligation.

For recombinant plasmids linearization, 10 μg of recombinant plasmids were added with 20 μl of 10X RE buffer, 10 μg of acetylated BSA and 25 U of SacI (Promega, Madison, WI). The reaction mixture was topped with ddH_2O to 200 μl and

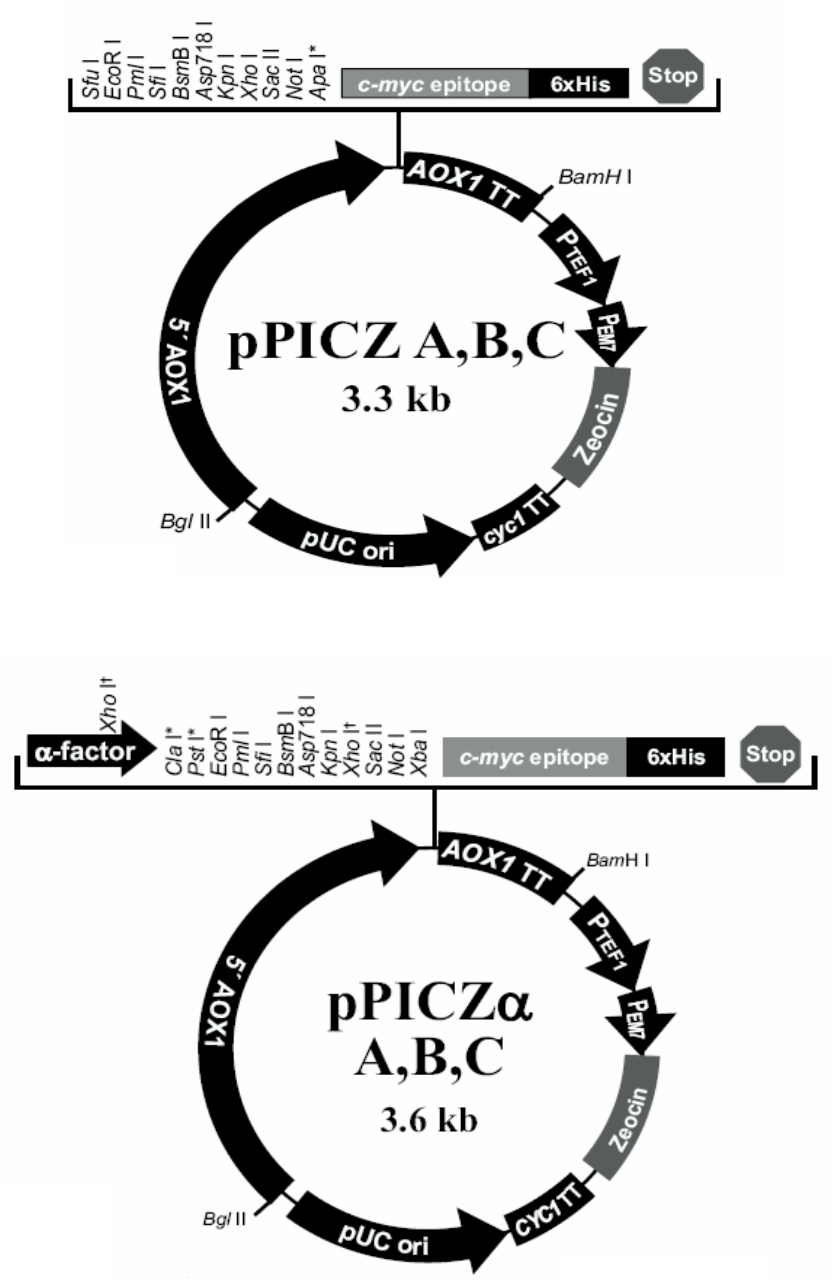


Figure 2.1. *Pichia* expression vectors for intracellular (A) and secretion (B) expression. pPICZB and pPICZalphaA were used in this project for protein expression. The gene of interest for intracellular expression was cloned into pPICZB between *Eco*RI and *Not*I restriction sites. For secretion expression, the gene was inserted into *Xho*I in α-factor sequence and *Not*I restriction sites of pPICZalphaA vector. The gene was cloned in frame with the C-terminal *myc* epitope tag and polyhistidine tag in both the expression vectors.

incubated at 37°C for 1.5 hour. Digested recombinant plasmids were gel-extracted.

2.20 Ligation and *E. coli* Transformation

One µl of vector DNA, 7 µl of insert DNA, 1 µl of 10X ligase buffer and 1 U of T4 DNA ligase (Promega, Madison, WI) were brought up to 10 µl. Ligase reactions were performed at room temperature for 3 hours.

Following the ligation, ligated DNA was transformed into TOP10F' competent cells. *E. coli* transformation was performed as by Ausubel *et al.* (1997). Competent cells were thawed rapidly and dispensed 100 µl immediately into 1.5 ml-Eppendorf tube containing the ligated DNA. The tube was gently swirled to mix and placed on ice for 10 min. Cells were heat-shocked by placing the tube into 42°C heat block for 2 min. After that, the cells were transferred into a green cap tube containing 1 ml low salt LB medium. The tube was incubated at 37°C for 1 hour with moderate shaking at 250 rpm. One hundred microliter of transformation culture was plated on low salt LB agar plates containing 25 µg/ml Zeocin (Invitrogen, Carlsbad, CA). Plates were incubated at 37°C for 12 to 16 hours.

2.21 Yeast Transformation

Pichia pastoris transformation was done according to manufacturer's recommendation. Wild type strain of *P. pastoris,* X33 was grown in 50 ml yeast extract peptone dextrose medium (YPD) at 30°C with shaking to an OD_{600} of 0.8-1.0. Cells were harvested and washed with 25 ml of sterile water followed by centrifugation at 1500 g for 10 min at room temperature. Water was discarded and

cells were resuspended with 1 ml of 100 mM LiCl. Cell suspension was transferred to a 1.5 ml Eppendorf tube and spun down at maximum speed for 15 s. Cells were resuspended in 400 µl of 100 mM LiCl. Fifty microliter of cell suspension was dispensed into a 1.5 ml Eppendorf tube for each transformation and used immediately.

One milliliter of 2 mg/ml denatured, fragmented salmon sperm DNA (Qbiogene, Morgan Irvine, CA) was boiled for 5 min and chilled on ice. Competent yeast cells were spun down and LiCl solution was removed. Two hundred and forty microliter of 50 % PEG (Sigma, St. Louis, MO), 36 µl 1 M LiCl, 25 µl 2 mg/ml salmon sperm DNA and 10 µg plasma DNA in 50 µl sterile water were added accordingly into the yeast competent cells. The tube was vortexed vigorously until the cell pellet was completely mixed and incubated at 30^{o}C for 30 min. Heat shocked was done in a heat block at 42^{o}C for 20-25 min. Tube was centrifuged at 8000 rpm for 15 s to remove the transformation solution. The pellet was resuspended in 1 ml of YPD and incubated at 30^{o}C with shaking at 200 rpm. After 1 hour and 4 hours, 100 µl of transformation culture was plated on YPD plates containing 100 µg/ml Zeocin. The plates were incubated at 30^{o}C for 2-3 days.

2.22 Direct PCR Screening of *Pichia pastoris* Clones

Direct PCR screening of pichia pastoris clones were done as described by Linder *et al*. (1996) with slight modification. A single colony was picked and resuspended in 10 µl of sterile water. Twenty five unit of lyticase (Sigma, St. Louis, MO) was added into the cell suspension and incubated at 30^{o}C for 10 min to 20 min. The sample was then frozen at -80^{o}C for 10 min. A 50 µl PCR reaction was set up for

a hot start by adding 10 µl of 5X colourless GoTaq Flexi buffer, 3 µl of 25 mM $MgCl_2$, 1 µl 10 mM dNTPs mix, 1 µl of 10 µM AOX1 5' primer (5'-GACTGGTTCCAATTGACAAGC-3') and AOX1 3' primer (5'-GCAAATGGCATTCTGACATCC-3') each followed by 5 µl of sample. The reaction mixture was topped up with sterile water to 50 µl and put into the cycler to heat them at 95°C for 5 min before 1.25 U of GoTaq (Promega, Madison, WI) was added into the tube. PCR was performed in a Mastercycler® ep Gradient S thermal cycler (Eppendorf, Hamburg, Germany) under the amplification profile of 95ºC for 1 min, 54°C for 1 min, 72ºC for 1 min, for 30 cycles followed by final extension at 72°C for 7 min. PCR products were analyzed by running a 10-µl aliquot of reactions on 1.2 % TAE agarose gel.

2.23 Mut Phenotype Determination

Five to six Zeocin-resistant colonies were picked and streaked on an minimal methanol (MM) plate first followed by an minimal dextrose (MD) plate. Plates were incubated at 30°C for 2 days. Mut^+ strain will grow normally on both plates. Mut^S strain will grow normally on MD plates but show little or no growth on MM plates.

2.24 Small Scale Expression Studies

A single colony of Mut^+ strain was inoculated into 5 ml buffered glycerol-complex (BMGY) medium in a 50 ml Falcon tube (CLP, San Diego, CA) to screen clones or 50 ml BMGY in 250 ml-conical flask for small scale expression. The culture was grown overnight at 30°C in a shaking incubator (250 rpm) until the culture

reached OD_{600} of 2 to 6. The cells were harvested by centrifugation at 3000 g for 5 min at room temperature. Supernatant was discarded and cell pellet was resuspended in buffered methanol-complex (BMMY) medium to an OD_{600} of 1.0 to induce expression. 100 % methanol was added into the culture to a final concentration of 0.5 % methanol every 24 hours to maintain induction.

To screen for optimum expression time point, at 0 hr, 24 hr, 48 hr and 72 hr time point, 1 ml of expression culture was transferred to a 1.5 ml Eppendorf tube. Culture was spun down at 13.4 rpm for 3 min at room temperature. Supernatant was transferred to a separate clean tube. Both cell pellets and supernatants were stored at – 80^{o}C until ready to assay.

2.25 Extraction of Yeast Protein

Cell pellets were thawed quickly and placed on ice. For 1 ml sample, 100 µl breaking buffer was added to the cell pellet. An equal volume of 0.5 mm glass beads (BioSpec Products, Bartlesville, OK) were added. The samples were vortexed for 30 s and then incubated on ice for 30 s. This is repeated for a total of 8 cycles. Samples were centrifuged at 16000 g for 10 min at 4^{o}C. Supernatants were transferred to fresh Eppendorf tubes.

2.26 NAD^{+} Cycling Assay

β-NAD^{+} was measured by the cycling enzymatic assay described by Graeff and Lee (2002), the sensitivity of which is in the nanomolar range. Briefly, reactions were conducted in black opaque 96-well plates. Forty nanomolar of β-NAD^{+} was

incubated with 0.1 unit/ml recombinant proteins in 20 mM sodium phosphate, pH 7.0 for 30 min at 37^{o}C. Enzymes were removed by filtration with Centricon-3 filters and samples were recovered in the filtrate after centrifugation at 4^{o}C for 50 min using Beckman JA 20 rotor. To 100 µl sample, the cycling reagent (0.1 ml) was then added, which contained 2% ethanol, 100 µg/ml alcohol dehydrogenase, 20 µM resazurin, 10 µg/ml diaphorase, 10 µM FMN, 10 mM nicotinamide, 0.1 mg/ml BSA and 100 mM sodium phosphate, pH 7.0. The cycling reaction was allowed to proceed for 2 hours and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically (15 min interval) using SpectraMax Gemini Fluorescence Reader (Molecular Devices, Sunnyvale, CA, USA). Standard solutions of β-NAD^{+} (ranging from 0-40 nM) were prepared in 20 mM sodium phosphate, pH 7.0, and taken through the same steps as the samples. The assay was done in triplicates.

2.27 Statistical Analysis

Differences between samples were analyzed from raw data using an unpaired two-tailed Student's *t*-test. $P < 0.05$ was taken as showing significance. All results are expressed as the mean ± SD and n refers to the number of determinations.

3. RESULTS

3.1 Comparison of NADase Activity between Mycelia and Conidia of *N. crassa*

In order to find out which stage of the life cycle of *N. crassa* contains the most abundance of NADase, mycelial and conidial proteins were extracted. Assays of enzyme activities of mycelial and conidial NADase were carried out and compared. The results showed that conidia had significantly higher specific enzyme activity of NADase than mycelia. The specific NADase activity of conidia and mycelia were 520.2 ± 36.3 μmol/min/mg protein and 0.112 ± 0.05 μmol/min/mg protein, respectively (Table 3.1). The specific enzyme activity of conidia was about 5000-fold higher than the specific enzyme activity of mycelia. Figure 3.1 showed the mobilities of the conidial and mycelial NADase on SDS-PAGE by catalyzing the NADase reaction using etheno-NAD^+ (e-NAD^+) as substrate to generate a fluorescent product, etheno-ADPR (e-ADPR). Mycelial NADase showed a smear band at the high molecular weight range, ranging from 250 kDa to 100 kDa, and a faint band at 42 kDa (Figure 3.1A). However, conidial NADase showed intense and sharp bands at 42 kDa and 75 kDa (Figure 3.1B). Figure 3.1C is the positive control using rat liver CD38, which is also a NADase.

3.2 Comparison of Conidial NADase with Partial Purified NADase from Sigma

Conidial NADase was also compared with partial purified NADase from Sigma in in-gel substrate staining. The partial purified NADase from Sigma is currently used as an endogenous NAD^+ remover in the cycling assay (Graeff and Lee, 2002). The conidial NADase had higher specific enzyme activity than the partial

Table 3.1. Specific enzyme activity of mycelial and conidial NADase from *N. crassa.* The data represent the mean ± SEM from three independent experiments performed in duplicates.

NADase	Specific enzyme activity (µmol/min/mg protein)
Mycelia	0.1118 ± 0.05
Conidia	520.2 ± 36.3

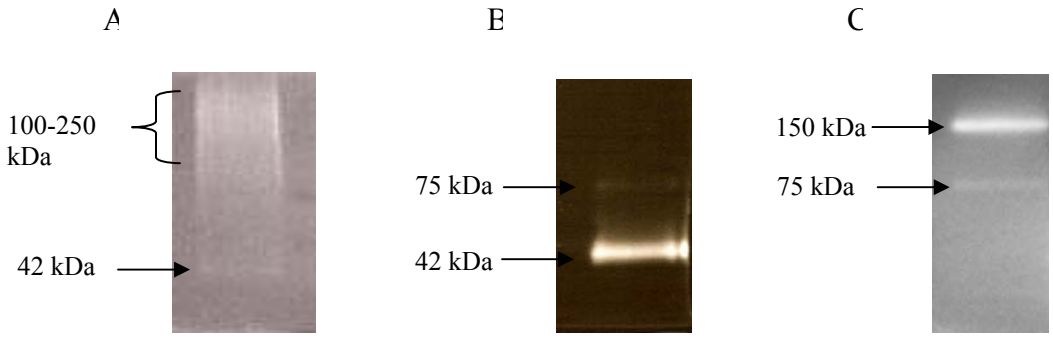


Figure 3.1. In-gel substrate staining of mycelia and conidia NADase. Twenty micrograms of mycelia crude enzyme (A) and conidia crude enzyme (B) were resolved in 8% non-reducing SDS PAGE. After electrophoresis, gels were washed with 50 mM Tris-HCl, pH 7.2 and 0.5% LDAO. Thereafter, gels were incubated with 50 mM Tris-HCl, pH 7.2, 0.03% LDAO and 150 µM e-NAD^+ as described in the Materials and Methods. Rat liver CD 38 (C) was used as a positive control.

Table 3.2. Specific enzyme activity of conidia NADase from *N. crassa* and partial purified NADase from Sigma. The data represent the mean ± SEM from three independent experiments performed in duplicates.

NADase	Specific enzyme activity (µmol/min/mg protein)
Conidia	520.2 ± 36.3
Sigma	6.540 ± 0.089

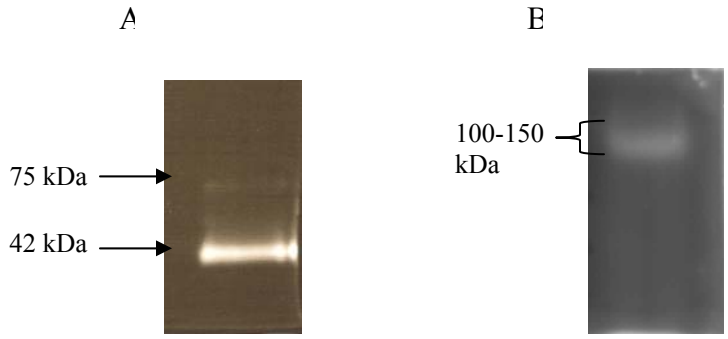


Figure 3.2. In-gel substrate staining of conidia NADase and partial purified NADase from Sigma. Twenty micrograms of conidia crude enzyme (A) and 15 µg of partial purified NADase from Sigma (B) was resolved in 8% non-reducing SDS PAGE. The gel was subjected to in-gel substrate staining thereafter.

purified NADase from Sigma, 520.2 ± 36.3 μmol/min/mg protein of conidial NADase compared to 6.540 ± 0.089 μmol/min/mg protein of Sigma NADase (Table 3.2). From Figure 3.2B, Sigma NADase showed a diffused band at high molecular weight range, similar to mycelial NADase.

3.3 Purification of Conidial NADase by Affinity Column

3.3.1 Cibcron Blue Agarose Purification

Conidia protein was extracted using KCl salt wash as described in the Materials and Methods. The crude protein was then applied to Cibacron Blue 3GA agarose affinity column. Table 3.3 demonstrated that after the purification step, the enzyme purity was increased 1.6-fold only with the loss of about 90% of the total enzyme activity. From Figure 3.3A lane 1, crude extract lane showed positive substrate staining at 42 kDa and 75 kDa band. The 42 kDa band had higher intensity than 75 kDa band. After the protein was purified by cibacron blue 3GA agarose, the staining spread across a wide range of molecular weight and this lane had higher intensity than crude extract lane even though same amount of protein was loaded into each lane (Figure 3.3A, lane 2). On the basis of the results in lane 1, the smear was contributed predominantly by 42 kDa and 75 kDa bands in lane 2. The purification factor is not high, but the purification step enabled NADase to be concentrated.

From the silver staining gel (Figure 3.3B, lane 2), even though the enzyme was partially purified, the proteins were quite well separated. It is clearly seen that the 75 kDa band was weakly stained as compared to 42 kDa band. The 42 kDa protein was more abundant than the 75 kDa protein. The staining of 42 kDa band in this lane

Table 3.3. Purification table for cibacron blue agarose purified NADase.

	Total protein (mg)	Total enzyme activity (µmol/15min)	Specific enzyme activity (µmol/15min/mg protein)	Purification (fold)	Yield (%)
Crude extract	6.74	8645.38	1282.7	1	100
Cibacron blue agarose	0.1416	281.15	1990.45	1.6	3.25

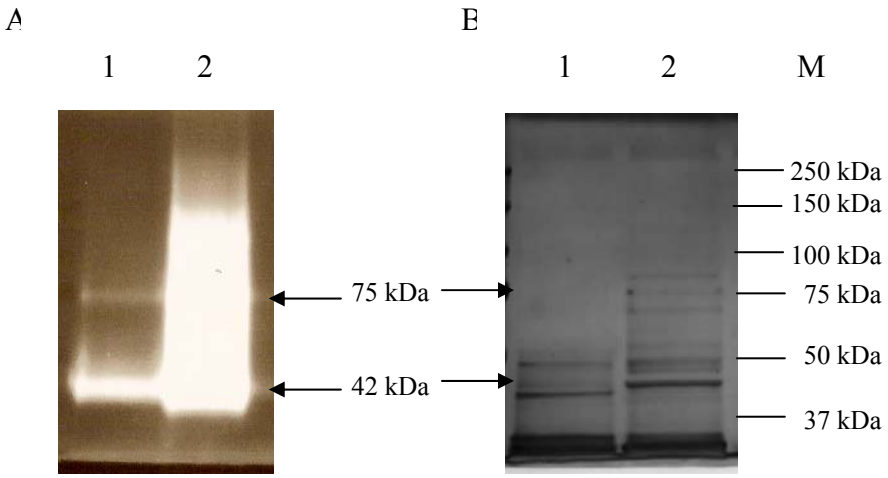


Figure 3.3. SDS PAGE analysis of cibacron blue purified NADase. Twenty micrograms of each crude extract (lane 1) and cibacron blue purified proteins (lane 2) were loaded into the 7.5% non-reducing SDS PAGE. (A) The gel was subjected to in-gel substrate staining. (B) Gel was stained with silver staining after in-gel substrate staining. Numbers on the right indicate the positions of marker proteins (M).

(Figure 3.3B, lane 2) was more intense than the 42 kDa band in crude extract lane (Figure 3.3B, lane 1). This further illustrated that the protein was concentrated after purification. However, the 75 kDa band cannot be seen in the crude extract lane (Figure 3.3B, lane 1) as in-gel substrate staining in Figure 3.3A, lane 1. The 42 kDa and 75 kDa bands (Figure 3.3B, lane 2) were cut out and subjected to mass spectrometry analysis.

3.3.2 Blue Sepharose CL6B Purification

The protein was also purified using blue sepharose (Amersham Biosciences) affinity chromatography. From the purification table, it showed that the specific enzyme activity was 4.4-fold higher after purification and the yield was 46%. For blue sepharose purified NADase, the protein was separated by native gel, followed by in-gel substrate staining and silver staining. One positive staining band was detected in the in-gel substrate staining of native gel while the crude extract exhibited a diffused band (Figure 3.4A). From the silver staining gel (Figure 3.4B), the blue sepharose lane (lane 2) was fairly pure compared to crude extract lane (lane 1). Basically, there were only 3 bands in the blue sepharose lane. These 3 bands were cut out for mass spectrometry analysis.

3.4 Characterization of Conidial NADase

The molecular weight of conidial NADase in its native form, K_m, effect of pH and temperature on the enzyme, N-linked glycosylation pattern of the enzyme were characterized.

Table 3.4. Purification table for blue sepharose purified NADase.

	Total protein (mg)	Total enzyme activity (Δfluo int/15min)	Specific enzyme activity (Δfluo int/15min /mg protein)	Purification (fold)	Yield (%)
Crude extract	10.35	$1.62x10^7$	$1.57x10^6$	1	100
Blue sepharose	1.09	$7.5x10^6$	$6.88x10^6$	4.4	46

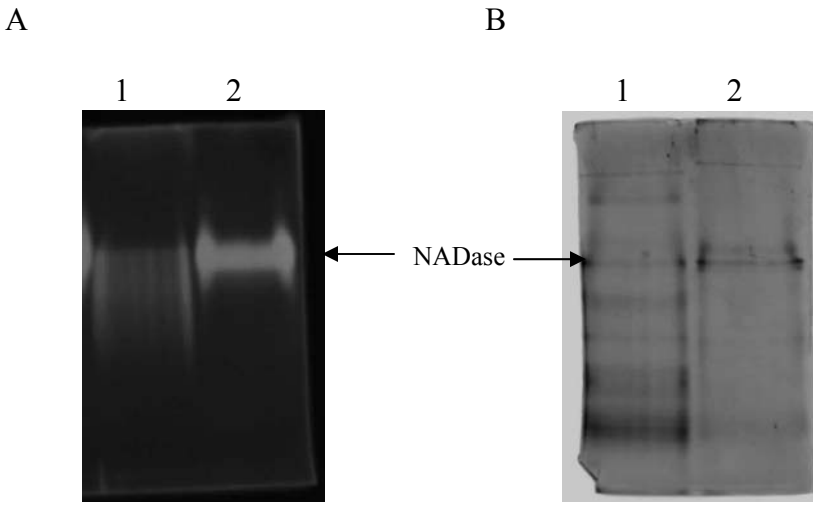


Figure 3.4. Analysis of blue sepharose purified protein by native PAGE. Crude extract (lane 1) and blue sepharose (lane 2) purified proteins were analyzed by 8% native PAGE. Five micrograms of proteins were loaded into each lane. After the electrophoresis was stopped, the gel was subjected to in-gel substrate staining (A) and then silver staining (B).

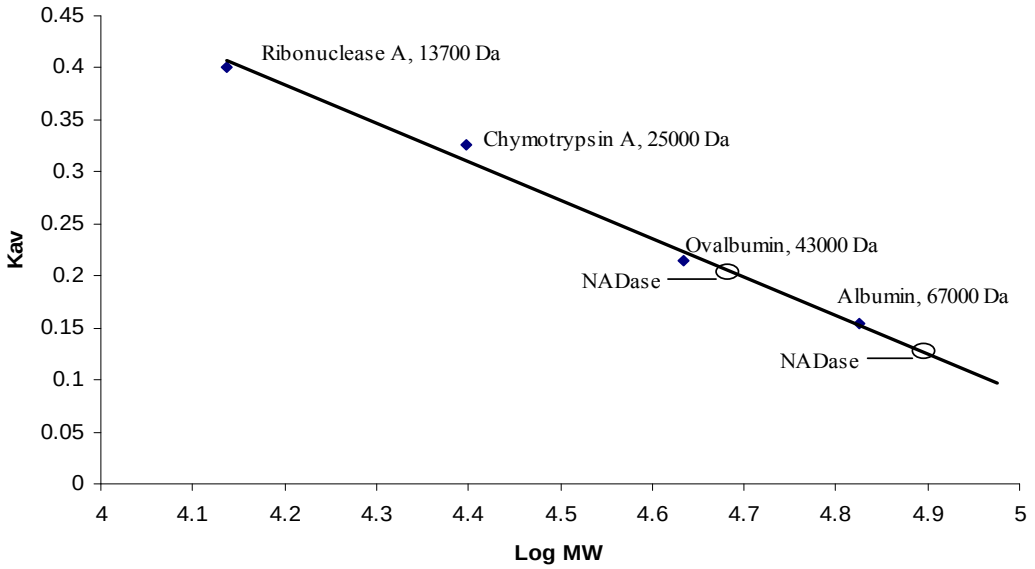


Figure 3.5. Calibration of Superdex 75 column and molecular weights of conidial NADase from *N. crassa*. The curve of K_{av} versus log molecular weight was plotted. Void volume for Blue Dextran (2000 kDa) and each elution volume were estimated by absorption at 280 nm. NADases were assayed using fluorimetric method. The blue colour diamond represents the standards while open circle represents the NADase.

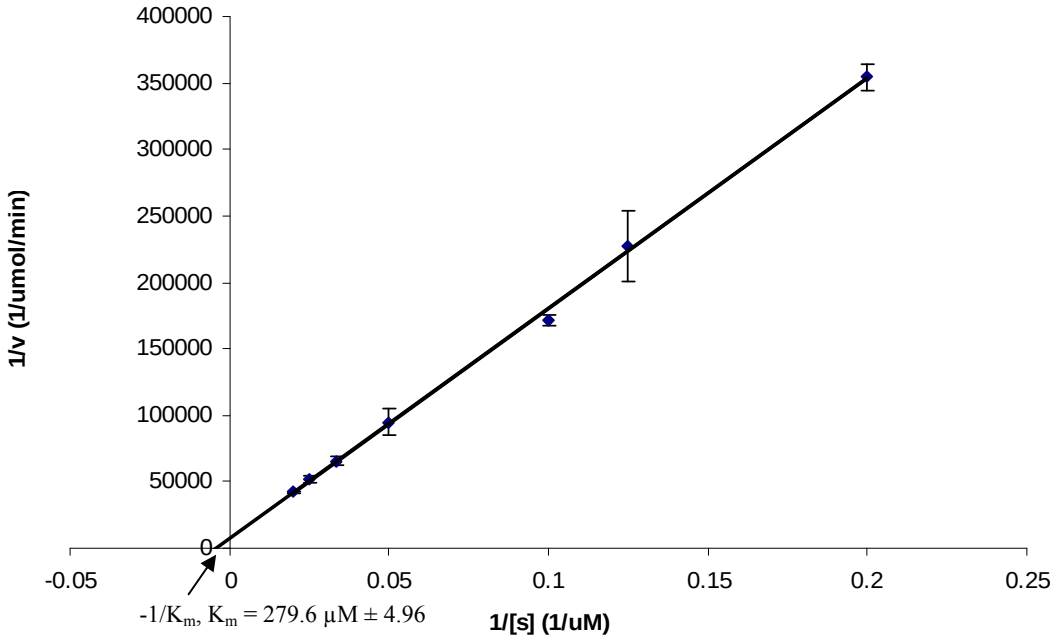


Figure 3.6. K_m determination of conidial NADase. $-1/K_m$ is intercept at x-axis of the Lineweaver-Burke plot. V: velocity; S; substrate.

3.4.1 Molecular Weight Determination of Conidial NADase

In order to determine the molecular weight of conidial NADase under native conditions, a FPLC gel filtration column, Superdex 75 was equilibrated with 0.05M sodium phosphate buffer, pH 7.5 in 0.15 M sodium chloride. The column was calibrated with ribonuclease A (13700 Da), chymotrypsin A (25000 Da), ovalbumin (43000 Da) and albumin (67000 Da). From the Figure 3.5, it illustrated that NADase corresponded to a molecular weight of around 80 kDa and 45 kDa in gel filtration. However, 80 kDa protein is the predominant form as this fraction has higher enzyme activity than 45 kDa protein fraction (data not shown).

3.4.2 Determination of K_m of the Conidial NADase

The K_m of NADase for β-NAD^+ was determined by measuring the enzyme activity of NADase using different concentrations of the substrate as described in the Materials and Methods. From the Lineweaver-Burke plot (Figure 3.6), the K_m was found to be 279.6 ± 4.95 μM.

3.4.3 Effect of pH and Temperature on Enzyme Activity

The NADase was assayed in a wide range of pH, from pH 2 to pH 11, to see the effect of pH on the enzyme activity. From Figure 3.7, pH 7 is the optimum pH for NADase. The enzyme activity began to fall below and above pH 7. The enzyme activity dropped sharply from pH 3 to pH 2. The enzyme could not function totally at pH 2 as no enzyme activity was detected. At pH 11, there was about 30 % of the enzyme activity remained.

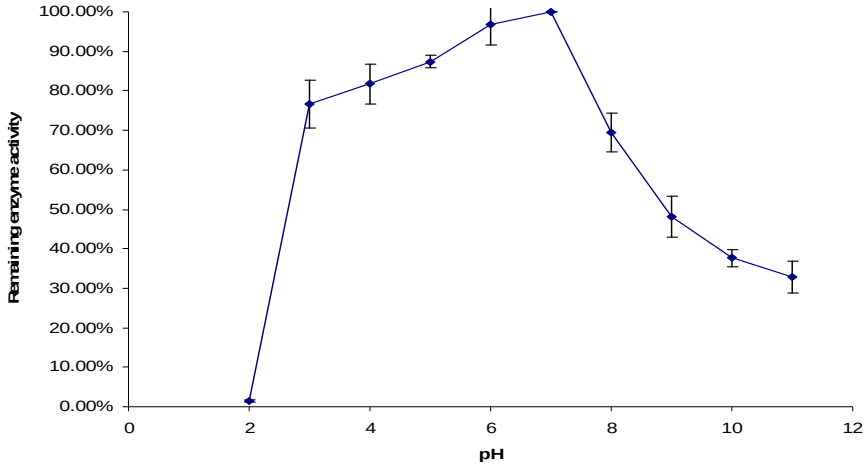


Figure 3.7. Effect of pH on NADase enzyme activity. NADase activity was tested in wide ranges of pH (pH 2 to pH 11). The enzyme activities were compared with enzyme activity carried out at pH 7. (n=3)

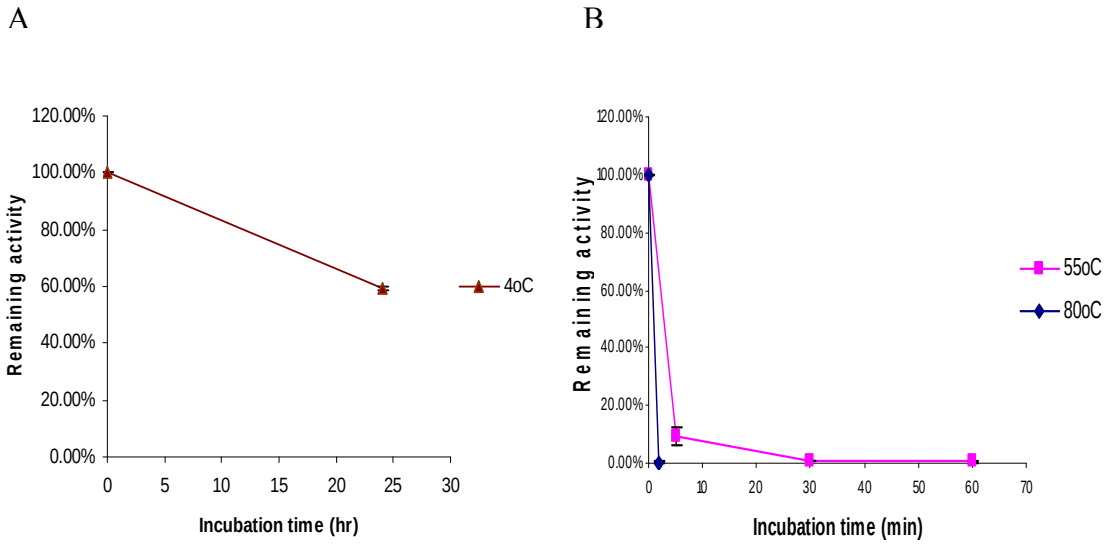


Figure 3.8. Effect of temperature on NADase enzyme activity. NADases were incubated in different temperature (4°C, 55°C and 80°C) and NADases were assayed as described in the Materials and Methods.

The effect on temperature on the enzyme was also investigated. The enzyme incubated at 4^{o}C for overnight, 55^{o}C for 1 hour and 80^{o}C for 10 min. Enzymes were aliquoted out for the determination of the enzyme activities at certain time points during incubation. After incubation at 4^{o}C for overnight, the enzyme activity decreased to 60% (Figure 3.8A). The enzyme was totally inactivated after an incubation at 55^{o}C for 30 min (Figure 3.8B). However, an incubation of the enzyme at 80^{o}C for 2 min was sufficient to inactivate the enzyme.

3.4.4 N-Linked Deglycosylation

The N-linked carbohydrate is the most common form of glycosylation. To investigate whether the conidial NADase is N-link glycosylated, endoglycosidase H was used to remove asparagine-linked oligomannose and hybrid, but not complex, oligosaccharides from glycoproteins. It cleaves between the two N-acetylglucosamine residues in the diacetylchitobiose core of the oligosaccharide, generating a truncated sugar molecule with one N-acetylglucosamine residue remaining on the asparagine. Endoglycosidase H reduced the size of NADase about 10 kDa, from 42 kDa to 32 kDa (Figure 3.9). One hour incubation completely removed the N-link glycosylation because incubation up to 3 hours did not reduce the size of NADase further. Deglycosylation did not affect the enzyme activity of the NADase.

3.5 Mass Spectrometry Analysis

The 42 kDa and 75 kDa bands from the cibacron blue purified protein lane and 3 bands from native PAGE of blue sepharose purified protein lane as described in

Section 3.3, were cut out and sent for MALDI-TOF-TOF (Matrix-assisted laser desorption/ionization- Time of Flight) mass spectrometry to identify the protein. The trypsin-digested proteins were mixed with matrix and subjected to MALDI-TOF. Set of peptides obtained was matched with *N. crassa* database for peptide mass fingerprinting. From the mass spectrometry results summary table (Table 3.4), gi| 85106032 (hypothetical protein) was the only candidate protein that appeared 3 times in all the analyses, from the 42 kDa band, 75 kDa of cibacron blue agarose purified gel and middle band from blue sepharose purified protein lane in native gel. A search from National Center for Biotechnology Information (NCBI) was performed on this candidate protein, the identified gene is located in on linkage group IV (chromosome IV) of the genome and it is a secreted protein.

Other protein candidates identified from MALDI-TOF analysis were known proteins which were not likely to be the NADase. Most of the enzymes matched were dehydrogenases which take part in energy metabolism. One common similarity shared by these protein candidates is that they use NAD^+/NADH as cofactor.

Table 3.5. Summary for mass spectrometry results.

Cibacron blue agarose		Blue sepharose	
42 kDa band	75 kDa band	Middle band	Top and bottom bands
NADP-specific glutamate dehydrogenase	glutathione-disulfide reductase	Protein similar to alcohol dehydrogenase	Gi|85089847 hypothetical protein
protein similar to 6-phosphogluconate dehydrogenase	Gi|85106032 hypothetical protein	Gi|85106032 hypothetical protein	Gi|85091612 hypothetical protein
Gi|85106032 hypothetical protein	related to alpha-glucosidase b		
	protein similar to cellobiose phosphorylase		

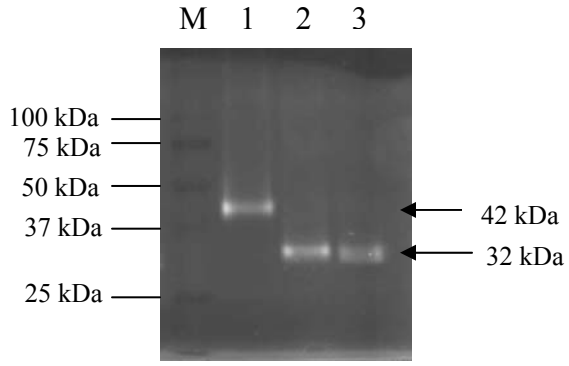


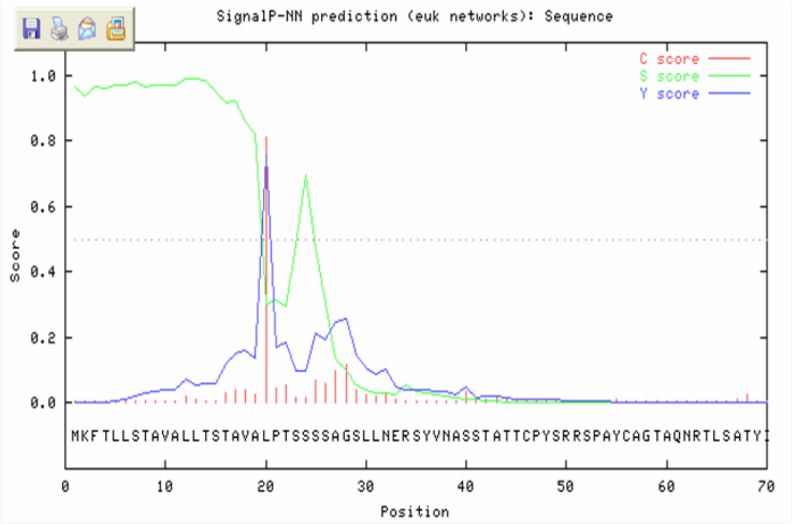
Figure 3.9. Endoglycosidase H treatment of conidia NADase. Blue sepharose purified proteins were subjected to N-link deglycosylation by endoglycosidase H. After 1 hour and 3 hours incubation, the size of NADase was checked by running the 8% SDS-PAGE. Lane 1, untreated conidia NADase; lane 2, NADase treated with endoglycosidase H for 1 hour; lane 3, NADase treated with endoglycosidase H for 3 hours; lane M, marker.

3.6 Construction of Gi|85106032 Recombinant Proteins in *P. pastoris*

To identify the signal peptide of the identified protein, SignalIP prediction tool was used to predict the secretion signal sequence. The probability of this protein being a secreted protein is 1.0 and the cleavage site is between the alanine and isoleucine amino acid residues (Figure 3.10). The full length sequence and mature sequence of Gi|85106032 was shown in Figure 3.11. The mature cDNA sequence was fused to the prepro region of the α-factor mating secretion signal in the vector pPICZαA. To express the intracellularly expressed protein, the full length cDNA with native secretion signal was cloned into vector pPICZB.

Total RNA isolated from the conidia culture which had been grown for 18 hrs in liquid media, was transcribed to first strand cDNA and amplified by PCR using specific primers for gi| 85106032 as described in Section 2.16 in the Materials and Methods. Figure 3.12 showed the PCR products for the intracellular and extracellular expression. The cDNA encoding the intracellular expressed protein had the size of 747 bp while the cDNA encoding secreted protein was about 696 bp. PCR products were ligated into respective linearized vectors and transformed into TOP10 F' (*E. coli* strain). Cloned plasmids were isolated and RE digested to confirm the size and sequence. All the intracellular and extracellular clones isolated had the correct sizes of both vector and cDNAs. The extracellular expressed plasmids had 3.6 kb vector and 686 bp insert (Figure 3.13A). The intracellular expressed plasmids had 3.3 kb vector and 736 bp insert (Figure 3.13B).

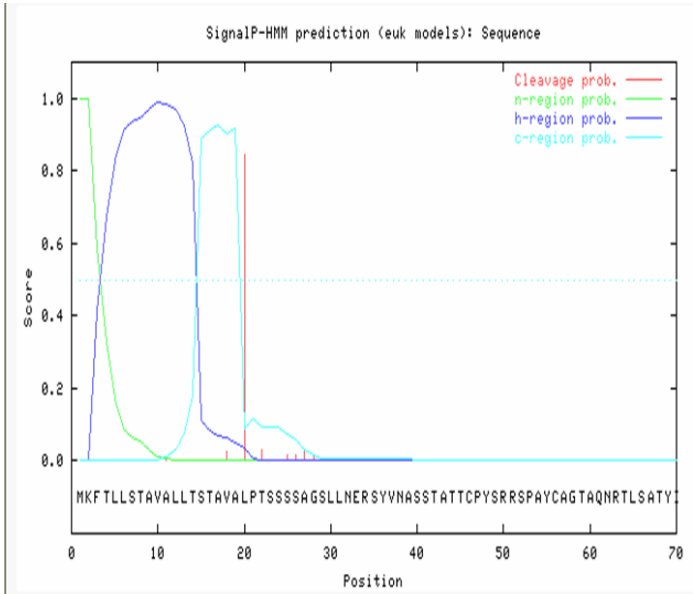
A




```
# data

>Sequence                length = 70
# Measure  Position  Value  Cutoff  signal peptide?
  max. C    20       0.810   0.32   YES
  max. Y    20       0.760   0.33   YES
  max. S    13       0.989   0.87   YES
  mean S     1-19    0.951   0.48   YES
       D     1-19    0.855   0.43   YES
# Most likely cleavage site between pos. 19 and 20: AVA-LP
```

B




```
# data

>Sequence
Prediction: Signal peptide
Signal peptide probability: 1.000
Signal anchor probability: 0.000
Max cleavage site probability: 0.843 between pos. 19 and 20
```

Figure 3.10. Signal peptide prediction of gi|85106032. The signal peptide sequence of gi|85106032 was predicted using a prediction software, SignalIP in EXPASY website. There were 2 prediction methods used in this software, SignalIP-NN and SignalIP-HMM methods.

1 atgaagttca ccctcctctc caccgccgtc gccctcttga ccagcaccgc cgtcgcc**ctt**
61 **cccactagct cctcctctgc tggaagtctc ctcaacgagc gctcctacgt caacgcctcc**
121 **tccaccgcca ccacctgccc ctacagccgg cgctccccgg cttactgcgc cggcaccgcg**
181 **caaaaccgga cgctctcggc aacctacatc tgcggcgact cgcgcctggg gcccgtcgtg**
241 **ctgccgcagt tctttttgcc gctggatccc attctcgaca tctacgaccg cttcggcggg**
301 **ctgtgcccgg gcgccttttt ggaaaagtgg ttcaaccaga cgggcagcgg ctggtgggac**
361 **tacccgcccc aaaacggctt cagtgtcgat gatgaaggga acatcatcgc ggccaacttg**
421 **acgctgcaga cgggcacgtt tgtggaccgg ttcggcagcg agtatggcag tttcctggcg**
481 **ccggcggcgg cgccgtatct gcagaggagt ttgccgccta gtaatttgaa tggggatgcc**
541 **aagttcccgt ggaactacca cgtttacagc gtcatcaagc cctttgctgt ccttgctgga**
601 **cccatcgccc cgtggttcgg ccagcctggt cagggcgtgc agtaccagac gtatgagaac**
661 **gtcgcgacgc tgattgctga tgggtatctg aaagctgagg atccccagag gctggttcct**
721 **agaaactact ag**

Figure 3.11. Full length sequence of gi|85106032. The mature sequence of gi|85106032 is in bold while the stop codon is in red.

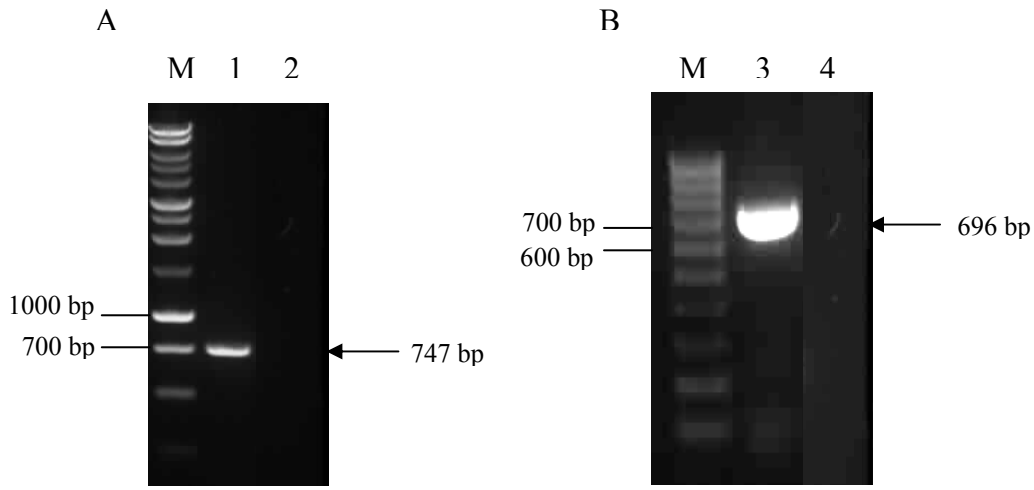


Figure 3.12. PCR products of gi|85106032. Sequences for intracellular expression (A) was amplified from full length cDNA while secretion expression (B) were amplified from the mature sequence. The sequences were amplified using Finnzyme, proof-reading DNA polymerase as described in the Materials and Methods. Lane 1, PCR products for intracellular expression; lane 3, secretion expression PCR products; lane 2 and 4, H_2O control; lane M, marker.

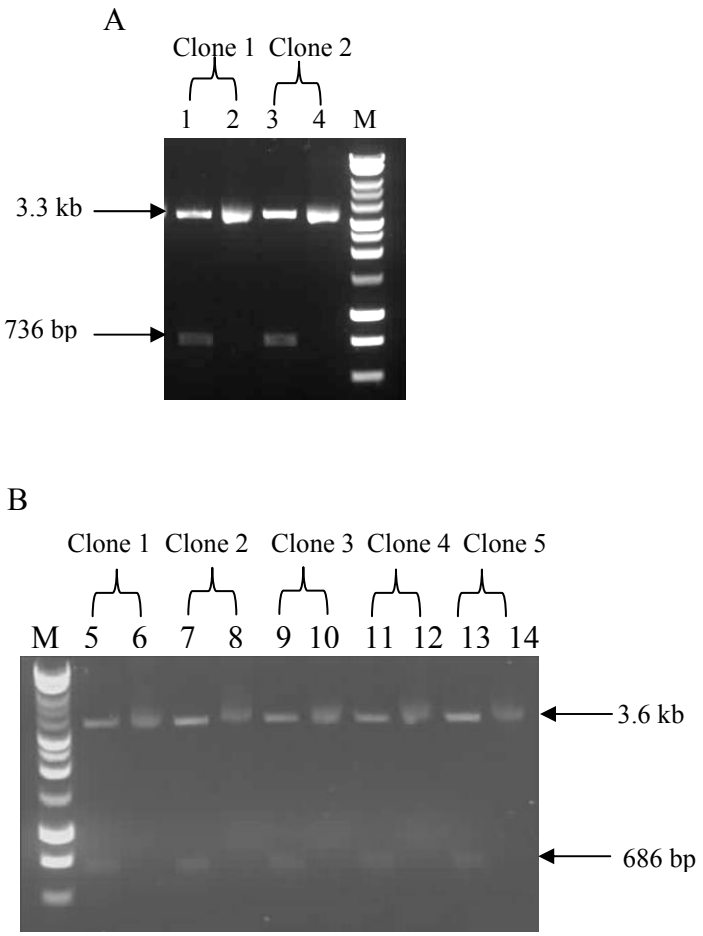


Figure 3.13. Analysis of *E. coli* transformants. Recombinant plasmids were isolated from *E. coli* transformants and subjected to restriction enzyme digestion. (A) pPICZB-gi|85106032 recombinant plasmids were RE digested by *EcoR*I and *Not*I. (B) pPICZalphaA-gi|85106032 recombinant plasmids were digested by *Xho*I and *Not*I restriction enzymes. Lane 1,3,5,7,9,11,13, plasmids treated with restriction enzymes; lane 2,4,6,8,10,12,14, uncut plasmids; lane M, marker.

A B

Clone 1	Clone 2	Clone 3	Clone 4
Clone 5		Positive control	

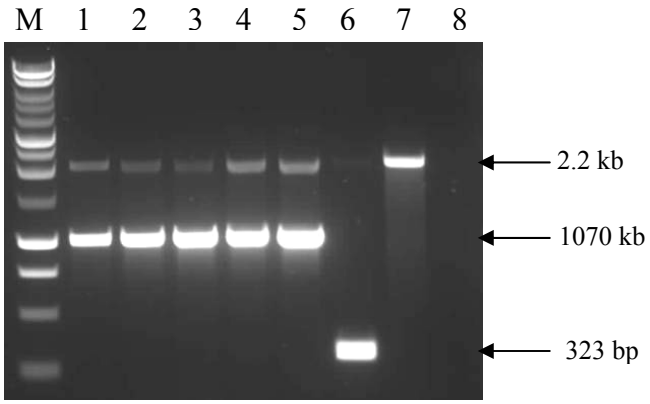
Figure 3.14. Mut phenotype determination of intracellular expression clones, pPICZB-gi|85106032. Zeo^R clones were patched on minimal dextrose agar plate followed (A) by minimal methanol agar plates (B). Mut^+ clones will be able to grow on both agar plates.

A B

Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
Clone 6	Clone 7	Clone 8		Positive control

Figure 3.15. Mut phenotype determination of secretoion expression clones, pPICZalphaA-gi|85106032. Zeo^R clones were patched on minimal dextrose agar plate followed (A) by minimal methanol agar plates (B). Mut^+ clones will be able to grow on both agar plates.

A



B

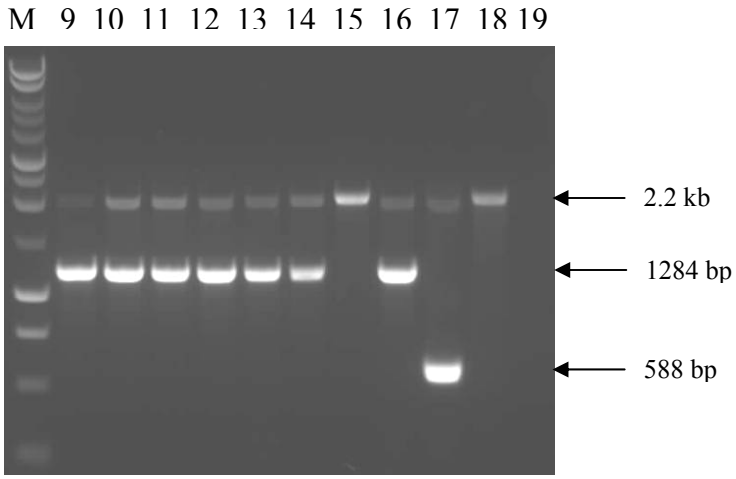


Figure 3.16. Direct yeast colony PCR. Five colonies of pPICZB-gi|85106032 *P. pastoris* transformants (A) and 8 pPICZalphaA-gi|85106032 colonies (B) were subjected to colony PCR. Mut^+ clones would have the foreign gene insert and an additional band at 2.2 kb. Lane 1-5, pPICZB-gi|85106032 yeast transformants; lane 9-16, pPICZalphaA-gi|85106032 transformants; lane 6 and 17, yeast transformed with empty plasmids; lane 7 and 18, untransformed yeast; lane 8 and 19, H_2O controls.

Recombinant plasmids were amplified by TOP10 F' and transformed into X33 using chemical transformation. The Mut^+ phenotype of the clones were confirmed by Mut phenotype determination. Figure 3.14 and Figure 3.15 showed that all clones were Mut^+ clones as all grew on MM and MD plates.

Five clones for intracellular expression and 8 clones for secreted expression were randomly selected to perform the yeast colony PCR and check for Mut^+ clones. All the 5 clones for intracellular expression were Mut^+ clones as all had the gi| 85106032 insert and 2.2 kb AOX1 gene (Figure 3.16A). However, 1 out of the 8 secretion expression clones did not contain the insert (Figure 3.16B). The remaining clones were Mut^+ clones.

3.7 Small Scale Expression of pPICZB-gi| 85106032 and pPICZalphaA-gi|85106032 Recombinant Proteins

In both of the expression vectors, the heterologous NADase protein is expressed under the control of AOX1 promoter. When the recombinant yeast is cultured in the media containing methanol, the heterologous protein is expressed. As seen from the western blot of intracellular expression, all clones expressed the protein with molecular weight at 37 kDa (Figure 3.17A). The positive control was a recombinant protein containing α-c-myc tag (Invitrogen, Carlsbad, CA). But, clone 1 and clone 5 expressed significant amounts of proteins compared to clone 2 to clone 4. After the recombinant proteins were purified with His-tag column, the band intensities increased (Figure 3.17B).

Surprisingly, there were detectable amount of recombinant proteins detected in the supernatants of all intracellularly expressed clones and the protein had the molecular weight of 50 kDa (Figure 3.18A). This suggested that the secretion signal from the *N. crassa* can be recognized by the *P. pastoris*. From the Figure 3.18B, all 7 secreted expression clones also expressed the recombinant proteins with clone 5 expressed the highest amount of protein. The molecular weight of secreted expressed protein was not the same as molecular weight of intracellularly expressed recombinant protein but was about the molecular weight of proteins detected in the supernatant of intracellular expression. Two forms of proteins were expressed, 50 kDa and 55 kDa proteins. For the negative controls in Figure 3.18, no bands were detected. The negative controls used were the X33 transformed with empty plasmid, pPICZB (Figure 3.18A, lane 6) or pPICZalphaA (Figure 3.18B, lane 14).

In order to follow the time course of pPICZB-gi| 85106032 and pPICZalphaA-gi|85106032 expression and to determine the optimum time to terminate the yeast culture, 1 ml aliquot of culture media were removed daily during 4 days after methanol induction. Western blot analysis was performed to detect the abundance of recombinant protein. Clone 1 of intracellular expression and clone 5 of secretion expression were used in the time course study. Clone 1 of intracellular expression expressed the highest amount of proteins at 24- and 48-hr (Figure 3.19A). Extending beyond 2 days resulted in a decrease of recombinant protein expression. However, for clone 5 of secretion expression, the optimum time for expression is at 72-hr (Figure 3.19B). At 96-hr, there was an insignificant decrease in protein amount. Thus, the

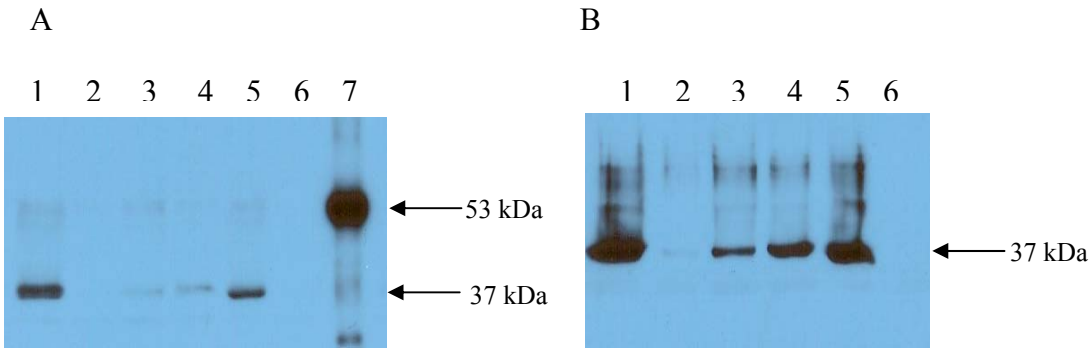


Figure 3.17. Western blot analysis of intracellularly expressed recombinant proteins expressed by *P. pastoris.* Thirty micrograms of crude extract lysate (A) and His-tag column purified proteins (B) were loaded into 8% SDS PAGE. Proteins were detected by incubating the membrane with α-c-myc antibody. The amount of positive control for α-c-myc tag loaded was 2.5 µg. Lane 1, clone 1; lane 2, clone 2; lane 3, clone 3; lane 4, clone 4; lane 5, clone 5; lane 6, clone 6; lane 7, positive control (recpmbinant protein containing α-c-myc tag). Data represent 3 independent experiments.

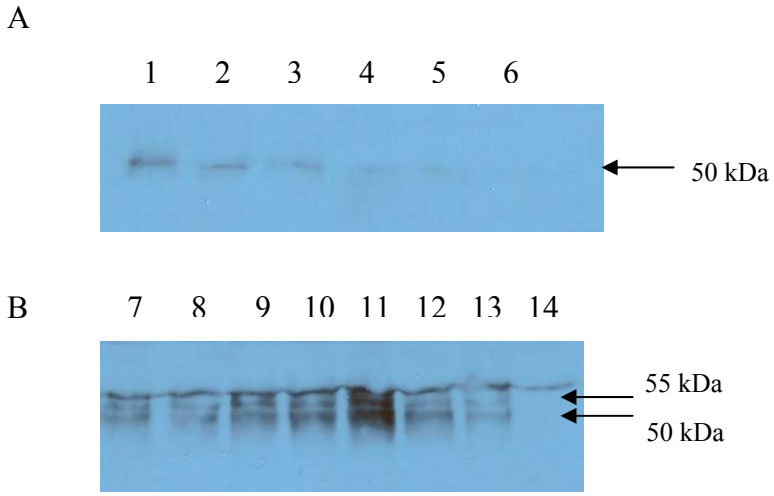


Figure 3.18. Western blot analysis of supernatant of intracellular expression and secretion expression recombinant proteins expressed by *P. pastoris.* Cultures were spun down and 40 µl of supernatant of intracellular expression (A) and secretion expression (B) were resolved in 8% SDS PAGE. Lane 1-5, intracellular expression clone 1-5; lane 7-13, secretion expression clone 1-6 and 8; lane 6 and 14, negative controls (X33 transformed with respective empty vectors). Data represent 3 independent experiments.

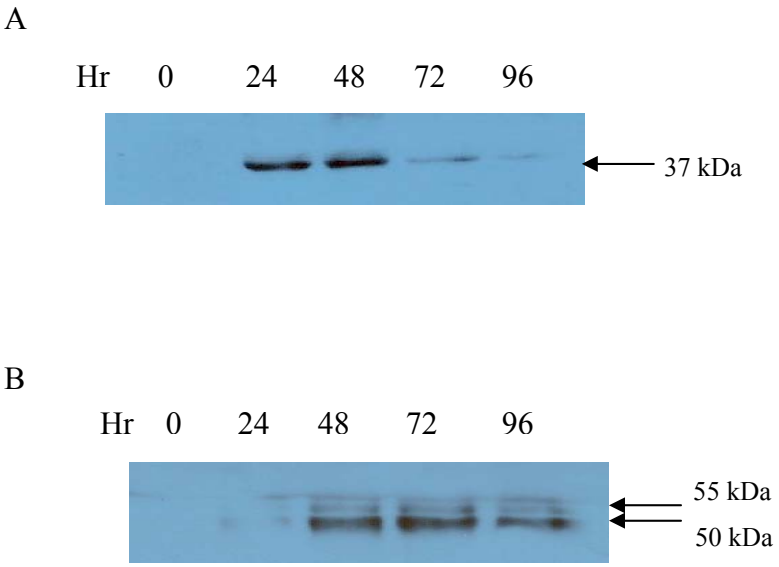


Figure 3.19. Time course of intracellularly expressed and secreted recombinant proteins. Both intracellular expression clone 1 (A) and secretion expression clone 5 (B) were cultured for 96 hours. One milliliter of culture was collected every 24 hour. Thirty micrograms of crude extract lysate and 20 µl of supernatant of secretion culture were resolved in 8% SDS PAGE and detected using α-c-myc antibody. Data represent 3 separate determinations.

optimum time for expression is at 24-hr and 72-hr for intracellular and secretion expression, respectively.

3.8 Enzyme Activity Check

Both intracellularly and extracellularly expression proteins were subjected to in-gel substrate staining to check the enzyme activities and size of the recombinant proteins in non-reducing condition. Seen from Figure 3.20, all clones, including the supernatants of intracellular expression, showed positive substrate staining. Under non-reducing condition, the stained bands had the molecular weight of about 100 kDa. The enzyme activity was also confirmed by fluorimetric NADase enzyme assay. All recombinant proteins catalyze the breakdown of e-NAD^+ by showing an increase in the fluorescence intensity over the time as a result of the formation of fluorescent product, e-ADPR (Figure 3.21). No cyclase activity was detected from the recombinant proteins samples (Figure 3.22). This further confirmed that the recombinant proteins only possess NADase activity.

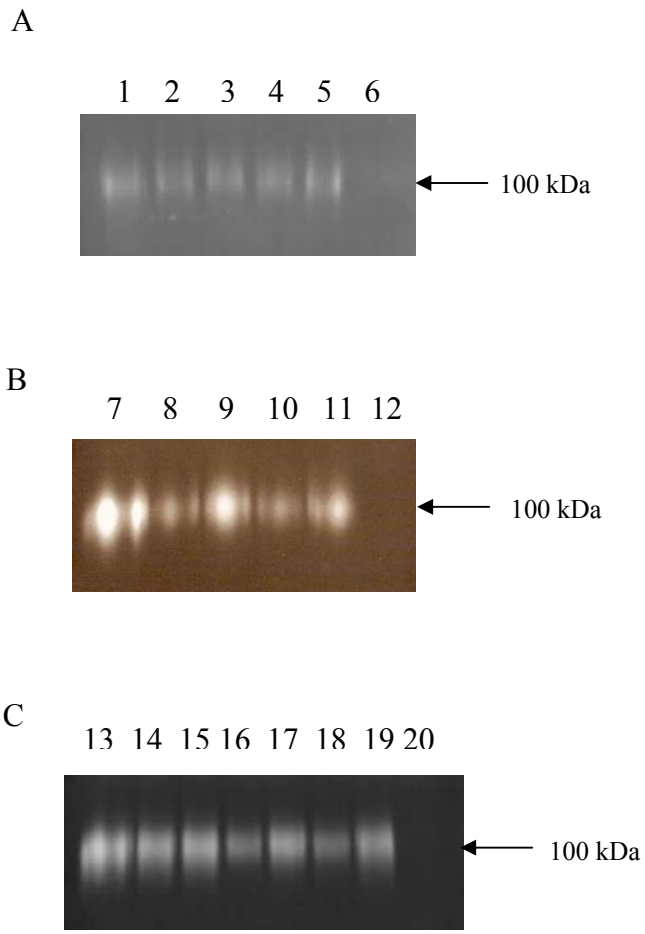


Figure 3.20. Enzyme activity check by in-gel substrate staining. Thirty micrograms of intracellularly expressed crude extract lysate (A), 40 µl of supernatant of intracellular expression (B) and supernatant of secretion (C) expression proteins were loaded into 10% SDS PAGE. Lane 1-5, clone 1-5; lane 7-11, clone 1-5; lane 13-19, clone 1-6 and 8; lane 6, 12 and 20, negative controls.

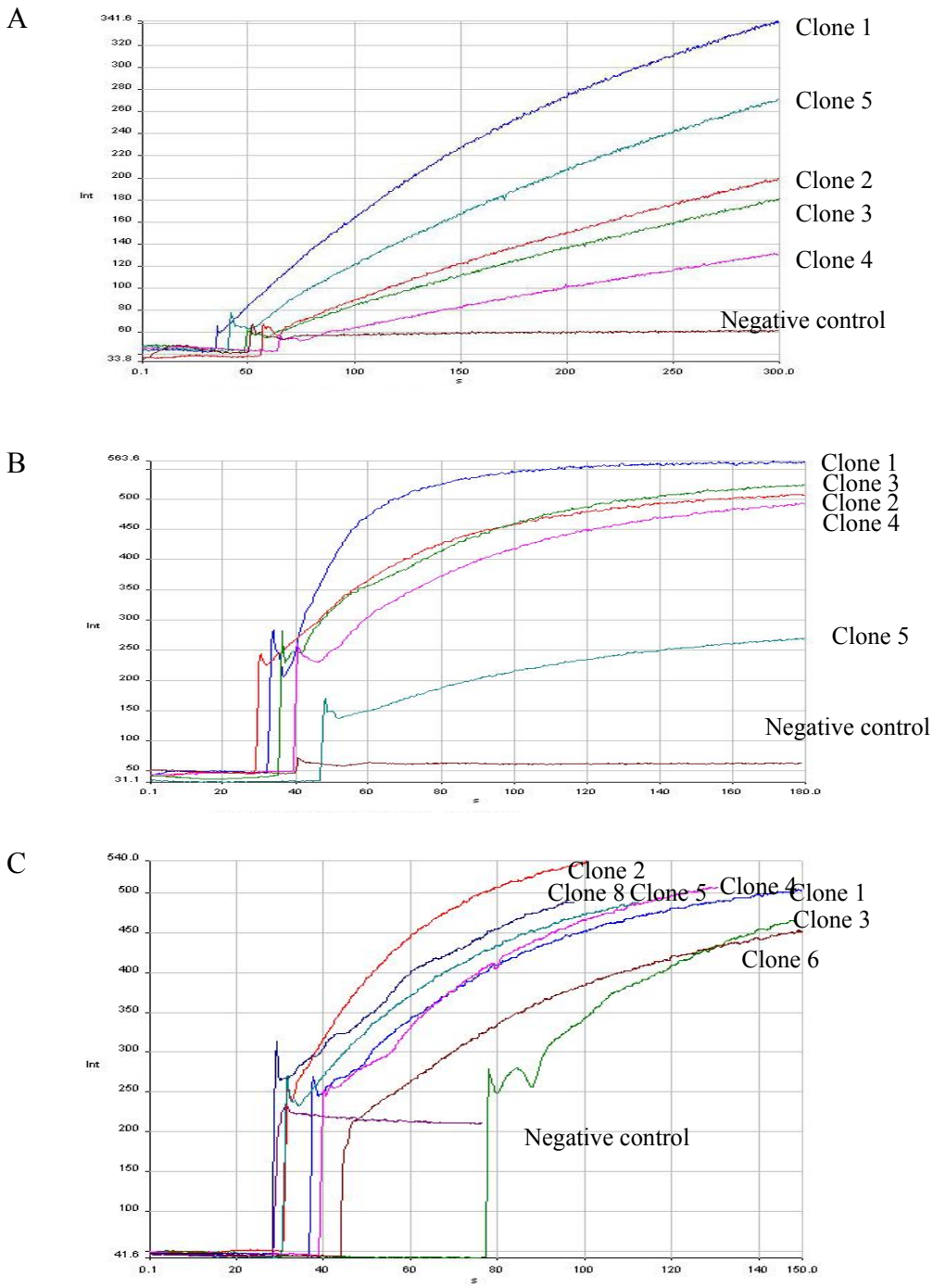


Figure 3.21. Fluorimetric NADase enzyme assay. The crude extract lysate (A), supernatant of intracellular expression (B) and supernatant of secretion expression were checked for enzyme activity using e-NAD+ as substrate as described in the material and methods. The excitation and emission wavelength were 310 nm and 410 nm respectively. Data represent 3 independent experiments.

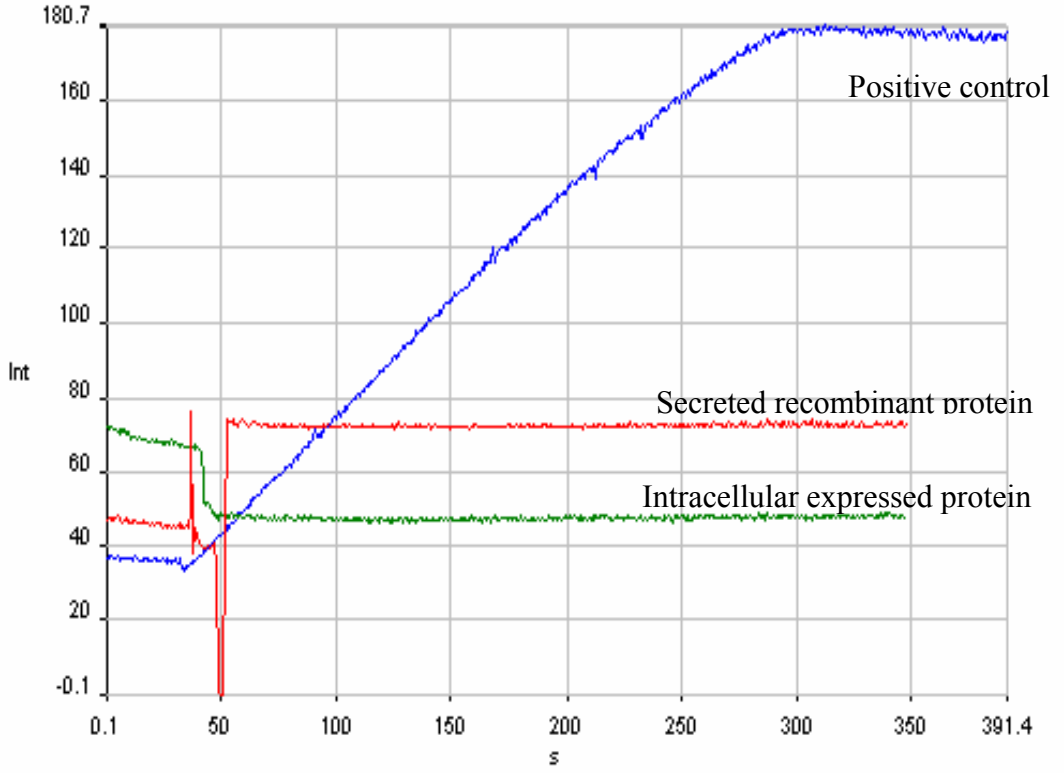


Figure 3.22. Analysis of ADP-ribosyl cyclase activity. Representative tracing of GDP-ribosyl cyclase activity (expressed in fluorescence intensity in arbitrary units) versus time (seconds) in the presence of 100 µM NGD^+ with 20 µl of crude lyaste of intracellular clone 1 or supernatant of serection clone 5 incubated at 37ºC in 20 mM Tris-HCl pH 7.2. Lysate of CD 38 transfected Hela cells was used as positive control. No cyclase activity was detected in recombinant proteins. Data represent 3 independent experiments.

3.9 NAD^+ Cycling Assay

The recombinant proteins which contained the NADase activity was applied in the NAD^+ cycling assay to test its effectiveness in catalyzing conversion of β-NAD^+ into ADP-ribose and nicotinamide. Known amount of NAD^+, 40 μM of NAD^+, was incubated with recombinant proteins followed by conversion of residual NAD^+ into resorufin fluorescence by a coupled-enzyme cycling reactions. Figure 3.23 showed that NAD^+ was successfully removed as residual NAD^+ left was less than 3% in the incubation with secreted protein while it was less than 1% in the incubation with intracellularly expressed protein. The partial purified Sigma NADase was used as positive control and no enzyme was added in the negative control.

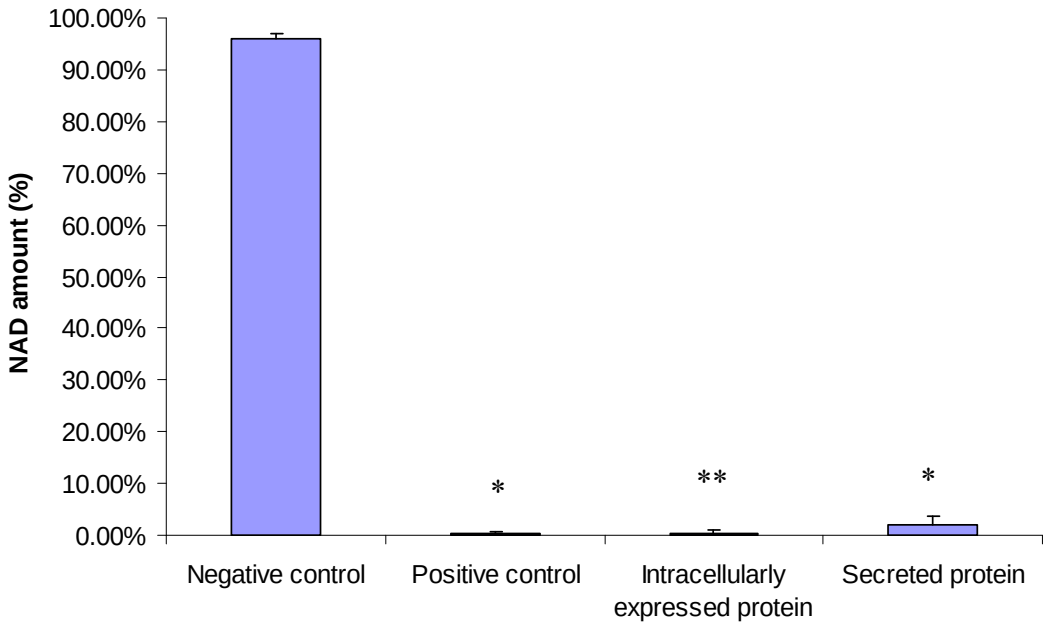


Figure 3.23. Cycling assay for NAD^+. Forty nanomolar of β-NAD^+ was incubated with intracellularly expressed and secreted recombinant protein. The mixtures were filtered and supernatants were added with cycling reagents which incorporated residual NAD^+ into cycling reactions and generated fluorescent products. Values are mean ± SEM of 3 independent experiments performed in triplicate (n = 3). *$P < 10^{-4}$, ** $P < 10^{-5}$.

4. DISCUSSION

4.1. Isolation and Characterization of Conidial NADase from *N. crassa*

Conidial NADase appeared to possess the highest specific enzyme activity among the three NADases tested, ie. mycelial NADase, conidial NADase and the commercial partial purified NADase from Sigma (Menegus and Pace, 1981). In addition, mycelial NADase and Sigma NADase appeared as a smear band in in-gel substrate staining rather than a clear band as in the case of conidial NADase. Sigma NADase is the product purified from mycelia of *N. crassa*. Therefore, it is not surprising that the isolated mycelial NADase from this study and Sigma NADase possess the same migration pattern, i.e. smear band, in non-reducing SDS-PAGE. NADase is a glycoprotein and the carbohydrate moiety on mycelial NADase is different from that on conidial NADase. The NADase purified from mycelia is unusual in having high content of carbohydrates (Everse *et. al.*, 1975). The conidial NADase has lower hexose content than mycelial NADase (Menegus and Pace, 1981). NADase from conidia contains 20% carbohydrates while the enzyme from mycelia has 80% carbohydrates (Pace *et. al.*, 1998).

The conidial NADase was purified using affinity chromatography, either with cibacron blue 3GA agarose or blue speharose CL-6B. However, the yield for each purification step was extremely low, especially when using cibacron blue agarose. As a result, the protein is usually not sufficient for the subsequent purification step. This could be due to the fact that conidial NADase is rather hydrophobic (Menegus and Pace, 1981) such that the enzyme is adsorbed tightly by alkyl-sepharose columns with sorption affinity rising as the alkyl group is increased from 3 to 8 carbon atoms (Er-el

et. al., 1972). As a result of the high hydrophopbicity, significant amount of enzyme activity of the enzyme was lost.

The reported molecular weight of conidial NADase is 33 kDa (Menegus and Pace, 1981), while the mycelial NADase was reported to be 37 kDa (Cho *et. al.*, 1998). However, the molecular weights of conidial NADase found in this study were 42 kDa and 75 kDa in SDS-PAGE or 45 kDa and 80 kDa in gel filtration, which are corresponding to the monomer and dimer form of NADase, respectively. NADase is present as a dimer when the enzyme is concentrated or when it is subjected to high temperature (for example 25^{o}C) while the monomer of NADase is the most active form (Pace *et. al.*, 1997). The molecular weight of the dimer form of conidial NADase is similar to the dimer form of mycelial NADase, which is 70 kDa (Cho *et. al*., 1998). Under native condition, the NADase dimer is the predominant form of protein (Cho *et. al*., 1998).

It is speculated that the dimer form of the enzyme is not inter-disulphide-bonded. This can be seen from non-reducing SDS-PAGE that 2 forms of NADase detected when the sample was very concentrated with monomer band protein is more abundant than the dimer form. Only one form of NADase i.e. monomer form of NADase was detected when the sample was diluted. When the dimer protein undergoes partial dissociation, it exits as monomer and dimer protein in SDS-PAGE. The enzyme forms a dimer through hydrophobic bonds (Pace *et. al.*, 1998). As the hydrophobic interactions are endothermic, the bonds are stronger when the temperature is raised. This results in the formation of enzyme dimer.

The conidial NADase is distinct from the enzyme purified from mycelial cultured in Zn-deficient medium (Menegus and Pace, 1981). The conidial NADase is different from mycelial NADase in terms of the carbohydrate moiety, effect of pH, temperature and K_m. The optimum pH for conidial NADase is pH 7.0. This is in contrast to mycelial NADase which is active over a wide range of pH, from pH 3 to pH 9 (Kaplan, 1955). The conidial NADase is sensitive to changes in temperature. An overnight incubation at 4^{o}C caused a significant loss of enzyme activity. The enzyme becomes inactivated slowly at 4^{o}C (Kaplan, 1955). An increase of the temperature from 4^{o}C to 55^{o}C and 80^{o}C denatures the enzyme and cause a loss of enzyme activity. At high temperature, for example at 80^{o}C, the protein was observed to precipitate out. This was due to the fact that at high temperature, hydrophobic bonds are promoted between the proteins. This results in the aggregation of protein and thus precipitation of protein. The K_m of conidial enzyme determined for this enzyme was 279.6 ± 4.95 μM which was different from the K_m of mycelial NADase, 500 μM (Kaplan, 1955). This is consistent with the finding that the specific enzyme activity of conidial NADase is higher than specific enzyme activity of mycelial NADase.

4.2 Identification of NADase Sequence

Conidial NADase has been purified and characterized in 1980s but the genomic sequence has not been revealed yet. The present study first identified the cDNA sequence of NADase, the sequence of which has been deposited in NCBI but the function remained uncharacterized. The full length cDNA sequence of gi|85106032, NADase is 732 bp with the deduced protein molecular weight of 27 kDa

without subjected to any post translational modifications. The mature sequence of NADase starts from the 58bp of the full length cDNA sequence.

The protein bands identified from the SDS-PAGE were subjected to mass spectrometry analysis. The peptide masses were matched against the *Neurospora crassa* database to reveal the identity of the proteins. *N. crassa* genome contains 7 linkage groups, which means 7 chromosomes. The genome has been sequenced and reported recently (Galagan *et. al.*, 2003; Mannhaupt *et. al.*, 2003; Mewes *et. al.*, 2004). The genome sequence has a total length of 38.6 Mb and contains 10,000 protein-coding genes. The genome sequences were searched against the public protein database to reveal or predict the function of uncharacterized sequences. Our mass spectrometry results matched some known proteins and hypothetical proteins. But the gi|85106032 hypothetical protein appeared the most frequent, 3 times out of 5 analyses. Gi|85106032 is a protein from linkage group IV and it is a secreted protein. All these information match the criteria of conidial NADase which is a secreted protein and its *nada* gene is located at linkage group IV (Nelson *et. al.*, 1975).

4.3 Cloning and Expression of Gi|85106032

mRNA of *N. crassa* was isolated in order to perform the reverse transcription. Initially, the mRNA from conidia was isolated and used to perform the reverse transcription. However, no products could be detected after PCR. In order to get the mRNA of gi|85106032, the culture method was changed. The conidia were cultured for 18 hours in media before mRNA extraction. With the use of mRNA after 18-hour culture, we could detect the PCR product encoding this protein. Even though highest

enzyme activities are detected in the conidia, it does not show any mRNA transcription in the mRNA of the conidia as the mRNA is a transcript during conidiophore differentiation. *N. crassa* NADase has been shown to be associated with the process of microconidiation (Stine, 1968; Urey, 1971; Zalokar and Cochrane, 1956). The NADase enzyme activity is the highest during the early phase of conidia germination (0-7 hour conidial growth in media) and when the conidiophores age (56-96 hour growth in media) (Stine, 1968). There is no or little enzyme activity detected during 24 to 48 hours growth in media. This may explain that during the logarithmic growth of conidia and during the differentiation of conidiophores, the protein is being under transcription and translation to prepare the needs for later stage of development. Therefore, mRNA of the gene can be detected after 18-hr culture, which corresponds to logarithmic growth of conidia. Accumulation of enzyme acitivities in conidia is an integral part of genetic program for the differentiation of aerial hyphae and macroconidia (Nelson *et. al.*, 1975).

The PCR products were ligated with the yeast expression vectors, pPICZB and pPICZalphaA. These 2 vectors utilize the AOX1 promoter to drive the transcription of foreign genes. The full length sequence of NADase was ligated with the intracellular expression vector. However, the cDNA encoding the mature NADase was ligated with the secretion expression vector, pPICZalphaA, since the vector contains the α-factor secretion signal from *S. cerevisiae*. The protein can be cloned in frame either with the native secretion signal or *S. cerevisiae* α-factor prepro-peptide. NADase enzyme activity can be detected from the supernatant of intracellular expression culture. Protein can be detected as well from the supernatant of the culture. This implies that

the secretion signal of the *N. crassa* can be recognized by the *P. pastoris* expression system (Digan *et. al.*1989; Tschopp *et. al.*, 1987). However, it was found that the level of secreted recombinant enzyme using native secretion signal was lower than using α-factor prepro-peptide signal sequence.

Clonal variation is observed within collections of transformants harboring the same number of expression cassettes. Therefore, it is necessary to screen a number of transformants to check their expression levels (Cregg *et. al.*, 1993). Our results showed that clone 1 from intracellular expression and clone 5 from secretion expression express more proteins than other clones. However, the reduction in the yield of recombinant proteins beyond the optimum expression time point might be due to the proteolysis of protein by *P. pastoris* protease. Secreted recombinant proteins are potentially subjected to proteolysis by extracellular protease, cell-bound protease and intracellular protease from lysed cells (Macauley-Patrick *et. al.*, 2005). But there was an insignificant reduction in yield from 72 hour to 96 hour. This could be due to the addition of casamino acids in the media which may protect the product from proteolysis by a carrier mechanism or may provide amino acids and energy for foreign protein synthesis and secretion (Cregg *et. al.*, 1993).

The molecular weights of intracellularly expressed and secreted proteins from intracellular expression culture are different. The intracellularly expressed protein has the molecular weight of 37 kDa while the secreted protein is 50 kDa. There are 2 forms of protein detected from secretion expression. They have the molecular weight of 50 kDa and 55 kDa. The difference in molecular weight between secreted protein and intracellular protein is attributed to different degree of glycosylation of the protein.

The intracellular proteins are rarely glycosylated or they have simpler sugar modifications. Unlike secreted proteins, they are glycoproteins. The different composition of glycosylation may be the reason for different molecular weight of the protein (Huo *et. al.* 2007; Sethuraman *et. al.*, 2006). The most common glycosylation is N-linked glycosylation where sugar components are added to asparagines found in Asn-X-Ser/Thr recognition sequences in protein. There are 4 potential N-linked glycosylation sites found in the amino acid sequence of NADase. Thus, it is possible that the secreted proteins go through the N-linked glycosylation in *Pichia pastoris* which mostly consists of short chain mannose N-acetylglucosamine residues. Regardless of the degree of glycosylation by *P. pastoris* expression system, it does not affect the enzyme activity of NADase.

Besides glycosylation, proteins are also disulfide-bonded. Under non-reducing condition, the secreted protein is about 2 times larger than the monomer protein. However, the intracellular recombinant protein is 3 times larger than its monomer. This indicates that yeast expressed protein is a dimer or trimer and the monomers are bonded together by interdisulfide bond. *P. pastoris* expression system is unique in such a way that it permits the protein that goes through the secretory pathway to undergo post-translational modifications including glycosylation, disulfide bond formation and proteolytic processing (Cereghino *et. al.*, 2002; Cregg *et. al.*, 2000). This is unlike prokaryotic system which has been unsuccessfully in the formation disulfide bond due to reducing environment.

4.4 NAD^+ Cycling Assay

Recombinant NADase was applied in the cycling assay for determination of NAD^+ to test its efficacy. The results showed that the recombinant NADases from intracellular and secretion expression can be applied in the cycling assay, which is able to measure cellular cADP-ribose with nanomolar sensitivity. Recombinant NADases can be produced in large scale to meet the need of cycling assay with relative economical cost since the defined media needed are inexpensive and commercially available. In addition, with the use of secretion expression, the purification of recombinant protein will be of ease since the yeast secretes low level of native proteins. It is known that the secreted heterologous protein comprises the majority of the total protein in the medium (Barr *et. al.*, 1992).

4.5. Conclusions

We have isolated and characterized the conidial NADase from *N. crassa.* Conidial NADase is different from mycelial NADase in certain aspects. Using mass spectrometry analysis, we have revealed the sequence of *N. crassa* NADase which has never been identified before even though it has been purified for a long time. The gene was cloned into the *Pastoris* expression vectors, pPICZB and pPICZalphaA. The recombinant proteins have been expressed successfully using the intracellular and secretion expression vectors. Furthermore, the recombinant proteins have been shown to be effective in removing NAD^+ in the cycling assay.

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6. APPENDIX

Vogel's minimal medium

Sucrose	20 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
biotin	5×10^{-6} g
sodium tetraborate	8.8×10^{-5} g
$(NH_4)_6Mo_7O_{24}$	6.4×10^{-5} g
$FeCl_3.6H_2O$	9.6×10^{-4} g
$CuCl_2$	2.7×10^{-4} g
$MnCl_2.4H_2O$	7.2×10^{-5} g
$ZnCl_2$	5×10^{-5} g
sodium tartarate	5.0 g
$NaNO_3$	1.0 g

Dissolve in 1 l water and autoclave for 20 min at 121^{o}C.

Zinc-deficient medium

Same recipe as Vogel's minimal medium except no $ZnCl_2$ added.

Protein extraction lysis buffer

0.1 M sodium phosphate buffer, pH 7.5
1 mM EDTA
0.1 mM DTT
1 mM PMSF
1% Triton X-100
Prepare fresh.

Buffer A

50 mM sodium phosphate buffer, pH 7.0
1 mM EDTA
1 mM DTT
1 mM PMSF
Prepare fresh.

Breaking buffer

50 mM sodium phosphate, pH 7.25
1 mM PMSF
1 mM EDTA
5% Glycerol
Prepare fresh.

His-tag binding buffer
20 mM sodium phosphate, pH 7.4
500 mM NaCl
20 mM imidazole

His-tag elution buffer
20 mM sodium phosphate, pH 7.4
500 mM NaCl
500 mM imidazole

Solution H6

1 M Tris-HCl, pH 6.8	60 mM	6 ml
SDS	12%	12 g
Sucrose	1.3 M	45 g

Dissolve in 100 ml deionised water. Warm to dissolve SDS. Store at room remperature.

5X non-reducing sample buffer

H6 solution	400 µl
Deionised water	80 µl
2% Bromophenol blue	20 µl

5X reducing sample buffer

H6 solution	400 µl
B-mercaptoethanol	25 µl
Deionised water	75 µl
2% Bromophenol blue	20 µl

SDS-PAGE

Reagent	8% Resolving Gel	10% Resolving Gel	12% Resolving Gel	4% Stacking Gel
30% acrylamide solution (29:1) (Bio-rad Cat #161-0156)	2.7 ml	3.3 ml	4.0 ml	0.4 ml
0.75 M Tris.HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	--
0.5 M Tris.HCl, pH 6.8	--	--	--	0.38 ml
Deionised water	4.6 ml	4 ml	3.3 ml	2.3 ml
10% SDS solution	0.1 ml	0.1 ml	0.1 ml	30 µl
10% APS solution	0.1 ml	0.1 ml	0.1 ml	30 µl
TEMED	6 µl	4 µl	4 µl	3 µl
Total volume :	10.0 ml	10.0 ml	10.0 ml	3.0 ml

1X SDS running buffer

Glycine	0.192 M	144.1 g
Sodium dodecyl sulfate	0.1%	10 g
Tris base	0.25 M	30.3 g

Top up to 1 L with deionised water. The pH is about 8.3. Store at room temperature.

Native PAGE

Reagent	8% Resolving Gel	4% Stacking Gel
30% acrylamide solution (29:1) (Bio-rad Cat #161-0156)	2.7 ml	0.4 ml
0.75 M Tris.HCl, pH 8.8	2.5 ml	--
0.5 M Tris.HCl, pH 6.8	--	0.38 ml
Deionised water	4.7 ml	2.4 ml
10% APS solution	0.1 ml	30 µl
TEMED	6 µl	3 µl
Total volume :	10.0 ml	3.0 ml

1X Running buffer

Tris base	3.0 g
Glycine	14.4 g

Dissolve in 1 l deionised water and adjust the pH to 8.3.

5X loading dye for Native PAGE

1 M Tris-HCl, pH 6.8	15.5 ml
1% Bromophenol blue	2.5 ml
Glycerol	25 ml
Deionised water	7.0 ml

Silver Staining solution

a. Fixer

Ethanol	40 ml
Glacial acetic acid	10 ml

Top up with deionised water to 100 ml.

b. Sensitizer

Ethanol	30 ml
Sodium thiosulfate	0.2 g
Sodium acetate	6.8 g

Dissolve in 100 ml deionised water.

c. Silver reaction

Silver nitrate	0.25 g

Dissolve in 100 ml deionised water.

d. Developing solution

Sodium carbonate	2.5 g
Formaldehyde	40 µl

Dissolve in 100 ml deionised water.

e. Stopping solution

EDTA, sodium salt	1.46 g

Dissolve in 100 ml deionised water.

Transfer buffer

Tris base	3.1 g
Glycine	14.4 g
Methanol	200 ml

Top up with deionised water to 1 l. Store at 4^{o}C.

Blocking solution

Skim milk powder	2.5 g

Dissolve in 50 ml TBST/PBST. Store at 4^{o}C.

TBST

Tris base	2.4 g
NaCl	8 g
Tween 20	1 ml

Dissolve in 1 l deionised water and adjust the pH to 7.5 with concentrated HCl. Store at 4^{o}C.

PBST

Na_2PO_4	1.42 g
KH_2PO_4	0.25 g
NaCl	8.0 g
KCl	0.2 g
Tween 20	500 µl

Dissolve in 1 l deionised water. Store at room temperature.

Stripping buffer

1 M Tris-HCl, pH 6.7	62.5mM	3.125 ml
10% SDS	2%	10 ml
14.3 M B-mercaptoethanol	100 mM	350 µl

Top up the volume to 50 ml using deionised water.

Diethylpyrocarbonate (DEPC) Treated Water

DEPC	0.1%	1 ml

Top up to 1 L with deionised water and stir overnight. Autoclave and store at room temperature.

RNA lysis buffer

Tris	100 mM	0.61 g
NaCl	0.6 M	1.6 g
EDTA	100 mM	1.46 g
SDS	4%	2 g

Dissolve in DEPC-treated water, adjust the pH to 8.0 and top up to 50 ml with DEPC-treated water.

1.2% denaturing agarose gel

10X MOPS	5 ml
Agarose powder	0.6 g

Top up with DEPC-treated water to 50 ml. After melting the agarose, cool to about 65°C. Add 900 µl 37% formaldehyde solution and 0.5 µl 10 mg/ml ethidium bromide solution to mix. Pour to set at room temperature.

10X MOPS Buffer

EDTA	10 mM	2.92 g
MOPS	200 mM	41.85 g
Sodium acetate	50 mM	4.10 g

Top up to 1 L with DEPC treated water and adjust to pH 7.0 with NaOH. Store at 4ºC in the dark.

1X MOPS Running Buffer

10X MOPS	10 ml
Formaldehyde (37%)	2 ml

Top up to 100 ml with DEPC treated water and store at room temperature.

5X RNA Loading Buffer

0.5 M EDTA solution	80 µl
10X MOPS buffer	4 ml
Formaldehyde (37%)	720 µl
Formamide	3.084 ml
Glycerol	2 ml
Saturated bromophenol blue	16 µl

Top up to 10 ml with DEPC treated water. Store at 4°C.

10 mM dNTP Mix for RT & PCR

100 mM dATP	100 µl
100 mM dCTP	100 µl
100 mM dGTP	100 µl
100 mM dTTP	100 µl
DEPC treated or deionised water	600 µl

Store at –20°C.

Agarose gel
Add the required amount of agarose powder into 1X TAE (For example 2% gel, 2.0 g in 100 ml buffer). Microwave to melt the agarose. Once melted, cool the agarose to about 50°C and add about 0.4 μg of ethidium bromide (10 mg/ml stock) per ml of gel volume. Pour to set at room temperature.

1X TAE buffer

Tris base	48.46 g
EDTA, disodium salt	3.72 g
Acetic acid	12.01 g

Dissolve in 1 l deionised water. Store at room temperature.

10X YNB (13.4%)
Dissolve 134 g YNB with ammonium sulfate and without amino acids in 1000 ml deionised water and filter sterilize. The shelf life of the solution is approximately one year.

500X Biotin (0.02%)
Dissolve 20 mg biotin in 100 ml deionised water and filter sterilize. Store at 4°C. The shelf life of the solution is approximately one year.

10X Dextrose (20%)
Dissolve 200 g of D-glucose in 1 l deionised water. Autoclave for 15 min or filter sterilize. The shelf life of the solution is approximately one year.

10X Methanol (5%)
Mix 5 ml of methanol with 95 ml of deionised water. Filter sterilize and store at 4°C. The shelf life of the solution is approximately two months.

1 M potassium phosphate buffer, pH 6.0
Combine 132 ml of 1 M K_2HPO_4 with 868 ml of 1 M KH_2PO_4. Autoclave for 20 min. Store at room temperature. The shelf life of the solution is greater than one year.

10X Glycerol (10%)
Mix 100 ml of glycerol with 900 ml of deionised water. Autoclave for 20 min or filter sterilize. Store at room temperature. The shelf life of the solution is greater than one year.

YPD

Yeast extract	1%	10 g
Peptone	2%	20 g
10X Dextrose	2%	100 ml

Dissolve yeast extract and peptone in 900 ml deionised water. Add 20 g of agar if making YPD plates. Autoclave for 20 min at 121°C. Cool the solution to ~60 °C and add 100 ml 20% dextrose. Add 1.0 ml of 100 mg/ml Zeocin, if needed. Store plates

and mediua with Zeocin at 4°C in the dark. Plates containing Zeocin can be stored for 2 weeks.

MM

10X YNB	1.34%	100 ml
500X Biotin	4 x 10^{-5}%	2 ml
10X Methanol	0.5%	100 ml

Autclave 800 ml of deionised water with 15 g of agar for 20 min at 121°C. Cool the solution and add the recipes above. Mix the solution well and pour into plates immediately. Store at 4°C.

MD

10X YNB	1.34%	100 ml
500X Biotin	4 x 10^{-5}%	2 ml
10X Dextrose	2%	100 ml

Autclave 800 ml of deionised water with 15 g of agar for 20 min at 121°C. Cool the solution and add the recipes above. Mix the solution well and pour into plates immediately. Store at 4°C.

BMMY

Yeast extract	1%	10 g
Peptone	2%	20 g
1 M potassium phosphate, pH6.0	100 mM	100 ml
10X YNB	1.34%	100 ml
500X Biotin	4 x 10^{-5}%	2 ml
10X Methanol	0.5%	100 ml

Dissolve yeast extract and peptone in 700 ml deionised water and autoclave for 20 min at 121°C. Add the potassium phosphate buffer, YNB, biotin and methanol. Mix the solution well and store at 4°C.

BMGY

Yeast extract	1%	10 g
Peptone	2%	20 g
1 M potassium phosphate, pH6.0	100 mM	100 ml
10X YNB	1.34%	100 ml
500X Biotin	4 x 10^{-5}%	2 ml
10X Glycerol	1%	100 ml

Dissolve yeast extract and peptone in 700 ml deionised water and autoclave for 20 min at 121°C. Add the potassium phosphate buffer, YNB, biotin and glycerol. Mix the solution well and store at 4°C.

Low salt LB medium

Tryptone	1%	10 g
Yeast extract	0.5%	5 g
NaCl	0.5%	5 g

Dissolve in 950 ml deionised water and adjust to pH 7.0 with NaOH. Add 15 g/l agar to prepare plates. Top up the volume to 1 l. Autoclave for 20 min at 121^{o}C. Cool the solution to ~55^{o}C and add 250 µl if desired. Store at 4^{o}C.