***In vitro* metabolic engineering for the salvage synthesis of NAD⁺**

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Abstract

Excellent thermal and operational stabilities of thermophilic enzymes can greatly increase the applicability of biocatalysis in various industrial fields. However, thermophilic enzymes are **generally** incompatible with thermo-labile substrates, products, and cofactors, since they **show the maximal activities** at high temperatures. Despite their pivotal roles in a wide range of enzymatic redox reactions, NAD(P)⁺ and NAD(P)H exhibit relatively low stabilities at high temperatures, tending to be a major obstacle in the long-term operation of biocatalytic chemical manufacturing with thermophilic enzymes. In this study, we constructed an *in vitro* artificial metabolic pathway for the salvage synthesis of NAD⁺ from its degradation products by the combination of eight thermophilic enzymes. The enzymes were heterologously produced in recombinant *Escherichia coli* and the heat-treated crude extracts of the recombinant cells were directly used as enzyme solutions. When incubated with experimentally optimized concentrations of the enzymes at 60°C, the NAD⁺ concentration could be kept almost constant for 15 h.

Keywords: NAD⁺ | thermophilic enzyme | *in vitro* metabolic pathway

1. Introduction

Enzymes from thermophilic microorganisms exhibit remarkable **thermostability** and **show the maximal activities** at high temperatures. In addition, many thermophilic enzymes display higher tolerance towards denaturants, such as detergents and organic solvents, than their mesophilic counterparts (Atomi, 2005; Owusu and Cowan, 1989). Such excellent operational stabilities of thermophilic enzymes can greatly increase the applicability of biocatalysis in various industrial fields, including food processing, biochemical analysis, and chemical manufacturing (Atomi et al., 2011; Bruins et al., 2001). On the other hand, there is an inherent technical limitation in the application of thermophilic enzymes, namely their incompatibility with thermo-labile substrates, products, and cofactors. In particular, the relatively low thermal stabilities of NAD(P)⁺ and NAD(P)H (Wu et al., 1986) tend to be a major obstacle in the application of thermophilic enzymes. **The KEGG database search revealed that nicotinamide cofactors are involved in more than 2000 enzymatic reactions, some of which have already been playing important roles in industrial chemical manufacturing processes (e.g., the enantioselective reductions of prochiral ketones by carbonyl reductases (Kataoka et al., 2003), asymmetric saturations of C=C bonds by enoate reductases (Stuermer et al., 2007), and regioselective hydroxylation of non-activated carbon atoms by cytochrome P450 monooxygenases (Urlacher et al., 2004)). In addition, since nicotinamide cofactors can be easily determined spectrophotometrically and electrochemically, they have been widely used as indicator reagents in enzymatic diagnoses and other biochemical analyses (Tachibana et al., 2006; Jena and Raj, 2006). Thus, nicotinamide cofactors have a wide range of practical application and their stabilization is one of the key issues to reinforce the industrial applicability of thermophilic enzymes. Recently,**

we have developed a novel approach to engineer an *in vitro* artificial metabolic pathway using thermophilic enzymes and demonstrated the one-pot conversion of glucose to lactate (Ye et al., 2012), malate (Ye et al., 2013), and 1-butanol (Krutsakorn et al., 2013). The biocatalytic conversion through the *in vitro* pathways could be accomplished at sufficient production yields (60-100 mol%) and conversion rates (0.5-1.2 $\mu\text{mol mL}^{-1} \text{h}^{-1}$). However, despite the use of highly stable enzymes, the bioconversion with these *in vitro* systems stopped within several hours at 50°C. The conversion rate could be recovered by the re-addition of NAD^+ and/or NADH to the reaction mixture, clearly indicating that the thermal decomposition of these cofactors hampered the long-term implementation of the *in vitro* bioconversion (Ye et al., 2012; Krutsakorn et al., 2013). Zhang and coworkers (2014a) demonstrated that an *in vitro* hydrogen production system could be operated for 90 h at 40°C under anaerobic conditions; however, they also reported that increase in the reaction temperature to 60°C resulted in a considerable decrease in the operational stability of their system (Rollin et al., 2014b). Similarly, Wu et al. (2011) reported that the kinetic resolution of (*RS*)-phenylethanol using an enzyme couple of thermostable NAD^+ -dependent alcohol dehydrogenase and NADH oxidase gave an insufficient conversion yield at a high temperature owing to the instability of NAD(H). Since these cofactors are generally expensive, their stabilization and long-term use is a crucial challenge to developing an economically feasible biotransformation process with thermophilic enzymes. Replacement of the natural redox cofactors with stable and inexpensive biomimetic analogs has been proposed as a possible solution to this problem (Rollin et al., 2013). Yet, the $k_{\text{cat}}/K_{\text{m}}$ of natural enzymes for these compounds are 1-3 orders of magnitude lower than those for natural cofactors (Ansell Lowe, 1999), indicating that protein engineering efforts are

indispensable to improve the affinity of enzymes to the biomimetic cofactors (Rollin et al., 2013).

These limitations motivated us to develop an alternative approach for the long-term use of the redox cofactors at high temperatures. The thermally unstable nature of nicotinamide cofactors generates a demand for the molecular apparatus for salvaging their degradation products in thermophilic microorganisms. Thus, these microorganisms would seem to be equipped with a series of enzymes involved in the resynthesis of the cofactors from their degradants. Identification and assembly of these enzymes enable us to construct an *in vitro* pathway for the salvage synthesis of the cofactors. In this study, we focused on NAD⁺ and constructed an *in vitro* salvage synthetic pathway of the cofactor using thermophilic enzymes.

2. Materials and methods

2.1. Reagents. NAD⁺, NADH, NADP⁺, ATP, ADP, and AMP were obtained from Oriental Yeast (Osaka, Japan). Nicotinamide, ADP-ribose, and other intermediates were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Thermotolerant glucose-1-dehydrogenase (GDH; code number, GCD-41-01) and glucose-6-phosphate dehydrogenase (G6PDH; code number, GLD-75-01) were products of Thermostable Enzyme Laboratory (Kobe, Japan). Polyphosphate was chemically synthesized as follows: 35 g of NaH₂PO₄ (anhydrous, Wako Pure Chemical, Osaka, Japan) and 0.3 g of Na₂HPO₄ (anhydrous, Wako Pure Chemical) were mixed by grinding in a mortar. The mixture was placed in a platinum pot and heated at 800°C for 150 min in an electric furnace. The resulting glassy material was used as polyphosphate.

2.2. Expression plasmids for thermophilic enzymes. The expression plasmids encoding nicotinate mononucleotide adenylyltransferase (NaMAT_{Tt}; gi|55981749), nicotinate phosphoribosyltransferase (NaPRT_{Tt}; gi|55980586), ADP-ribose pyrophosphatase (ADPRP_{Tt}; gi|29603631), ribose-phosphate pyrophosphokinase (RPK_{Tt}; gi|55981518), and adenylate kinase (ADK_{Tt}; gi|55981640) were obtained from the RIKEN *Thermus thermophilus* HB8 expression plasmid library (Yokoyama et al, 2000). The expression vectors for pyruvate kinase (gi| 55979972), lactate dehydrogenase (gi|55981082), and glucokinase (gi|55980268) from *T. thermophilus* HB8, which were used for the determination of the activities of ADK_{Tt} and polyphosphate kinase from *T. thermophilus* HB27 (PPK_{Tt}; gi|46198945), were also obtained from the same library. The codon-optimized genes encoding NAD⁺ synthase from *T. thermophilus* HB8 (NADS_{Tt}; gi|55981869) and that from *Bacillus stearothermophilus* (NADS_{Bs}; gi|696475119) were synthesized by GeneArt (Regensburg, Germany) and introduced to the *NdeI/XhoI* restriction sites of pET21a (Novagen, Madison, WI, USA). The gene coding for nicotinamidase from *Thermoplasma acidophilum* (NADase_{Ta}; gi|499203341) was amplified by PCR using the chromosomal DNA of *T. acidophilum* NBRC15155 with the following oligonucleotide primers: 5'-cccatatgaaacctgcacttgtagttgtggaca-3' (the *NdeI* restriction site is underlined), and 5'-ggaagcttctacgtctctatctgatcttctctgc-3' (the *BamHI* restriction site is underlined). The PCR amplicon was digested with *NdeI* and *BamHI*, and then introduced to the corresponding site of pET21a. The expression vector for PPK_{Tt} was constructed as described elsewhere (Iwamoto et al., 2007). *E. coli* Rosetta2 (DE3) (Novagen) was used as the host strain for gene expression. Recombinant cells were

cultivated aerobically at 37°C in Luria-Bertani (LB) medium supplemented with 50 µg ml⁻¹ ampicillin and 30 µg ml⁻¹ chloramphenicol. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.2 mM in the late-log phase. The cells were further cultivated for 4 h. The cells were then harvested by centrifugation at 6,500 × g and 4°C for 10 min and washed once with 100 mM HEPES-NaOH buffer (pH 8.0).

2.3. Enzyme assays. Recombinant cells were suspended in an appropriate volume of 100 mM HEPES-NaOH (pH 8.0) and disrupted using a UD-201 ultrasonicator (TOMY, Osaka, Japan) at 20 W for 2 min. Cell debris was removed by centrifugation at 12,000 × g at 4°C for 10 min. The protein concentration in the crude lysates was measured by the Bradford assay using bovine serum albumin as the standard. The Bio-Rad assay system (Bio-Rad, Hercules, CA, USA) was used for protein determination. The resulting crude lysates were incubated at 70°C for 30 min to inactivate the host-derived enzymes and centrifuged again to remove denatured proteins. The supernatants were obtained as enzyme solutions. When necessary, the obtained enzyme solutions were concentrated using Amicon Ultra 10K (Merck Millipore, Darmstadt, Germany).

Enzyme activity was determined spectrophotometrically at 60°C in the basal buffer consisting of 400 mM HEPES-NaOH (pH 8.0), 60 mM NH₄Cl, 10 mM MgCl₂, and 1 mM polyphosphate. NADase_{Ta} activity was assessed by incubating the enzyme at an appropriate amount in the basal buffer supplemented with 0.2 mM ATP, 0.2 mM phosphoribosyl pyrophosphate (PRPP), 1 mM glucose, and excess amounts of NaPRT_{Ti}, NaMAT_{Ti}, NADS_{Bs}, and GDH. After the preincubation at 60°C for 2 min, the reaction was started by adding nicotinamide at a final concentration of 0.2 mM and the

formation of NADH was monitored at 340 nm. Similarly, the activity of NaPRT_{Tt} was determined by coupling the enzyme with excess amounts of NaMAT_{Tt}, NADS_{Bs}, and GDH, and using 0.2 mM nicotinate as the substrate. NaMAT_{Tt} and NADS_{Bs} activities were assessed similarly using 0.2 mM each of their substrate (NaMN and deamino-NAD⁺, respectively) except that PRPP was not added to the reaction mixture. ADPRP_{Tt} activity was spectrophotometrically assessed by coupling with RPK_{Tt}, NaPRT_{Tt}, NaMAT_{Tt}, NADS_{Bs}, and GDH. The enzymes were precubated at 60°C in the basal buffer containing 0.2 mM ATP, 0.2 mM nicotinate, and 1 mM glucose. The reaction was initiated by adding 0.2 mM ADP-ribose and monitored at 340 nm. RPK_{Tt} assay was performed in the same manner using 0.2 mM ribose-5-phosphate (R5P) as the substrate. ADK_{Tt} activity was determined by the coupling reaction with pyruvate kinase and lactate dehydrogenase from *T. thermophilus* HB8. ADK_{Tt} at an appropriate amount was incubated with excess amounts of the coupling enzymes in the basal buffer containing 0.2 mM AMP, 0.2 mM phosphoenolpyruvate, and 0.2 mM NADH. After the preincubation for 2 min, the reaction was started by adding 0.2 mM ATP and then the consumption rate of NADH was monitored at 340 nm. PPK_{Tt} activity was assessed through the coupling reaction with thermotolerant glucokinase and G6PDH. The enzymes were incubated in the buffer containing 0.2 mM ADP, 0.2 mM glucose, and 0.2 mM NADP⁺. The NADPH formation rate accompanied by the oxidation of glucose-6-phosphate by G6PDH was monitored at 340 nm. The millimolar extinction coefficient of 6.2 mM⁻¹ cm⁻¹ was employed for the determination of NADH and NADPH concentrations at 340 nm. One unit (U) of enzyme was defined as the amount catalyzing the consumption of 1 μmol of the substrate per minute under the assay conditions.

2.4. *In vitro* salvage synthesis of NAD⁺. The reaction mixture was composed of 400 mM HEPES-NaOH (pH8.0), 60 mM NH₄Cl, 10 mM MgCl₂, 1 mM polyphosphate, 3 mM ATP, and 4 mM NAD⁺. NADase_{Ta}, NaPRT_{Tt}, NaMAT_{Tt}, NADS_{Bs}, ADPRP_{Tt}, RPK_{Tt}, ADK_{Tt}, and PPK_{Tt} were put in the reaction mixture at final concentrations of 0.006, 0.052, 0.08, 0.5, 0.006, 0.02, 0.02, and 0.08 U ml⁻¹, respectively (Supplementary Table S1). The mixture with a final volume of 5 ml was incubated at 60°C. An aliquot was sampled at specific intervals and ultrafiltrated with Amicon Ultra 10 K (Merck Millipore). The filtrate was analyzed by HPLC.

2.5. Analytical methods. NAD⁺, ADP-ribose, nicotinamide, nicotinate, NaMN, deamino-NAD⁺, AMP, ADP, and ATP were analyzed by HPLC on a Cosmosil 5C₁₈-ARII column (ϕ 4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was composed of (A) 50 mM potassium phosphate buffer (pH 6.0) and (B) 25% (v/v) methanol containing 5 mM 1-sodium 1-octanesulfonate. The column was eluted with mobile phase A for the initial 5 min and then the volumetric ratio of mobile phase B was increased from 0 to 100% in the following 6 min. The column was further eluted only with mobile phase B for 6 min. The column temperature was maintained at 40°C and the total flow rate of the mobile phases was kept at 1.0 ml min⁻¹. The eluent was analyzed with a SPD-20AV UV-VIS detector (Shimadzu) at 254 nm.

3. Results

3.1. Identification of the thermal degradation products of NAD⁺

The decomposition of the oxidized nicotinamide cofactors, NAD^+ and NADP^+ , starts from a base-catalyzed hydrolysis of the C-N bond between nicotinamide and ribose moieties (Chenault and Whitesides, 1987). HPLC analysis of the degradation products of NAD^+ at pH 8.0 gave two major peaks and their retention times were identical to those of authentic nicotinamide and ADP-ribose. These compounds almost stoichiometrically accumulated in accordance with the degradation of NAD^+ (Fig. 1) although a trace amount of nicotinamide, which was likely to be an impurity in the commercial NAD^+ , was detected even at time zero. This observation indicated that nicotinamide and ADP-ribose are relatively stable at high temperatures. In fact, no significant degradation was observed for at least 6 h when nicotinamide and ADP-ribose were incubated at 60°C and pH8.0 (Supplementary Fig. S1). Based on these results, we selected these compounds as the initial substrates for the salvage synthesis of NAD^+ .

3.2. Enzymes involved in nicotinamide salvage

The single-gene-expression library of *Thermus thermophilus* HB8 (Yokoyama et al., 2000) was employed as a primary source of the genes encoding thermophilic enzymes. The enzymes that are likely involved in the nicotinamide salvage were selected on the basis of the annotation data of the *T. thermophilus* HB8 genome and then assayed to confirm their capability to catalyze expected reactions (Fig. 2, Supplementary Fig. S2). The enzyme assay revealed that the nicotinate mononucleotide adenylyltransferase of *T. thermophilus* HB8 (NaMAT_{Tt}) can use both nicotinamide mononucleotide (NMN) and nicotinate mononucleotide (NaMN) as substrates and catalyze their adenylylation to NAD^+ and deamino- NAD^+ , respectively. The specific enzyme activities in the crude extract of recombinant *E. coli* with NaMAT_{Tt} were estimated to be 0.177 and 3.72 U

mg⁻¹ total protein for NMN and NaMN, respectively. By contrast, although nicotinate
 phosphoribosyltransferase (NaPRT_{Tt}) catalyzed the phosphoribosylation of nicotinate to
 NaMN using PRPP, nicotinamide did not serve as the substrate of the enzyme. NAD⁺
 synthase from *T. thermophilus* HB8 (NADS_{Tt}) catalyzed the NH₃-dependent amination
 of deamino-NAD⁺ to NAD⁺. The enzyme required ATP as an energy cofactor and
 released AMP. However, the specific activity of NADS_{Tt} in the recombinant *E. coli*
 (5.48 × 10⁻⁴ U mg⁻¹ total protein) was considerably lower than those of other enzymes,
 such as NaMAT_{Tt} and NaPRT_{Tt} (4.12 × 10⁻² U mg⁻¹ total protein), under the standard
 conditions. Therefore, a literature search was done to find another thermophilic NAD⁺
 synthase and that from *Bacillus stearothermophilus* (NADS_{Bs}) was identified
 (Yamaguchi et al., 2002). The desired enzyme activity was found in the crude extract of
 the recombinant *E. coli* having NADS_{Bs} and the specific activity was determined to be
 0.19 U mg⁻¹ total protein.

By this method, we identified some thermophilic enzymes involved in the salvage
 synthesis of NAD⁺; however, those to be engaged in the initial reactions of nicotinamide
 salvage, namely, phosphoribosylation to NMN and deamination to nicotinate, were not
 found in the annotation data of the *T. thermophilus* HB8 genome. Among the
 bacterial/archaeal enzymes, nicotinamide phosphoribosyltransferase (NPRT) from
Haemophilus ducreyi has been demonstrated to catalyze the phosphoribosylation of
 nicotinamide to NMN (Martin et al., 2001). However, BLAST searches of the genomic
 sequences of *T. thermophilus* and other thermophiles yielded no hits when the amino
 acid sequence of *H. ducreyi* NPRT was used as a query. Indeed, the comparative
 genomics analysis by Gazzaniga et al. (2000) suggests that genes encoding putative
 NPRT are conserved only in a limited number of animals, bacteria, and viruses, but not

in thermophilic microorganisms. On the other hand, nicotinamidase, the enzyme catalyzing the deamination of nicotinamide to nicotinate, has been isolated from some thermophilic archaea, such as *Acidilobus saccharovorans* (Stekhanova et al., 2014), *Pyrococcus horikoshii* (Du et al., 2001), and *Thermoplasma acidophilum* (Luo et al., 2010). We cloned and expressed the gene encoding nicotinamidase from *T. acidophilum* (NADase_{Ta}) and confirmed its activity (Fig. S2). The specific activity of NADase_{Ta} in the crude extract of the recombinant *E. coli* was 0.11 U mg⁻¹ total protein. Thus, we employed the four-step pathway, consisting of deamination, phosphoribosylation, adenylation, and reamination, for the *in vitro* salvage of nicotinamide (Fig. 2).

3.3. ADP-ribose salvage and regeneration of ATP from AMP

ADP-ribose pyrophosphatase (ADPRP_{Tt}), which is engaged in the initial reaction of ADP-ribose salvage, from *T. thermophilus* HB8 has been well characterized with respect to its structure and function (Ooga et al., 2005; Yoshida et al., 2004). We also verified that the recombinant ADPRP_{Tt} expressed in *E. coli* can catalyze the hydrolysis of ADP-ribose (Fig. S2). The reaction products, *i.e.*, R5P and AMP, should be subsequently pyrophosphorylated for salvaging in NAD⁺ synthesis. The ribose-phosphate pyrophosphokinase from *T. thermophilus* HB8 (RPK_{Tt}) was selected for the pyrophosphorylation of R5P to PRPP and its activity was confirmed by a coupling reaction with NaPRT_{Tt}. When these enzymes were coincubated with R5P, ATP, and nicotinate, NaMN formation was observed (Fig. S2).

The *in vitro* pathway for the salvage synthesis of NAD⁺ from nicotinamide and ADP-ribose is illustrated in Fig. 3. For the synthesis of one molecule of NAD⁺ through the designed pathway, two molecules of ATP are required as phosphate and energy

sources in the reaction catalyzed by RPK_{Tt} and NADS_{Bs}, respectively. In addition, AMP released by ADPRP_{Tt} should also be pyrophosphorylated to be recycled as an adenylate donor of NaMAT_{Tt} reaction. Therefore, an ATP-regenerating system should be integrated into the *in vitro* pathway for the continuous recycling of the adenylate moieties. Polyphosphate is often used as an inexpensive phosphate donor in *in vitro* ATP regeneration and several polyphosphate-dependent enzymatic systems for the pyrophosphorylation of AMP to ATP have been reported (Kameda et al., 2001; Resnick and Zehnder, 2000; Satoh et al., 2004). We employed a coupling reaction of adenylate kinase and polyphosphate kinase (Satoh et al., 2004) owing to the well-characterized feature of the thermostable polyphosphate kinase from *T. thermophilus* HB27 (PPK_{Tt}) (Iwamoto et al., 2007). The thermophilic adenylate kinase (ADK_{Tt}) was obtained from the gene-expression library of *T. thermophilus* HB8 and used as the coupling partner. The thermal stabilities of the enzymes involved in the designed pathway are shown in Fig. S3. Although NADS_{Bs} and RPK_{Tt} lost 50% of their activity after the incubation at 60°C for 12 h, other enzymes retained more than 75% activity upon the heat treatment under the same conditions.

3.4. *In vitro* salvage synthesis of NAD⁺

NAD⁺ is relatively stable at an acidic pH while its reduced derivative, NADH, is more stable under alkaline conditions (Chenault and Whitesides 1987). Therefore, by stabilizing NAD⁺ at an alkaline pH, both oxidized and reduced forms of the cofactor can be stably maintained. On the basis of this assumption, we employed a reaction pH of 8.0 for the implementation of the *in vitro* salvage synthesis of NAD⁺.

The heat-treated (70°C for 30 min) crude extracts of recombinant *E. coli* with thermophilic enzymes were directly used as catalysts for the *in vitro* salvage synthesis (Supplementary Fig. S4). The flux through the *in vitro* pathway (*i.e.*, NAD⁺ production rate) was monitored in a real-time manner by spectrophotometric assays. NAD⁺ produced through the pathway was concomitantly reduced with a thermotolerant glucose dehydrogenase (GDH) and the formation of NADH was monitored at 340 nm. This real-time monitoring technique enabled us to experimentally optimize the enzyme concentration to achieve a desired flux. To achieve NAD⁺ production from nicotinamide at a rate of 0.006 $\mu\text{mol ml}^{-1} \text{ min}^{-1}$, which is identical to the initial degradation rate of NAD⁺ at pH 8.0 and 60°C (Fig. 1), we first used 0.006 U ml^{-1} each of NADase_{Ta}, NaPRT_{Tb}, NaMAT_{Tb}, and NADS_{Bs}. The NAD⁺ production rate observed under these conditions was, however, considerably lower than the expected value. In an *in vitro* reconstituted pathway, the actual concentrations of intermediates are kept lower than those used under the standard assay conditions (Krutsakorn et al., 2013). Consequently, the reaction rates of the enzymes, particularly those with high K_m values for their substrates, are lower than those observed under the standard assay conditions. The rate-limiting enzymes in the NAD⁺ synthetic pathway could be identified by increasing the concentration of each enzyme in the spectrophotometer cuvette, one by one. The concentration of an identified enzyme was then increased in a stepwise manner until the NAD⁺ production rate no longer increased. Subsequently, the next rate-limiting enzyme was identified and its concentration was modulated in the same manner. These procedures were repeated until the NAD⁺ production rate through the cascade reaction reached 0.006 $\mu\text{mol ml}^{-1} \text{ min}^{-1}$. As a result, the optimum concentrations of NADase_{Ta}, NaPRT_{Tb}, NaMAT_{Tb}, and NADS_{Bs} for the nicotinamide salvage were determined to be

0.006, 0.013, 0.08, and 0.25 U ml⁻¹, respectively. Similarly, the optimum concentrations of ADPRP_{Tt} and RPK_{Tt} were determined to be 0.006, and 0.01 U ml⁻¹, respectively, by using the above-mentioned concentrations of NaPRT_{Tt}, NaMAT_{Tt}, and NADS_{Bs}. The rate of ATP production catalyzed by PPK_{Tt} was also spectrophotometrically determined by coupling the enzyme with glucokinase from *T. thermophilus* and a thermotolerant glucose-6-phosphate dehydrogenase (G6PDH) and by monitoring the NADPH production rate through the cascade reaction. For the spectrophotometric determination of ADP production rate with ADK_{Tt}, the enzyme was coupled with pyruvate kinase and lactate dehydrogenase from *T. thermophilus*. For the salvage synthesis of NAD⁺, excess concentrations of ADK_{Tt} and PPK_{Tt} were used (0.02, and 0.08 U ml⁻¹, respectively) to ensure that the rate of salvage synthesis is not limited by the regeneration rate of ATP.

In addition to their crucial roles in cellular redox reactions, nicotinamide cofactors are involved in many biologically important reactions, such as ADP ribosylation of proteins (Gill et al., 1969), sirturin-mediated deacetylation of protein lysine (Imai et al., 2000), and bacterial DNA ligation (Wilkinson et al., 2001). These facts imply that the intracellular concentrations of nicotinamide cofactors are tightly controlled in living organisms by regulating their biosynthetic enzymes at transcriptional, translational, and post-translational levels. We investigated whether the activities of enzymes involved in our salvage pathway are allosterically modulated by NAD⁺ and found that NaPRT_{Tt} was inhibited by a high concentration of NAD⁺ (Fig. 4). In the presence of 4 mM NAD⁺, NaPRT_{Tt} lost nearly 60% of its original activity. Accordingly, in the *in vitro* reconstituted pathway, NaPRT_{Tt} was used at a fourfold higher concentration than that determined on the basis of the spectrophotometric assay. Besides NaPRT_{Tt}, the

concentrations of NADS_{Bs} and RPK_{Tt} were doubled by taking into consideration their relatively low thermal stability (Fig. S3).

To demonstrate the *in vitro* salvage synthesis of NAD⁺, the enzymes at their optimized concentrations (Supplementary Table S1) were incubated at 60°C with 4 mM NAD⁺ (Fig. 5). NAD⁺ concentration was kept almost constant in the presence of the salvage enzymes for 15 h although that in the control experiments without the enzymes decreased to 0.9 mM after the incubation for the same period.

4. Discussion

In this study, we identified the series of thermophilic enzymes involved in the salvage synthesis of NAD⁺ from its thermal degradation products, nicotinamide and ADP-ribose. We then assembled the enzymes to construct an *in vitro* salvage synthetic pathway of the cofactor. By the integration of the *in vitro* pathway, the degradation of NAD⁺ at a high temperature could be apparently prevented for 15 h. After the reaction for 15 h, decrease in NAD⁺ concentration accompanied by the accumulation of AMP became significant (Supplementary Fig S5). This observation indicated that the salvage synthesis stopped most probably due to the arrest of the ATP regeneration. The salvage synthesis of NAD⁺ is an energetically expensive reaction owing to the involvement of several water-releasing condensation steps (*i.e.*, those catalyzed by RPK_{Tt}, NaPRT_{Tt}, NaMAT_{Tt}, and NADS_{Bs}) and thus requires a continuous supply of ATP. Meanwhile, the ATP-regenerating enzymes, ADK_{Tt} and PPK_{Tt}, exhibited relatively high thermal stability (Fig S3). In addition, we used these enzymes at excess concentrations to maintain the higher rate of ATP regeneration than that of NAD⁺-salvage synthesis. These facts imply that the arrest of the ATP regeneration is more likely due to the

depletion of polyphosphate rather than the thermal inactivation of the enzymes. On the other hand, increased concentration of polyphosphate tends to inhibit the activities of metal-dependent enzymes owing to its chelating ability (Restiawaty et al., 2011). Therefore, addition of polyphosphate in a fed-batch manner would be effective to prolong the endurance time of the salvage synthesis of NAD⁺.

While the *in vitro* salvage synthesis of NAD⁺ can be a promising approach to extend the operational stability of biocatalytic conversion with thermophilic enzymes, the series of enzymes consisting of the *in vitro* pathway had to be separately prepared in this study. This time-consuming operation would be skipped by the coexpression of multiple genes encoding the series of thermophilic enzymes in a single recombinant strain. Recently, we constructed an artificial operon encoding nine thermophilic enzymes consisting of a non-ATP-forming glycolytic pathway and coexpressed them in a single recombinant *E. coli* (Ninh et al., 2015). The expression levels of thermophilic genes were controlled by modulating their sequential order in the operon. A simple heat-treatment of the crude extract of the resulting coexpression cells led to a one-step preparation of the cocktail of the thermophilic enzymes, which could be directly used for *in vitro* bioconversion of glucose to pyruvate and lactate. Similarly, development of a multiple-gene-expression strain harboring the thermophilic NAD⁺ synthetic enzymes would markedly increase the feasibility of the *in vitro* salvage synthetic system. Construction of the coexpression strain and its integration to another *in vitro* bioconversion pathway for chemical manufacturing is now in progress.

Another crucial challenge would be a construction of the salvage synthetic pathway for the phosphorylated derivative of the cofactor, NADP⁺, as well as those for reduced forms of nicotinamide cofactors. Ben-Hayyim et al. (1967) reported that the thermal

degradation of NADP^+ proceeds analogously to that of NAD^+ . Accordingly, 2'-phosphorylated ADP-ribose and nicotinamide are generated as degradants of NADP^+ . This fact indicates that the salvage synthesis of NADP^+ can likely be achieved with a similar enzyme system, which may involve an extra enzyme set for 2'-dephosphorylation and rephosphorylation of ADP-ribose 2'-phosphate.

On the other hand, completely different mechanisms have been proposed for the degradation of the reduced forms of nicotinamide cofactors. According to a conventionally accepted model, NADH decomposition involves hydration, epimerization, and cyclization; consequently, cyclic 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (cTHNAD) is formed as the degradation product (Chenault and Whitesides, 1987). More recently, Hofmann et al. (2010) used mass spectrometry to investigate the thermal degradation of NAD(P)H. Based on the results of their spectrometric analyses, they postulated that the degradation of reduced forms of nicotinamide cofactors proceeds via the oxidative ring-opening of a ribose moiety, release of nicotinamide, and dehydration. As a result, an ADP derivative with an incomplete ribose ring is generated. To our knowledge, enzymes involved in the metabolisms of these degradation products have not yet been reported, indicating that the *in vitro* salvage synthesis of NAD(P)H will be a more difficult challenge than that of the oxidized cofactors. However, the markedly high growth temperatures of thermophiles suggest the presence of unidentified enzyme systems for the salvage of these thermal degradation products for managing the thermo-labilities of NAD(P)H. The comprehensive elucidation of the salvage synthetic routes of nicotinamide cofactors in thermophiles would be an important issue for not only the biotechnological purposes

but also a deeper understanding of the underlying mechanisms in the unique physiology of thermophilic microorganisms.

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539

Figure legends

Fig. 1. Thermal degradation of NAD⁺. NAD⁺ (4 mM) was incubated at 60°C in 400 mM HEPES-NaOH (pH8.0). Samples were taken at every 1-h intervals and analyzed by HPLC. The time courses of the concentrations of NAD⁺ (filled square), nicotinamide (open circle), and ADP-ribose (open square) are shown. The assays were performed in triplicate; mean ± standard deviations (error bars) are depicted.

Fig. 2. Possible routes for the salvage synthesis of NAD⁺ from nicotinamide. The enzyme that was not identified in this study is shown by a white arrow. The nicotinamide-salvage route constructed in this study is highlighted in gray box.

Fig. 3. Design of the *in vitro* pathway for the salvage synthesis of NAD⁺ from nicotinamide and ADP-ribose. Blue arrows indicate ATP regenerating reactions, which are shown in detail in the inner panel.

Fig. 4. Inhibitory effect of NAD⁺ on NaPRT_{Tt} activity. The enzyme at an appropriate amount was incubated at 60°C in the reaction mixture consisting of 400 mM HEPES-NaOH (pH 8.0), 60 mM NH₄Cl, 10 mM MgCl₂, 1 mM polyphosphate, 1 mM PRPP, 1 mM nicotinamide, and indicated concentrations of NAD⁺. The mixture was analyzed by HPLC and the enzyme activities were calculated from the concentration of NaMN formed by the enzyme reaction. The assays were performed in triplicate; mean ± standard deviations (error bars) are depicted.

564 **Fig. 5.** *In vitro* salvage synthesis of NAD⁺. NAD⁺ (4 mM) was incubated at 60°C in 400
565 mM HEPES-NaOH (pH 8.0) with (closed square) and without (open square) the
566 enzymes. Samples were taken at every 3-h intervals, ultrafiltrated, and then analyzed by
567 HPLC. The assays were performed in triplicate; mean $\pm$ standard deviations (error bars)
568 are depicted

Figure 1

Figure 2

nicotinamide

NMN

NAD⁺

NADase_{Ta}

nicotinate

NaPRT_{Tt}

NaMN

NaMAT_{Tt}

deamino-NAD⁺

Figure 3

Figure 4

Figure 5