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Characterization of Thermostable Deblocking Aminopeptidases of Archaeon *Thermococcus onnurineus* NA1 by Proteomic and Biochemical Approaches

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Thermococcus onnurineus **NA1 is a hyperthermophilic archaeon that grows optimally at >80°C. The deblocking aminopeptidase (DAP) (TNA1-DAP1) encoded in Ton_1032 of *T. onnurineus* NA1 is considered a major DAP. However, four genes encoding putative DAP have been identified from a genomic analysis of *T. onnurineus* NA1. A proteomic analysis revealed that all four DAPs were differentially induced in YPS culture medium and, particularly, two DAPs (TNA1-DAP1 and TNA1-DAP2) were dominantly expressed in *T. onnurineus* NA1. The biochemical properties and enzyme activity of DAPs induced in an *E. coli* expression system suggested that the two major DAPs play complementary roles in *T. onnurineus* NA1.**

Keywords: *Thermococcus onnurineus* NA1, deblocking aminopeptidases, proteomic identification

Introduction

Deblocking aminopeptidases (DAPs) are exoproteases that release amino acids from peptides modified with various amino-terminal acyl-type blocking groups (Lee *et al.*, 2007). The functions of these enzymes are not well known but protein maturation and metabolism of peptides from proteasomes are considered major roles (Dura *et al.*, 2005; Mori and Ishikawa, 2005). The DAP of *Pyrococcus furiosus* has been used in Edman sequencing, because it can remove the deblocking amino acid (Kamp *et al.*, 1998; Tsunasawa, 1998). In particular, DAPs have been reported from several thermophilic archaea such as *Pyrococcus horikohsii*, *Pyrococcus furiosus*, and *Thermococcus kodakarensis* KOD1 (Kamp *et al.*, 1998; Ando *et al.*, 1999; Onoe *et al.*, 2002; Porciero *et al.*, 2005; Jia *et al.*, 2011). An interesting feature of archaea DAPs is the multitude of DAPs in a single cell, which was recently revealed by genome and biochemical analyses (Ando *et al.*, 1999; Mori and Ishikawa, 2005; Jia *et al.*, 2011). This characteristic raises a question about the role of multiple DAPs in archaea but, until now, not much is known.

Thermococcus onnurineus NA1 is another thermophilic archaeon with multiple DAP-encoding genes (Lee *et al.*, 2008). A thermostable DAP (Ton_1032) is considered the sole enzyme in *T. onnurineus* NA1 and has been biochemically characterized (Lee *et al.*, 2007). However, we found three other putative DAP encoding genes (Ton_0369, Ton_0570, and Ton_0627) in *T. onnurineus* NA1 through genome analyses.

In this study, shotgun proteomic analysis confirmed induction of all four genes with different expression levels in YPS culture media, suggesting that the four DAPs may be active in the cell and have specific roles in *T. onnurineus* NA1. Interestingly, two DAP genes (Ton_0369 and Ton_1032) were dominantly expressed in *T. onnurineus* NA1. Because of their abundance, the physicochemical properties of the two major DAPs were analyzed, and their biology in *T. onnurineus* NA1 is discussed. Two other DAP genes (Ton_0570 and Ton_0627) were also induced in an *E. coli* expression system for characterization. Our results suggest that two enzymes (Ton_0369 and Ton_1032) are major DAPs in *T. onnurineus* NA1 and play complementary roles.

Materials and Methods

Proteomic analysis and DAP identification

The proteomic analysis method was described previously (Yun *et al.*, 2011b). Proteomic analysis of the crude extract was performed by 1-DE/MS-MS analysis and protein identification and quantification were performed using the MASCOT program (Matrix Science, UK).

Cloning and expression of the DAPs

Cloning and overexpression of Ton_0369 (TNA1-DAP2) was described in a previous report (Yun *et al.*, 2011a). Two other genes (Ton_0570; TNA1-DAP3 and Ton_0627; TNA1-DAP4) were amplified from genomic *T. onnurineus* NA1 DNA by polymerase chain reaction (PCR). The primers for TNA1-DAP3 (sense [5′-CATATGCTCGTTGAAGAGCTGAGGGA-3′] and antisense [5′-GGATCCTCACAGCTCGAAAGCTATCGCTT-3′]), TNA1-DAP4 (sense [5′-CATATGGAGAGAGTCGTTAGGATACTCA-3′] and antisense [5′-GGATCCTCATTCAAGGCGGTATTTACTCT-3′]) were designed. The *Nde*I and *Bam*HI sequences are underlined, respectively.

The amplified DNA fragments of the DAP genes were cloned

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into the TOPcloner Blunt V2 vector and used to transform *E. coli* DH5α cells. The TNA1-DAP genes (Ton_0570 and Ton_0627) from the TOPcloner Blunt V2 vectors were excised by *Nde*I and *Bam*HI digestion, ligated into the pTYB2 vectors, and then transformed into *E. coli* DH5α. The true transformants were selected by colony PCR, and the nucleotide sequences of their vectors were confirmed. These vectors were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL for overexpression. Transformants were grown to an optical density of 0.6 at 600 nm and 37°C in LB medium containing 50 μg/ml chloramphenicol and kanamycin or ampicillin. The culture was then cooled on ice and isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.5 mM. The TNA1-DAP3 and TNA1-DAP4 transformants were incubated for 24 h at 20°C.

Purification of recombinant DAPs

Purification of TNA1-DAP2 was described previously (Yun *et al*., 2011a). TNA1-DAP3 and TNA1-DAP4-expressing *E. coli* BL21 was collected by centrifugation, resuspended in lysis buffer (20 mM HEPES; pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton-X100), disrupted by sonication, and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were loaded on chitin beads (New England Biolabs, USA) and washed in the same buffer. The column was then quickly flushed with 30 mM DTT cleavage buffer (20 mM HEPES; pH 8.0, 500 mM NaCl, 0.1 mM EDTA). The flow was stopped, and the column was maintained at 4°C overnight. TNA1-DAP3 and TNA1-DAP4 were eluted with cleavage buffer without DTT. Purified proteins were dialyzed with 20 mM Tris-HCl (pH 7.5), and their biochemical characteristics were examined. Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific, USA) and bovine serum albumin was used as the standard.

Enzyme activity assay

DAP and deacetyl aminopeptidase activities were measured by the amount of *p*-nitroaniline released at a wavelength of 406 nm. The assay mixture (400 μl) contained 50 mM Tris-HCl buffer (pH 7.5), 0.1 mM $CoCl_2$, 0.2 M NaCl, and 2 mM of substrates (Leu-*p*NA and Met-*p*NA) and was pre-incubated at 80°C for 5 min. The reaction was started by adding the enzyme (50 μl). The reaction mixture was incubated at 80°C for 5 min, and the reaction was stopped by adding 1 M trichloroacetic acid (50 μl). Activity toward the *p*NA derivatives was calculated using the absorption coefficient ε_{406}=9.91 mM^{-1} of nitroaniline released.

Measurement of DAP molecular weight using matrix-assisted laser desorption/ ionization time of flight mass spectroscopy (MALDI TOF MS)

All samples were prepared by mixing equal volumes of protein solution and sinapinic acid (10 mg/ml in 50% CAN/0.1%

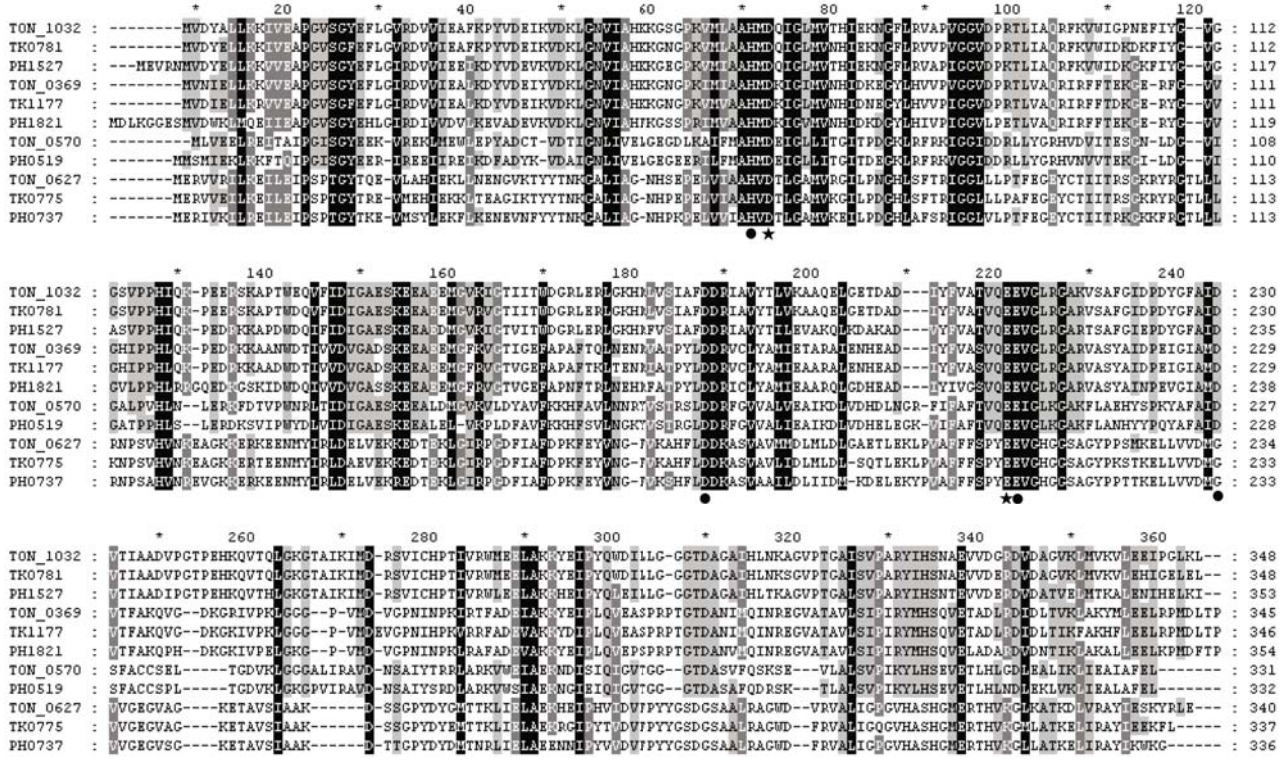

Fig. 1. Alignment of amino acid sequences of *T. onnurineus* NA1 deblocking aminopeptidases (DAPs) with those of other DAPs. Multiple sequence alignment was performed using the ClustalW program. Catalytic residues are indicated as stars, and metal binding residues are indicated as black circles. Conserved amino acid residues are indicated by gray or black shading. *T. kodakarensis* KOD1 DAPs (TK0781, TK1177, and 0775) (Fukui *et al*., 2005) and those of *P. horikoshii* OT3 (PH0519, PH0775, PH1527, and PH1821) (Kawarabayasi *et al*., 1998) are included in the alignment.

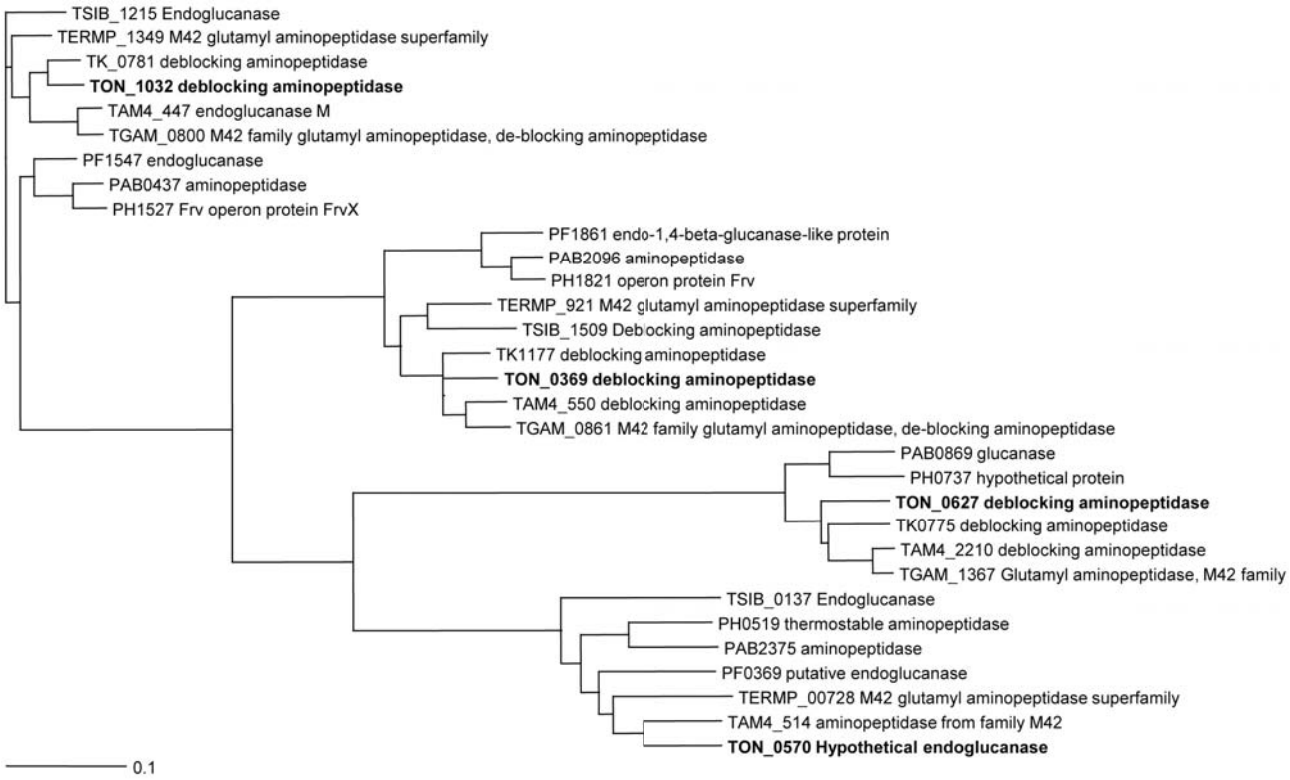

Fig. 2. Phylogenic tree of deblocking aminopeptidases (DAPs) constructed using the TreeView program. DAPs from nine archea were compared in this analysis (Maeder *et al.*, 1999; Mardanov *et al.*, 2009; Zivanovic *et al.*, 2009; Oger *et al.*, 2011; Vannier *et al.*, 2011).

TFA) (Pimenova *et al.*, 2009). One microliter of the mixture was spotted onto a MALDI plate and dried in air at room temperature. A MALDI-TOF mass spectrometer (ultrafleXtreme, Bruker, Germany), equipped with a high-mass detector (HM2 tuvo, CovalX AG, Switzerland), was employed. Typical acceleration voltages were 20 kV, and the gain voltage on the detector was set to 2.8 kV. Mass spectra were acquired by averaging 300 laser shots at different locations within a sample (Bich *et al.*, 2010). The instrument was externally calibrated with the C150 and C450 calibration kits (CovalX AG). The mass accuracy range was 10–500 kDa. Mass spectra were background subtracted and smoothed using a dedicated software package (Complex Tracker 2, CovalX AG).

Results

T. onnurineus NA1 has four differently originating genes encoding the DAP

Four genes (Ton_0369, Ton_0570, Ton_0627, and Ton_1032) were assigned as DAPs or as an endoglucanase based on the *T. onnurineus* NA1 genome analysis. We designated these proteins as TNA1-DAP2, TNA1-DAP3, TNA1-DAP4, and TNA1-DAP1, respectively. All DAPs have common active catalytic residues (Asp^{73} and Glu^{221}) and metal binding residues (His^{71}, two $Asp^{188, 244}$, and Glu^{222}), except for TNA1-DAP4 (Fig. 1). However, a sequence analysis showed that homology between DAPs was not significantly high (sequence homology range, 19–52%), and that the genes were scattered

Table 1. Identification of deblocking aminopeptidase of *T. onnurineus* NA1 by MS/MS analysis using FT-ICR MS spectrometer

Gene no.	Description	Cellualr localization (psortB v 3.0)	Molecular weight (Da)	No. of detection	1st Analysis			2nd Analysis		
					emPAI	Peptides	Unique peptides	emPAI	Peptides	Unique peptides
TON_0369	Deblocking aminopeptidase (TNA1-DAP2)	Cytoplasmic	38061	2	31.52	274	21	38.25	327	24
TON_0570	Hypothetical endoglucanase (TNA1-DAP3)	Cytoplasmic	36573	2	6.1	79	14	4.84	108	15
TON_0627	Deblocking aminopeptidase (TNA1-DAP4)	Cytoplasmic	37465	2	0.61	15	4	0.61	14	4
TON_1032	Deblocking aminopeptidase (TNA1-DAP1)	Cytoplasmic	37917	2	26.19	306	24	23.74	538	22

Table 2. Kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) of TNA1-DAP1 and TNA1- DAP2

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
TNA1-DAP1[a]	Leu-*p*NA	0.8	37.5	4.7×10^4
	Met-*p*NA	0.2	10.9	5.5×10^4
TNA1-DAP2	Leu-*p*NA	2.65	108	4.1×10^4
	Met-*p*NA	0.46	101	2.2×10^5

[a] Kinetic parameters of TNA1-DAP1 was quoted from the previous study (Lee *et al.*, 2007)

in the *T. onnurineus* NA1 genome. These results ruled out the possibility of gene duplication from a unique gene and suggest different origins. Phylogenic analysis of TAN1-DAPs with other DAPs also supported that each TRAN1-DAP had peculiar homology groups among DAPs from various archea (Fig. 2).

Proteomic analysis revealed that two DAPs (TNA1-DAP1 & TNA1-DAP2) are dominantly induced in *T. onnurineus* NA1

Global proteomic analysis provides valuable information about the proteins induced under specific culture conditions and their quantitative values, which are very important for understanding the physiology. Crude protein extract mixtures of *T. onnurineus* NA1 cultured in YPS medium are used to identify and quantify the proteins to understand the physiology (Kwon *et al.*, 2009; Yun *et al.*, 2011b; Moon *et al.*, 2012). We extracted and summarized the proteomic data for the four DAPs from the raw proteomic data (Table 1). The results showed that all *T. onnurineus* NA1 DAPs were expressed in YPS culture medium. In particular, TNA1-DAP2 (average emPAI: 34.89) and TNA1-DAP1 (average emPAI: 24.96) were induced abundantly, but TNA1-DAP3 (average emPAI: 5.47) and TNA1-DAP4 (average emPAI: 0.61) were only traceably induced. This result suggests that two enzymes (TNA1-DAP2 and TNA1-DAP1) play a major biological role as DAPs.

Biochemical characterization of the three other DAPs

Because TNA1-DAP1 was characterized previously (Lee *et al.*, 2007), the three other DAPs were overexpressed, purified, and characterized in the present study. A comparative analysis of the two enzymes was performed, as TNA1-DAP1 and TNA1-DAP2 are major DAPs. A kinetic analysis (k_{cat}/K_m ($M^{-1}s^{-1}$)), using Leu-pNA and Met-pNA as substrates, showed that the two enzymes had different substrate specificities. The hydrolysis activity of TNA1-DAP1 to Leu-*p*NA was slightly higher than that of TNA1-DAP2. However, TNA1-DAP2 had about four-fold higher hydrolysis activity to Met-*p*NA than that of TNA1-DAP1 (Table 2). The effects of metals on the two DAPs were similar, except for Mn^{2+} and Ni^{2+} (Fig. 3A). TNA1-DAP2 had a 90°C optimal temperature and half-life enzyme activity at 96°C was about 2 h (Figs. 3B and 3C). The molecular weight of native TNA1-DAP2 was about 539.6 kDa by MADLI-TOF MS, indicating that native TNA1-DAPs was composed of 14 subunits (Fig. 4B).

Other minor DAPs (TNA1-DAP3 and TNA1-DAP4) were also expressed in the *E. coli* expression system. Although expression of these enzymes was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4A), enzyme

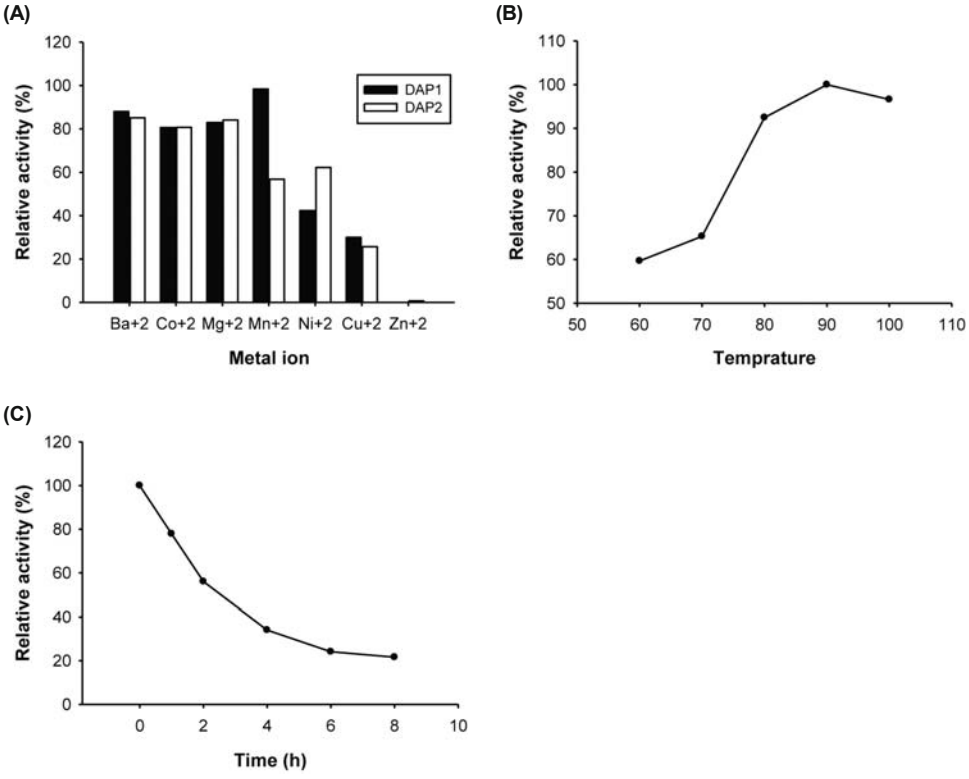

Fig. 3. Biochemical properties of *T. onnurineus* NA1 deblocking aminopeptidase2 (TNA1-DAP2). Metal effects on enzyme activity (A), optimal temperature for enzyme activity (B), and thermostability (C) of TNA1-DAP2.

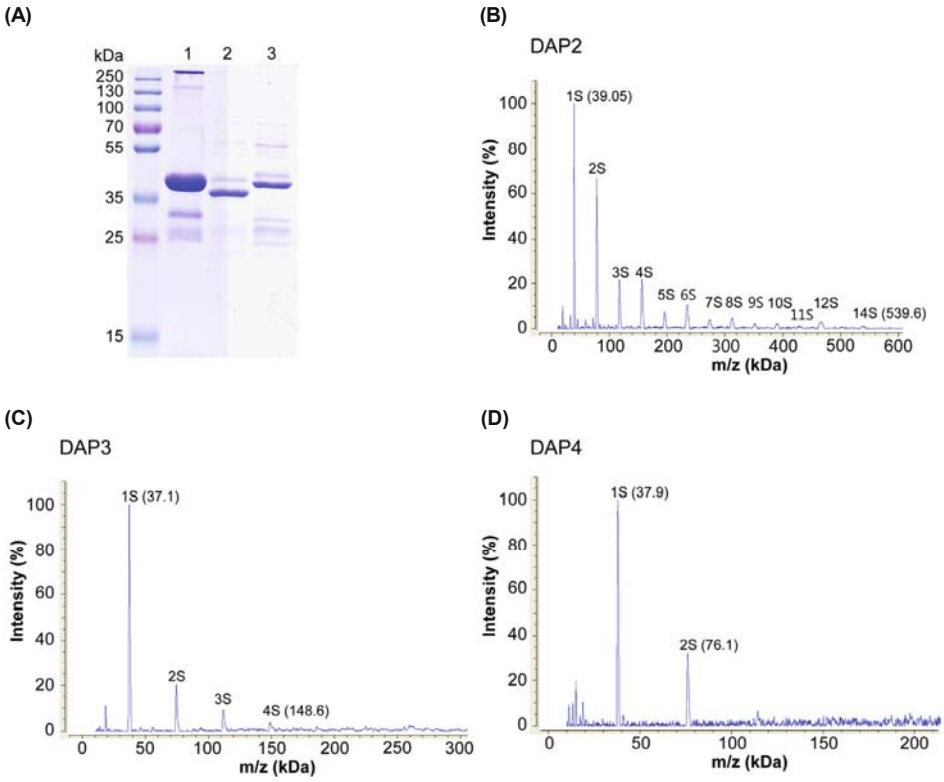

Fig. 4. Molecular weights of native *T. onnurineus* NA1 deblocking aminopeptidases (DAPs) measured by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS). Overexpressed DAPs on SDS-PAGE (A). Lanes: 1, TNA1-DAP2; 2, TNA1-DAP3; 3, TNA1-DAP4. *T. onnurineus* NA1 deblocking aminopeptidase2 (TNA1-DAP2) (B), TNA1-DAP3 (C), and TNA1-DAP3 (D). Theoretical molecular weights of TNA1-DAP2, DAP3, and DAP4 were calculated to be 37.95, 36.47, and 37.36 kDa, respectively. The His-tag (×6) was not removed from TNA1-DAP2.

activity was very low. TNA1-DAP4 enzyme activity was detectable by UV-spectrometer, but TNA1-DAP3 activity was not detected. The molecular weights of native TNA1-DAP3 and TNA1-DAP4 were calculated as 148.6 kDa and 76.1 kDa, which suggest a tetramer and dimer, respectively (Figs. 4C and 4D).

Discussion

Four genes encoding DAPs were identified in the *T. onnurineus* NA1 genome, and their expression was confirmed by proteomic analysis. The proteomic results showed that two DAPs (TNA1-DAP1 and TNA1-DAP2) were highly expressed, suggesting their major roles as DAPs in the cell. The enzyme activity of these DAPs, expressed in the *E. coli* system, also confirmed their role in *T. onnurineus* NA1. The substrate specificity assay showed that TNA1-DAP2 had high affinity to Met-*p*NA compared to that of TNA1-DAP1, suggesting that the two DAPs are complementary during hydrolysis of various substrate proteins in *T. onnurineus* NA1. Induction of other minor DAPs (TNA1-DAP3 and TNA1-DAP4) was very low in the *E. coli* expression system and in *T. onnurineus* NA1. Multiple thermostable DAPs have been reported in archea (Ando *et al.*, 1999; Mori and Ishikawa, 2005; Jia *et al.*, 2011). Representative archea with multiple DAPs are *Thermococcus kodakarensis* KOD1 and *Pyrococcus horikoshii* OT3. In the case of *T. kodakarensis* KOD1 with three DAP genes, two DAPs are upregulated by heat and oxidative stress but induction and enzyme activity of the third DAP is not detectable (Jia *et al.*, 2011). *P. horikoshii* OT3 has three active DAPs with different specificities and activation factors (Mori and Ishikawa, 2005). Sequence analysis of multiple DAPs from the three archea revealed that four *T. onnurineus* NA1 DAPs belong to four different groups, of which DAPs have high sequence homology in the range of 75–94%, suggesting that each group has specific roles in the cell. However, further substrate specificity and mutagenesis studies are needed to understand the roles of the multiple DAPs more clearly.

Proteomics was as an effective method for detecting and quantifying multiple differentially induced proteins from protein mixtures of DAPs in *T. onnurineus* NA1 in this study. We have previously enriched and identified the hyperthermostable proteins using heat treatment and proteomic analysis (Yun *et al.*, 2011a). We found from a re-analysis of this proteomic data that three (TNA1-DAP1, 2, 4) of the four *T. onnurineus* NA1 DAPs remained soluble and did not precipitate at 100°C. These two studies showed that at least three DAPs (except for TNA1-DAP3) are active in the *E. coli* expression system and in *T. onnurineus* NA1 under high temperature conditions (>90°C).

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