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ORIGINAL PAPER

Enrichment and proteome analysis of a hyperthermostable protein set of archaeon *Thermococcus onnurineus* NA1

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Abstract *Thermococcus onnurineus* NA1 is a hyperthermophilic archaeon that can be used for the screening of thermophilic enzymes. Previously, we characterized the metabolic enzymes of the cytosolic proteome by two-dimensional electrophoresis/tandem mass spectrometry (2-DE/MS–MS). In this study, we identified a subset of hyperthermostable proteins in the cytosolic proteome using enrichment by in vitro heat treatment and protein identification. After heat treatment at 100°C for 2 h, 13 and 149 proteins were identified from the soluble proteome subset by 2-DE/MS–MS and 1-DE/MS–MS analysis, respectively. Representative proteins included intracellular protease I, thioredoxin reductase, triosephosphate isomerase, putative hydroperoxide reductase, proteasome, and translation initiation factors. Intracellular protease, deblocking aminopeptidases, and fructose-1,6-bisphosphatase were overexpressed in *Escherichia coli* and biological activity above 85°C was confirmed. The folding transition temperature (Tm) of identified proteins was analyzed using the in silico prediction program TargetStar. The proteins enriched with the heat treatment have higher Tm than the homologous proteins from mesophilic strains. These results suggested that the heat-stable protein set of hyperthermophilic *T. onnurineus* NA1 can be effectively fractionated and enriched by in vitro heat treatment.

Keywords Archaea · *Thermococcus* · Hyperthermostable · Proteome

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Introduction

Hyperthermophiles are valuable living things for the elucidation of adaptation mechanism of microorganisms inhabiting in extreme environment. Recently, proteomic approaches have been applied to understand metabolic characteristics of hyperthermophiles. Proteomic mining has been used to comprehensively investigate the proteins of hyperthermophiles (Barry et al. 2006; Burghardt et al. 2008; Kwon et al. 2009; Lee et al. 2008a; Yamazaki et al. 2006; Wang et al. 2004). These results revealed the elemental metabolic characters of the strains. In the case of *Thermoanaerobacter tengcongensis* and *Thermus thermophilus*, comparative proteome analysis was performed to identify proteins associated with hyperthermophilic activity by using the strains cultured in different temperatures (Trauger et al. 2008; Wang et al. 2007); the results revealed which proteins were up- and downregulated according to stimulation by temperature of culture. However, proteomic approaches to screen heat-stable proteins of hyperthermophiles was not intensively performed. In recent, thermal and chemical perturbation methods have been used for screening of thermostable proteome (Prosinecki et al. 2006). This method lowered the complexity of the soluble

proteome of hyperthermophilic *Sulfurisphaera* sp. and allows enrichment of cytosolic hyperstable proteins (Prosinecki et al. 2006).

Thermococcus onnurineus NA1 is a hyperthermophilic archaeon that was isolated from a deep-sea hydrothermal vent area in the PACMANUS field (3°14′S, 151°42′E) of the East Manus Basin (Bae et al. 2006). Genome sequencing revealed a single circular chromosome (1847 kb) containing 1976 coding DNA sequences (CDS) (Lee et al. 2008a, b); genomic analysis identified metabolic pathways for organotrophic growth on peptides, amino acids, and sugars, and for carboxydotrophic growth in the presence of carbon monoxide. Recently, formate-utilizing activity of *T. onnurineus* NA1 was reported (Kim et al. 2010). In our previous study, key metabolic enzymes were confirmed by proteomic approach using two-dimensional electrophoresis/tandem mass spectrometry (2-DE/MS–MS) (Kwon et al. 2009). Besides the metabolic characteristics, the proteins thermostability of *T. onnurineus* NA1 is also of great interest because the optimum growth temperature is above 85°C. In particular, DNA polymerase, aminopeptidase, and α-amylase of *T. onnurineus* NA1 have been selected and biochemically analyzed for the bio-industrial application due to their characteristic thermophilic properties (Kim et al. 2007a; Lee et al. 2007; Lim et al. 2007).

In the present study, we screened for hyperthermostable proteins of *T. onnurineus* NA1 by combined approach; heat treatment, proteomic analysis, and in silico prediction. In vitro heat treatment was used to enrich hyperthermostable proteins, which were identified by 2-DE and 1-DE/MS–MS. In silico analysis using the program TargetStar was performed and the findings were compared with the proteomic results. Our data suggested that the hyperthermostable proteome can be enriched by in vitro heat treatment, and that proteomic approaches combined with in silico prediction can be useful for the screening and identification of novel hyperthermostable proteins from *T. onnurineus* NA1.

Experimental methods

Cultivation and protein sample preparation

Thermococcus onnurineus NA1 was isolated from a deep-sea hydrothermal vent (Bae et al. 2006). Culture and strain maintenance was performed according to the standard procedures described for archaea (Robb et al. 1995). A 25-mL serum bottle of yeast extract-peptone-sulfur (YPS) medium (Holden et al. 2001) was inoculated with a single colony of *T. onnurineus* NA1 from a phytagel plate and cultured anaerobically at 85°C for 20 h. YPS is composed of yeast extract (3 g/L), peptone (3 g/L), and sulfur (10 g/L). These seed cultures were added to 700 mL of YPS medium in a 1-L anaerobic jar for culture at 85°C for 20 h. Cytosolic proteins were prepared as described previously (Kwon et al. 2009). Once cultivated, cells were harvested by centrifugation (15000×*g* for 45 min), suspended in 20 mM Tris–HCl buffer (pH 8.0), and disrupted using a French pressure cell (SLM-Aminco, Urbana, IL) at 20000 lb/in^2. Supernatants (cytosolic protein extract) were prepared by centrifugation (15000×*g* for 45 min).

Heat treatment and separation of heat-stable proteins

Cytosolic protein extracts were subjected to heat treatment for the removal of thermolabile proteins using the modified method of Prosinecki et al. (2006). Protein samples containing 1 mg of protein in 20 mM Tris–HCl (pH 8.0), 1 mM PMSF were incubated for 10 min, 30 min, 1, 2, 3, 6, 10, and 24 h at 60, 80, and 100°C. Denatured and coagulated proteins were precipitated and removed by centrifugation at 18000×*g* for 20 min. The amount of each cytosolic protein fraction was assayed by the Bradford method. Proteins in the cytosolic fraction were separated by 2-DE and SDS–PAGE.

2-DE and 1-DE

After heat treatment at 80 and 100°C for 2 h, protein samples were prepared and used for gel analysis. The initial amount of each protein sample was 250 μg. A protein sample that did not undergo heat treatment was used as a control. Two-dimensional electrophoresis was performed according to the method described previously (Kwon et al. 2009). Protein samples were applied to immobilized pH 3–10 (nonlinear) or pH 4–7 (linear) gradient strips (18 cm) using IPGphor (Amersham Pharmacia Biotech, Uppsala, Sweden). In the second dimension, SDS–PAGE (12%) was performed using a Hoeffer Dalt system (Amersham Pharmacia Biotech). For each sample, 2-DE was carried out three or more times for verification of induced proteins. For 1-DE/MS–MS, SDS–PAGE (12%) was performed for fractionation of sample proteins according to their molecular size.

In-gel digestion

In-gel digestion was carried out using a previously described method (Kwon et al. 2009). Protein spots on the 2D gels were excised; following reduction and alkylation of cysteines, proteins were digested in trypsin solution for 12–16 h at 37°C. Digested peptides were extracted with a solution consisting of 50 mM ammonium bicarbonate, 50% acetonitrile, and 0.1% trifluoroacetic acid, and lyophilized in a vacuum concentrator. Dried tryptic peptides were dissolved

in 0.5% TFA and prepared using Zip Tip C18 cartridges (Millipore, Billerica, MA). Peptides were loaded onto a MALDI plate after mixing with α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/mL, 0.5% TFA/50% acetonitrile). One-dimensional gels were sliced into 26 fractions and used for tryptic digestion according to the method described previously.

Protein identification by MS/MS analysis using MALDI-TOF MS

A 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA) was used for MS/MS analysis. MASCOT ver.2.2 (Matrix Science, London, UK; http://www.matrixscience.com) was used for protein identification; peptide mass tolerance was 50 ppm. Carbamidomethylation of cysteines, oxidation of methionines, and pyroglutamylation of glutamates were considered in the protein identification procedure.

Identification of proteins in the thermostable fraction using the LCQ Deca XP

After heat treatment, prepared proteins were subjected to SDS-PAGE followed by in-gel digestion with trypsin. Tryptic-digested peptide mixtures were redissolved in 15 µL of buffer (0.1% formic acid, 0.02% acetic acid). Aliquots of 10 µL of the sample solutions were concentrated in MGU30-C18 trapping columns (LC Packings, Amsterdam, The Netherlands). Concentrated peptides were directed onto a C18 reverse-phase column (15 × 75 µm i.d., fused silica) at a flow rate of 120 nL min^{-1}. Peptides were eluted with an acetonitrile gradient (0–65% for 70 min), and sprayed into the analyzer. All MS and MS/MS spectra in the LCQ-Deca ESI ion trap mass spectrometer were acquired in data-dependent mode. For protein identification, nano LC–MS/MS spectra were searched with MASCOT (Matrix Science) using data from the genome of *T. onnurineus* NA1 obtained from NCBInr (http://www.ncbi.nih.gov) and the decoy sequence database. Search parameters for oxidation of methionine and carbamidomethylation of cysteines were applied, and one missed trypsin cleavage was considered. Allowed mass tolerance was 1.5 Da. The exponentially modified protein abundance index (emPAI) was generated using MASCOT (Matrix Science) (Ishihama et al. 2005).

Gene cloning, overexpression, and enzyme activity assay of hyperthermophilic proteins

The full-length genes of deblocking aminopeptidase (DAP; TON_0369) and fructose-1,6-bisphosphatase (FBPase; TON_1497) of *T. onnurineus* NA1 were amplified by PCR using the following primer pairs: sense 5′-CATATGGAG AGAGTCGTTAGGATACTCA-3′ and antisense 5′-GGAT CCTCATTCAAGGCGGTATTTACTCT-3′; sense 5′-CAT ATGGCCATTGGAGA GAAA ATAACGA-3′ and antisense 5′-GGATCCTCACTCAATGTCCTCAAAGCGCT-3′, respectively. The PCR products were digested with *Nde*I/*Bam*HI and cloned into *Nde*I/*BamH*I sites of the expression vector pET-28a(+) (Novagen, Madison, WI) yielding the recombinant DAP expression plasmids pDAP2 and pFBP1, respectively. The full-length sequence of the intracellular protease-encoding gene, flanked by *Nhe*I and *Xho*I sites, was amplified with the following primers: sense 5′-CGA CCCGGGCTAGCATGAAGGTACTGTTTCTGAGCGCG GATG-3′ and antisense 5′-CTCCACATCTCGAG TCAGT GGAGGAGCTTCACAAACTCCCTC-3′. The amplified DNA fragment was digested with *Nhe*I and *Xho*I, and ligated into *Nhe*I/*Xho*I-digested pET-24a(+) (Novagen). The nucleotide sequences of the plasmids were confirmed by DNA sequencing. To express these recombinant proteins, either the *E. coli* BL21-CodonPlus(DE3)-RIL or the *E. coli* BL21-Rosetta™(DE3)pLysS strain (Stratagene, La Jolla, CA,) transformed with the constructed plasmids was grown at 37°C in LB medium containing 50 µg/mL kanamycin to an optical density of 0.6 at 600 nm. Isopropyl-*β*-D-thiogalactopyranoside (IPTG) (37°C for 3 h) was added to induce protein expression. Purification and enzyme assays for DAB and FBPase were performed according to the methods described previously (Lee et al. 2007; Verhees et al. 2002). DAP activity was determined by analyzing hydrolysis of Leu-*p*NA (Bachem AG, Bubendorf, Switzerland), FBPase activity was determined using 0.5 mM fructose-1,6-bisphosphate as the substrate. The amount of fructose-6-phosphate formed was determined spectroscopically by measuring $NADP^+$ reduction (340 nm) in the presence of glucose-6-phosphate isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Verhees et al. 2002). Intracellular protease was purified through two-step chromatography (HiTrap Q HP and Superdex 200 10/300 GL columns) using the Äkta FPLC system, and activity was assayed by monitoring the production of MCA (7-amido-4-methylcoumarin) from the fluorogenic peptide, LLVY-MCA (leucine-leucine-valine-tyrosine-methylcoumarylamide) 0.15 mM; MCA fluorescence was measured at 360 ± 40 nm excitation, 460 ± 40 nm emission, on an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

In silico prediction of thermostable proteins using TargetStar software

The prediction program TargetStar (http://www.ensoltek.com) was used to predict the relative thermostabilities of the *T. onnurineus* NA1 proteome (Kim et al. 2007b). The

relative thermostability was described as the folding transition temperature (Tm). Tm is defined as the temperature at which the protein unfolding rate is 50%. Tm values of the identified proteins remained at 100°C were compared with the homologous proteins from the mesophilic and hyperthermophilic strains. Proteins having less than 0.001 *e* value in blastp (ver. 2.2.23+) were selected as homologous proteins from the mesophilic and hyperthermophilic strains. Five mesophilic and four hyperthermophilic strains were used as the control database; mesophilic strains (*Corynebacterium glutamicum* ATCC13032 (Ikeda and Nakagawa 2003), *Escherichia coli* K12 (Blattner et al.

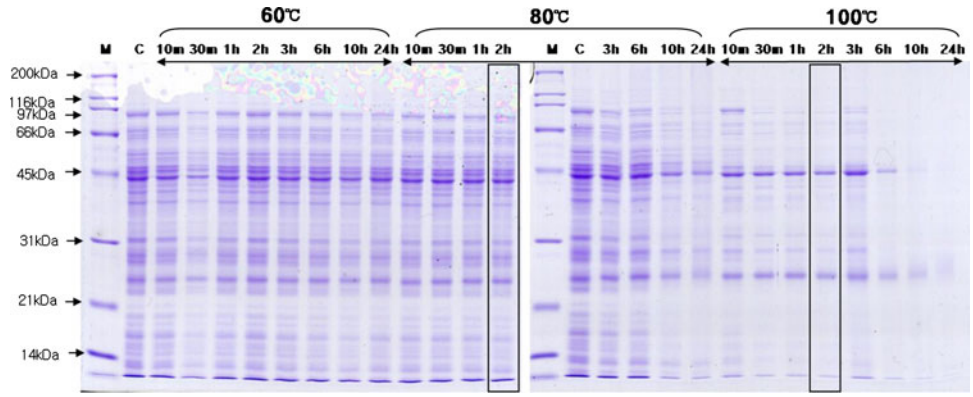

Fig. 1 In vitro heat treatment of soluble protein fractions of *T. onnurineus* NA1. Protein samples containing 1 mM PMSF was incubated for 10 min–24 h at 60, 80, and 100°C. After removal of precipitated proteins by centrifugation, soluble protein factions were loaded on the 12% SDS-polyacrylamide gel. *C* non-heat-treated protein sample. Boxed gel lanes were used for identification of thermostable proteins by 1DE/MS–MS analysis. The results of the analysis are shown in Table 2

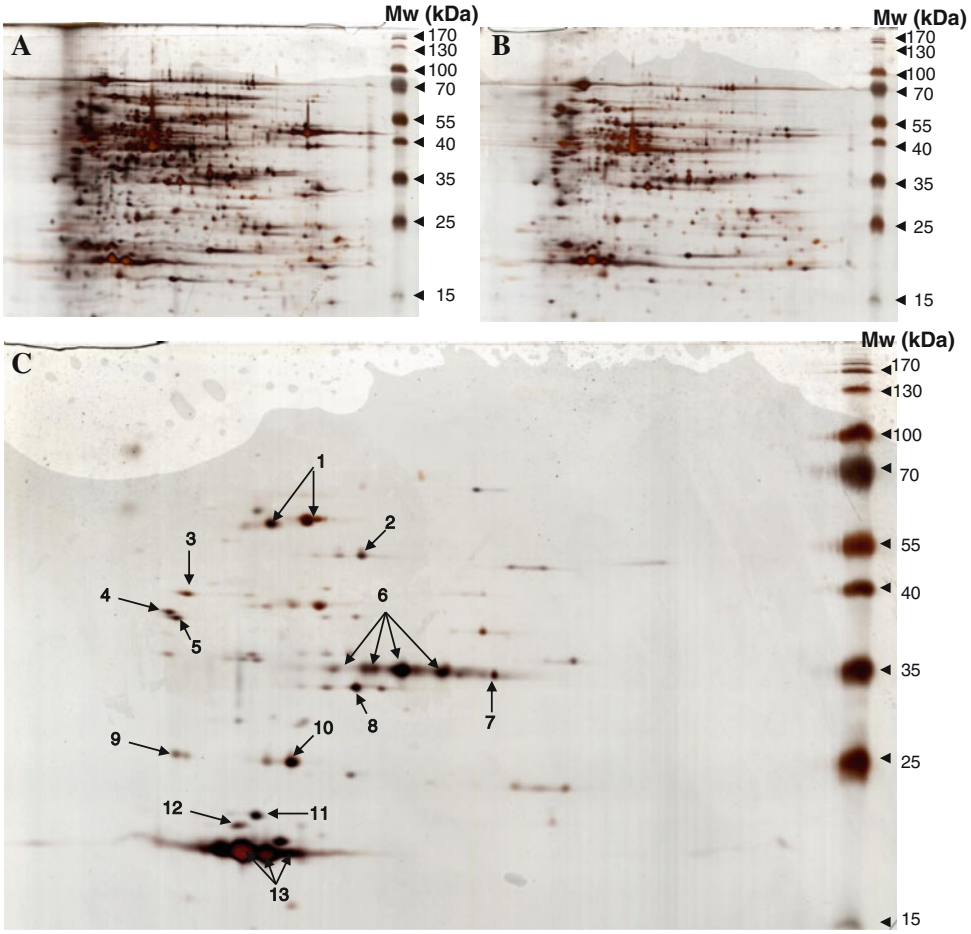

Fig. 2 Two-dimensional gel electrophoresis of thermostable proteins of *T. onnurineus* NA1. Aliquots of 250 μg of cytosolic protein samples were heat-treated at 80 and 100°C. After removal of coagulated proteins by centrifugation, enriched soluble proteins were used for 2-DE. **a** Control; **b** Enriched proteins after 80°C heat treatment; **c** Enriched proteins after 100°C heat treatment. Numbered protein spots were identified by MS/MS analysis (Table 1)

1997), *Listeria monocytogenes* EGD-e (Doumith et al. 2004), *Prochlorococcus marinus* SS120 (Dufresne et al. 2003), *Synechococcus* sp. WH8102 (Palenik et al. 2003)) and hyperthermophilic strains (*Archaeoglobus fulgidus* (Klenk et al. 1997), *Methanococcus jannaschii* (Bult et al. 1996), *Pyrococcus abyssi* (Cohen et al. 2003), and *Thermococcus kodakaraensis* KOD 1 (Fukui et al. 2005)). The ranges of optimal growth temperatures of the mesophilic and hyperthermophilic strains were 24–37°C and 80–100°C, respectively.

Results and discussion

Fractionation of hyperthermostable proteins

Proteins were defined as hyperthermostable if they were soluble after heat treatment at 100°C. The cytosolic fraction of *T. onnurineus* NA1 was prepared for fractionation of hyperthermostable proteins. Heat treatment of the cytosolic fraction was performed at 60, 80, and 100°C over a period of 10 min to 24 h. Denatured and coagulated proteins were removed by centrifugation, and the remaining soluble proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). We assumed that thermolabile proteins of *T. onnurineus* NA1 were coagulated and precipitated by heat treatment and that thermostable proteins remained and were enriched in the soluble protein fraction. However, some proteins were denatured and coagulated at lower temperatures even though the optimal growth temperature of *T. onnurineus* NA1 is 85°C. When compared with the untreated cytosolic fraction, 63.7 and 19.2% of cytosolic proteins remained in the soluble fraction after heat treatment for 2 h at 80 and 100°C, respectively (Fig. 1). These samples were used for 2-DE (Fig. 2). Most protein spots from the soluble fraction prepared by heat treatment at 100°C were well matched with protein spots from a reference 2-DE map indicating that enriched soluble proteins were not digested by proteases during heat treatment, and that they retained their native physicochemical properties (Kwon et al. 2009).

Table 1 Identification of protein spots on 2D gels by PMF and MS/MS analysis using MALDI-TOF/TOF MS

Protein no.	ORF ID	Annotation	MW/pI	PMF			MS/MS		
				Coverage (%)[a]	Score[b]	Match[c]	Coverage (%)[a]	Score[b]	Match[c]
1	TON_1285	Intracellular protease I	18704/5.36	47	53	8	33	268	4
2	TON_0305	NADH oxidase	48401/5.66	46	99	17	8	118	4
3	TON_1613	Enolase	46818/4.94	41	90	16	10	163	3
4	TON_1302	389aa long hypothetical nonspecific lipid-transfer protein (acetyl CoA synthetase)	40893/4.88	41	58	11	9	29	2
5	TON_0157	Glutamate dehydrogenase (GDH)	46784/5.48	44	125	20	9	112	3
6	TON_1603	Thioredoxin reductase	35812/6.28	71	171	24	7	336	3
7	TON_0639	Glyceraldehyde-3-phosphate dehydrogenase	37123/6.21	46	72	13	14	170	3
8	TON_1296	Translation initiation factor eIF-2B, delta subunit	36030/5.77	50	94	15	26	116	5
9	TON_0027	Proteasome alpha subunit (multicatalytic endopeptidase complex alpha subunit)	28735/4.87	38	40	8	14	155	3
10	TON_0332	Probable transcription regulator (DUF118 helix-turn-helix family)	33040/5.9	43	72	11	25	274	5
11	TON_1640	Triosephosphate isomerase	23890/5.36	27	26	6	12	67	2
12	TON_1426	Proteasome, beta subunit 2	21753/5.35	19	20	4	12	75	2
13	TON_0829	216aa long hypothetical alkyl hydroperoxide reductase	24726/5.4	91	220	21	23	427	5

[a] Sequence coverage calculated by the MASCOT program

[b] MASCOT score

[c] Number of matched peptide fragments

Table 2 Protein identification by MS/MS analysis using LCQ Deca XP in the soluble protein fraction prepared after in vitro heat treatment

ORF ID	Annotation	MW/pI	80°C 2 h		100°C 2 h	
			emPA[a]	Mol%	emPAI[a]	Mol%
TON_0829	216aa long hypothetical alkyl hydroperoxide reductase[b]	24726/5.4	24.47	14.25	207.93	52.90
TON_1603	Thioredoxin reductase[b]	35812/6.28	5.80	3.38	35.90	9.13
TON_1640	Triosephosphate isomerase[b]	23890/5.36	1.74	1.01	9.47	2.41
TON_0305	NADH oxidase[b]	48401/5.66	0.80	0.47	7.16	1.82
TON_1543	Acylamino acid-releasing enzyme	72566/5.33	1.20	0.70	5.77	1.47
TON_1613	Enolase[b]	46818/4.94	1.38	0.80	5.74	1.46
TON_1866	Translation elongation factor EF-1, beta subunit	10265/4.33	2.10	1.22	5.59	1.42
TON_0157	Glutamate dehydrogenase (GDH)[b]	46784/5.48	4.21	2.45	5.20	1.32
TON_1314	Unnamed protein product	24308/5.35	0.39	0.23	5.12	1.30
TON_0674	Hypothetical protein	27629/6.92	1.39	0.81	4.73	1.20
TON_1285	Intracellular protease I[b]	18704/5.36	1.33	0.77	4.44	1.13
TON_1764	ABC-type dipeptide/oligopeptide transport system, probable periplasmic component	89946/4.79	1.48	0.86	4.15	1.06
TON_1271	NADH oxidase	47354/8.5	0.19	0.11	4.10	1.04
TON_0639	Glyceraldehyde-3-phosphate dehydrogenase[b]	37123/6.21	0.12	0.07	3.61	0.92
TON_0934	Translation initiation factor eIF-2B, beta subunit	39663/6.19	N.D.	N.D.	3.18	0.81
TON_0319	Protein disulfide oxidoreductase	25512/4.67	1.57	0.91	3.11	0.79
TON_0033	Hypothetical protein, conserved	14746/9.1	N.D.	N.D.	2.79	0.71
TON_1362	Nicotinate-nucleotide pyrophosphorylase	43240/5.74	N.D.	N.D.	2.71	0.69
TON_1032	Deblocking aminopeptidase	37940/5.67	0.53	0.31	2.59	0.66
TON_0983	ABC-type iron(III) transport system, periplasmic component	54088/5.01	0.83	0.48	2.34	0.60
TON_0704	380aa long hypothetical protein	42791/6.09	0.33	0.19	2.11	0.54
TON_0314	Uracil phosphoribosyltransferase	24770/6.78	0.18	0.10	2.11	0.54
TON_1617	Hypothetical protein	17575/5.33	N.D.	N.D.	2.09	0.53
TON_1296	Translation initiation factor eIF-2B, delta subunit[b]	36030/5.77	2.43	1.42	2.07	0.53
TON_1354	Aor-2 tungsten-containing aldehyde ferredoxin oxidoreductase	67279/5.51	0.95	0.55	1.98	0.50
TON_1497	Thermophile-specific fructose-1,6-bisphosphatase	41875/5.27	0.79	0.46	1.90	0.48
TON_0594	Sugar binding protein	44175/4.65	2.62	1.53	1.75	0.45
TON_0129	NADH: polysulfide oxidoreductase	48658/6.65	N.D.	N.D.	1.73	0.44
TON_1302	389aa long hypothetical nonspecific lipid-transfer protein (acetyl CoA synthetase)[b]	40893/4.88	0.49	0.29	1.70	0.43
TON_1722	Histidyl-tRNA synthetase	49556/5.36	N.D.	N.D.	1.68	0.43
TON_1651	Purine-nucleoside phosphorylase	29042/6.46	0.15	0.09	1.63	0.41
TON_1914	Inorganic pyrophosphatase	20812/4.77	0.47	0.27	1.61	0.41
TON_0369	Deblocking aminopeptidase	38084/5.33	0.24	0.14	1.60	0.41
TON_1815	Xanthine/guanine phosphoribosyltransferase	17609/5.46	N.D.	N.D.	1.45	0.37
TON_0644	Small nuclear ribonucleoprotein, putative	8628/6.08	0.55	0.32	1.41	0.36
TON_0372	Predicted acetyltransferase, isoleucine patch superfamily	19022/5.5	0.23	0.13	1.31	0.33
TON_0560	*S*-adenosylmethionine synthetase	44728/5.1	0.09	0.05	1.26	0.32
TON_1623	Molybdopterin converting factor, subunit 1	9921/4.69	1.18	0.69	1.18	0.30
TON_1301	Acyl carrier protein synthase	38042/7.07	0.70	0.41	1.10	0.28
TON_1358	Hypothetical protein, conserved, DUF43 family	40183/4.83	N.D.	N.D.	1.03	0.26
TON_0027	Proteasome alpha subunit (multicatalytic endopeptidase complex alpha subunit)[b]	28735/4.87	N.D.	N.D.	1.01	0.26

Table 2 continued

ORF ID	Annotation	MW/pI	80°C 2 h		100°C 2 h	
			emPA[a]	Mol%	emPAI[a]	Mol%
TON_0435	Ornithine carbamoyltransferase	35080/5.95	0.41	0.24	1.00	0.25
TON_0184	DUTPase	17580/7.88	N.D.	N.D.	0.97	0.25
TON_0690	Molybdenum cofactor biosynthesis protein B	18629/7.85	N.D.	N.D.	0.89	0.23
TON_0397	Threonine 3-dehydrogenase	52314/9.13	0.26	0.15	0.86	0.22
TON_0951	Nucleoside diphosphate kinase	19649/5.81	0.22	0.13	0.84	0.21
TON_1065	Geranylgeranylglyceryl diphosphate synthase	27180/5.69	N.D.	N.D.	0.81	0.21
TON_0176	Cell division GTPase	41153/4.81	N.D.	N.D.	0.80	0.20
TON_1335	Hypothetical protein	13527/5.57	N.D.	N.D.	0.78	0.20
TON_0527	137aa long hypothetical protein	13719/6.34	N.D.	N.D.	0.77	0.20
TON_1426	Proteasome, beta subunit 2[b]	21753/5.35	0.20	0.12	0.73	0.19
TON_0239	Hypothetical protein, conserved, DUF75 family	29818/4.78	0.14	0.08	0.71	0.18
TON_1847	Predicted nucleic acid-binding protein, contains PIN domain	15265/4.76	N.D.	N.D.	0.67	0.17
TON_1292	Predicted stress-inducible protein, OsmC/Ohr family	15297/6.1	N.D.	N.D.	0.67	0.17
TON_0938	ArgE/DapE-related deacylase	39543/4.58	N.D.	N.D.	0.67	0.17
TON_1303	Hypothetical protein, conserved, DUF35 family	15609/6.9	0.29	0.17	0.65	0.17
TON_0987	Metallophosphoesterase, calcineurin superfamily	73622/5.08	0.25	0.15	0.65	0.17
TON_0925	DNA-binding protein, putative	15665/5.28	N.D.	N.D.	0.65	0.17
TON_0332	Probable transcription regulator (DUF118 helix-turn-helix family)[b]	33040/5.9	0.63	0.37	0.63	0.16
TON_0801	226aa long hypothetical aspartate racemase	25982/6.88	0.36	0.21	0.59	0.15

N.D Not detected

[a] The exponentially modified protein abundance index (emPAI) was calculated according to Ishihama et al. (2005)

[b] Proteins identified by 2-DE after in vitro heat treatment at 100°C

Identification of hyperthermostable proteins

To identify thermostable proteins, proteomic analysis of the proteins that remained in solution was performed using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and LCQ ion-trap MS. 19 protein spots on the 2D gel were successfully identified by MS/MS analysis and 13 proteins were unique proteins because intracellular protease (protein no. 1), thioredoxin reductase (protein no. 6), and hypothetical alkyl hydroperoxide reductase (protein no. 13) were multi-spots (Fig. 2, Table 1); 12 had been isolated previously (Kwon et al. 2009), and the remaining spot corresponded to triosephosphate isomerase (TIM). We assumed that the removal of thermolabile proteins by heat treatment allowed detection of this enzyme. The major proteins on the 2D gel were intracellular protease 1, thioredoxin reductase 6, transcription regulator 10, and hypothetical alkyl hydroperoxide reductase (Fig. 2; Table 1); hypothetical alkyl hydroperoxide reductase was the most enriched, and is highly homologous with the putative alkyl hydroperoxide reductase of *Pyrococcus horikoshii* OT-3 (Kawakami et al. 2004), which is known to be heat-stable at 90°C.

One-dimensional electrophoresis/liquid chromatography/tandem mass spectrometric (1-DE/LC/MS–MS) analysis and LCQ ion-trap MS were used for identification of more hyperthermostable proteins; 284 and 149 proteins were detected in protein samples prepared with heat treatment at 80 and 100°C, respectively (Additional file 1). Protein abundance was estimated based on exponentially modified protein abundance index (emPAI) values and mol%; proteins that had >0.15 mol% at 100°C are listed in Table 2. The 13 proteins isolated in 2-DE/MS–MS analysis, and several others including acylamino acid-releasing enzyme (Ton_1543), ABC-type dipeptide/oligopeptide transport system (Ton_1764), NADH oxidase (Ton_1271), and deblocking aminopeptidase (DAP; Ton_0369), were identified as abundant proteins by 1-DE/LC/MS–MS. These observations suggest that some proteins may be removed during sample preparation for 2-DE. The abundances of various ribosomal proteins (Ton_1103, Ton_0085, Ton_0087, etc.), and the metabolic enzymes, glutamate dehydrogenase (Ton_0157), sugar binding protein (Ton_0594), phosphoenolpyruvate synthetase (Ton_0311), and ribulose 1,5-bisphosphate carboxylase (Ton_1234), were significantly decreased with heat treatment.

Hyperthermostability of DAP, fructose-1,6-bisphosphatase, and intracellular protease

We selected three putative hyperthermophilic enzymes from the identified proteins for verification of thermostability: deblocking aminopeptidase (DAP), fructose-1,6-bisphosphatase (FBPase), and intracellular protease. Deblocking aminopeptidases have aminopeptidase activity, but are also known to act at the amino-termini of polypeptide chains and to release amino acids from proteins and peptides modified with various amino-terminal acyl-type blocking groups. Three DAP genes were identified in the *T. onnurineus* NA1 genome database; one of the DAPs (TNA_DAP) was reported previously, and its gene product showed thermostable enzyme activity in the 90–100°C range (Lee et al. 2007). In the present study, DAP 2 (TON_0369) was amplified by PCR and the expressed enzyme was purified from a soluble cell extract of *Escherichia coli*. Purified DAP 2 exhibited a band of the expected size (39 kDa) and was homogenous, as indicated by the single band on SDS-PAGE (Fig. 3). It showed optimum activity at 90°C, and its half-life ($t_{1/2}$) was 2.5 h. FBPase is an essential regulatory enzyme in the gluconeogenic pathway, and also converts D-fructose-1,6-bisphosphate to D-fructose-6-phosphate, an important precursor in several biosynthetic pathways (Nishimasu et al. 2004). FBPase (TON_1497) was expressed and purified using the method described above; it had the expected size of 42 kDa (Fig. 3), showed optimum activity at 100°C, and its $t_{1/2}$ was 8.3 h. Intracellular proteases are presumably used for peptide utilization in hyperthermophilic organisms (Halio et al. 1997). The intracellular protease (Ton_1285) of this organism showed optimum activity at the relatively low temperature of 80°C, and its $t_{1/2}$ was 17.5 h (Fig. 3).

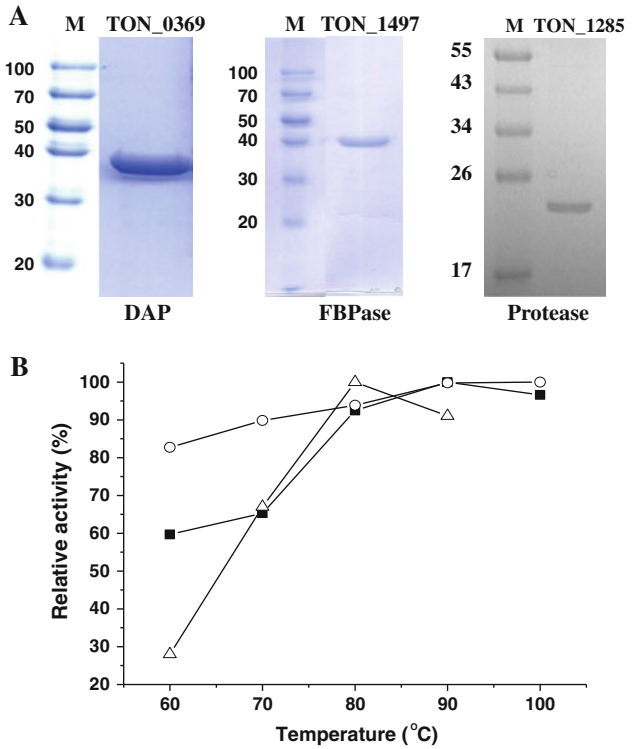

Fig. 3 **a** Overexpression and purification of recombinant deblocking aminopeptidase (DAP, Ton_0369), fructose-1,6-bisphosphatase (FBPase, Ton_1497), and intracellular protease (Ton_1285). **b** Relative activity of recombinant enzymes according to temperature. FBPase (*open circle*); DAP (*closed square*); Intracellular protease (*open triangle*)

In silico prediction of heat-stable proteins using TargetStar software

Thermostability prediction based on Tm was carried out using the TargetStar program. The result showed that Tm values of enriched proteins after heat treatment were relatively higher than the Tm values of homologous proteins from the mesophilic stains (Fig. 4). Proteins having higher Tm values include thioredoxin reductase (Ton_1603), alkyl hydroperoxide reductase (Ton_0829), triosephosphate isomerase (Ton_1640), intracellular protease I (Ton_1285), which were identified as abundant proteins from 2-DE analysis. Additionally, deblocking aminopeptidase (Ton_0369, Ton_1032) and elf-2B (Ton_0934), which were identified from LCQ MS analysis, were also found to have higher Tm values (Additional file 2). Especially, two overexpressed proteins (deblocking aminopeptidase, Ton_0369; intracellular protease, Ton_1285) were confirmed to have high Tm values. This result suggests that Tm can play as an index for heat stability of proteins and TargetStar can be applied as a heat stability prediction tool.

Conclusions

In this study, we identified a set of hyperthermostable proteins from *T. onnurineus* NA1 by in vitro heat treatment and proteome analysis using 2-DE/MS–MS and 1-DE/MS–MS. Although the optimal growth temperature of *T. onnurineus* NA1 is 85°C, not all of the proteins induced were soluble under in vitro heat treatment conditions; 36.3 and 80.8% of total proteins lost their solubility and precipitated at 80 and 100°C, respectively. It is reasonable that enzymes are more stable at in vivo condition because they can be well protected or controlled by chaperones or heat shock proteins in the cell. Other interesting parameter is salt condition. We used 20 mM Tris–HCl (pH 8.0) as standard buffer for enzyme assay and did not consider other salt effect for protein stabilization. However, several reports suggested that K+, NH4+, SO42−, and HPO42− are more efficient (Vieille and Zeikus 2001). Substrate effect and presence of intracellular proteases should be considered.

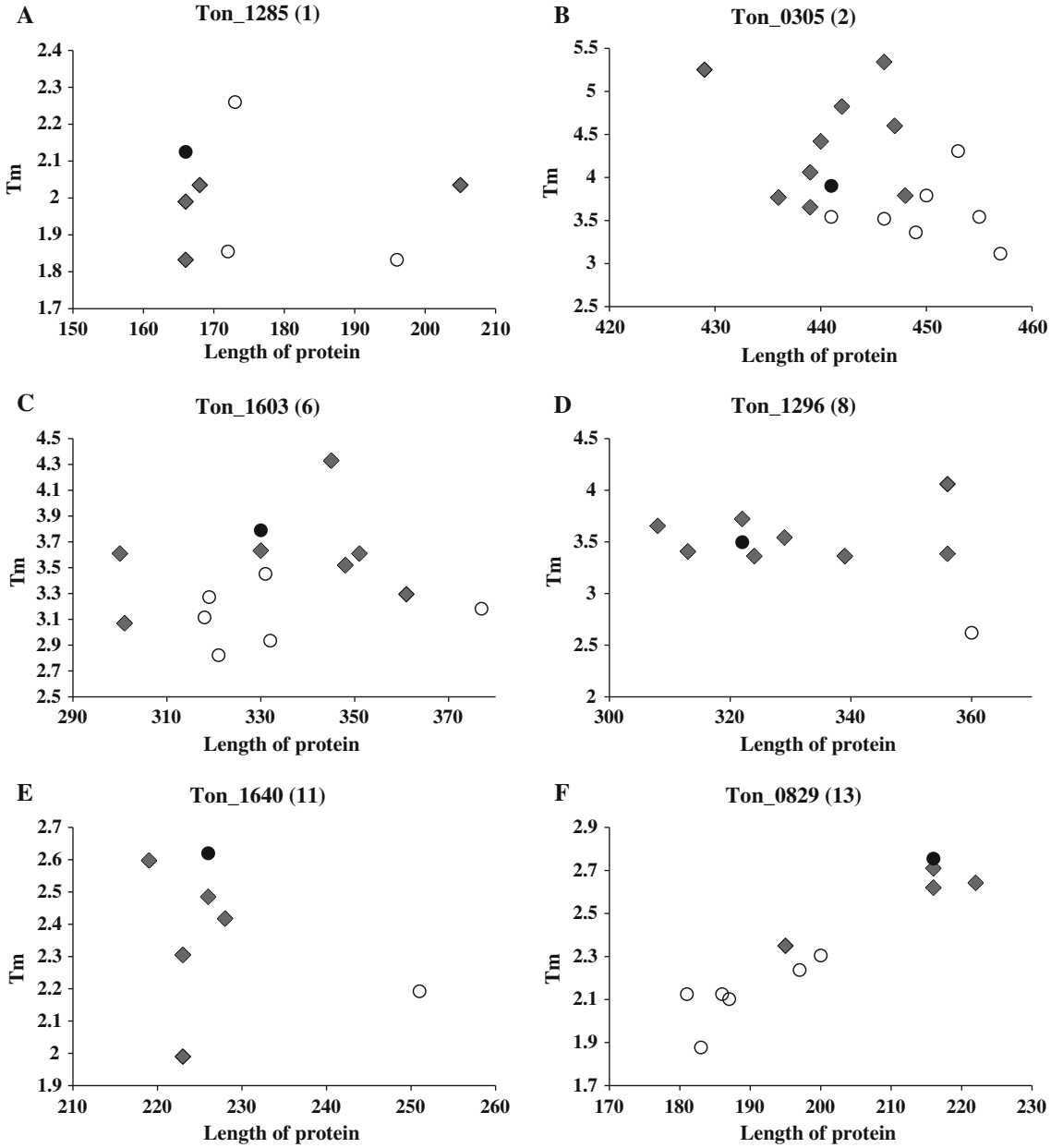

Fig. 4 Calculation of the folding transition temperature (Tm) of hyperthermostable proteins from *T. onnurineus* NA1 (*closed circle*) identified on 2-D gel. Other hyperthermophilic and mesophilic microorganisms are indicated as *closed diamond* and *open diamond*, respectively. Each number indicates the spot number on the 2D gel (Table 1). Ton_1285, intracellular protease I; TON_0305, NADH oxidase; Ton_1603, thioredoxin reductase; Ton_1296, translation initiation factor eIF-2B, delta subunit; Ton_1640, triosephosphate isomerase; Ton_0829, 216aa long hypothetical alkyl hydroperoxide reductase

The soluble proteins (19.2% of total proteins) at 100°C could be grouped as a hyperthermostable protein set and were analyzed by two different proteomic approaches (2-DE/MS–MS and 1-DE/MS–MS); 2-DE/MS–MS identified abundantly induced hyperthermostable proteins, and 1-DE/MS–MS was useful for the detection of all putative hyperthermostable proteins regardless of their abundance. We induced expression of three enzymes (FBPase, DAP, intracellular protease) from the identified proteins in an *E. coli* expression system (Fig. 3) and characterized them using an enzyme activity assay. All of these enzymes showed thermostability at high temperatures (80°C and 100°C), suggesting the usefulness of proteomic screening. Although most of the thermolabile proteins were removed during the heat treatment process, several were detected by MS/MS analysis due to its high sensitivity, indicating that careful verification of proteomic screening of hyperthermostable proteins by 1-DE/MS–MS is required.

Overexpression and enzyme activity assay can be used for verification but these methods are time-consuming. As an alternative approach, we used the in silico thermostable prediction program TargetStar to calculate the Tm values of the *T. onnurineus* NA1 proteins.

TargetStar was designed to calculate the relative thermostability based on its amino acid sequence and the results were displayed as Tm (Kim et al. 2007b). Our result suggests that Tm can be used as an index of thermostability. Putative hyperthermostable proteins enriched from heat treatment have higher Tm values than homologous proteins of mesophilic strains. Additionally, other parameters must be considered to provide more accurate predictions (Walden et al. 2001; Boutz et al. 2007). One of them is the complexity of proteins. Even though Tm value of triosephosphate isomerase (TIM) of *T. onnurineus* NA1 was relatively low (2.62), TIM of *T. onnurineus* NA1 (Ton_1640) was detected as a major soluble protein at 100°C (Table 1; Fig. 2). TIM of *Pyrococcus woesei* is known for its extreme thermostability due to the adoption of a compact tetrameric structure (Walden et al. 2001). The high degree of sequence homology of TIMs between *P. woesei* and *T. onnurineus* NA1 suggests that TIM of *T. onnurineus* NA1 (Ton_1640) may form a similar protein complex and, therefore, be thermostable.

In conclusion, using a combination of proteomic approaches (in vitro heat treatment, 1-DE/LC/MS–MS, and in silico prediction of thermostability), we identified a large number of potential, novel, and hypothetical hyperthermophilic proteins from *T. onnurineus* NA1.

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