Identification of Temperature Dependant Proteins by Proteomics

[1]MASOOD AHMED SIDDIQUI*, [2]SINSUKE FUJIWARA AND [3]TADAYUKI IMANAKA
[1]*Department of Chemistry, University of Balochistan, Quetta, Pakistan.*
[2]*Department of Bioscience, Nanobiotechnology Research Center, School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen Sanda, Hyogo 669-1337, Japan.*
[3]*Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-8501, Japan.*

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Summary: The cells of hyperthermophilic archaeon *Thermococcus kodakaraensis* grow between (70- 95 °C). Cells were cultivated at low growth limit (70 °C) optimum (85 °C) and high growth (95 °C) temperatures. Protein sample were extracted from the cells by using ProteoPrep Total Extraction Sample kit. 900 μg protein was used for first-dimension isoelectric focusing IEF and electrophoresis was performed using IPGphor. Protein spots images were acquired by an imaging densitometer and amino acid sequences were determined by Applied Biosystem Procise sequencer. Comparative analysis indicated that proteome of *T. kodakaraensis* changed with change in growth temperature and several proteins were specifically expressed at 70 °C optimum 85 °C and high growth 95 °C temperatures. Among them, three efficiently expressed spots were chosen for further study and were identified as peroxiredoxin, molecular Chaperonins (CpkA) and (CpkB).

Introduction

At present, the data of fully sequenced genome of many organisms is available. Depending on the type of organism, only a part of sequence can be directly correlated with a biological function. Furthermore, it has also been found, that there is very low correlation between abundance of mRNA formation as compared to small quantity of protein synthesis [1, 2], therefore, it has become necessary to analyze the total protein also known as "proteome" of a genome [3]. Proteomics is the large-scale study of proteins and is the next step in the study of biological systems, after genomics [4, 5]. An organism's genome is constant, but proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment. Another major difference is the complexity of proteins relative to nucleic acids, *e.g.*, according to rough estimation there are about 25,000 identified genes present in human genome which code for more than 500,000 proteins. The reason for this increased complexity has been explained due to mechanisms such as alternative splicing, protein modification (glycosylation, phosphorylation) and protein degradation [6].

In 1988 for the determination of proteome, a basic protocol of 2-D electrophoresis with immobilized pH gradient (IPG) was described [7]. Since that the procedure has become the standard procedure of two dimensional gel electrophoresis with immobilized pH gradient (IPG-Dalt) for protein research [8], permitting higher resolution [7, 9] and high loading capacity [10] for micro-preparative 2-D electrophoresis with subsequent spot identification [11].

Now proteomics is a rapidly growing field of research that is becoming increasingly important. For example, it is extensively applied to the study of proteins involved in carcinogenesis, as well as to discover biomarkers for clinical use [12]. Disease markers are widely used for screening, diagnosis, monitoring response to treatment and detection of recurrent diseases [13].

The Phylogenetic tree based on 16S rRNA or proteins sequences show that all organisms have a common ancestor. All hyperthermophiles that grow at temperatures near and above 100°C are represented as the deepest and shortest lineages, suggesting that hyperthermophiles are very primitive and the common ancestor might be a hyperthermophile. In the course of evolution, hyperthermophilic ancestor was considered to adapt to lower temperature environment. Hyperthermophiles therefore appears more closely related than all other organisms to the

*To whom all correspondence should be addressed.

ancestor of all extant life, having evolved when the earth was much hotter than it is at present. All living organisms respond to the stress by inducing a class of conserved proteins [14, 15]. There are many types of stressors such as temperature, pH, metals, alcohols, antibiotics and osmotic pressure [16, 17]. These stress proteins including temperature dependant specific proteins can be identified by the techniques used in proteomics. In order to obtain information of proteome change at different growth temperatures and to know the characteristics of temperature dependant proteins, we cultivated hyperthermophilic archaea at lower limit, optimum and high growth temperatures and performed proteome analysis using archaeon *T. kodakaraensis* [18] as a model strain.

Results and Discussion

Total *T. kodakaraensis* protein samples were prepared from three different batches of cells grown at 70 °C, 85 °C and 95 °C for 2-D gel electrophoresis in order to confirm reproducibility of proteins at specific temperature. The cells were lysed by sonication and proteins were solubilized in lysis buffer A [19]. For the reduction of disulfide bond and complete unfolding of protein instead of dithiothretol (DTT), tributylphosphine [20] and iodoacetamide were used because DTT held responsible for point streaking and smearing [21]. About 900 μg protein was used for 2-D electrophoresis. At first 13 cm IPG strips with pH gradient 4 – 7 were used for IEF. Coomassie blue was used for staining. Silver staining might have detected more spots, but was not used since it could have posed problems during naked eye protein identification or by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS analysis) [22]. Protein spots from the gel were transferred on PVDF membrane by blotting.

Comparative analysis indicated that several proteins were specifically expressed at 70 °C, 85 °C and 95 °C (Fig. 1). Among them, three efficiently expressed spots were chosen for further study. One of them which were expressed specifically at 70 °C but not at 85 °C and 95 °C was identified as peroxiredoxin by N-terminal sequencing [23] and submitting the obtained sequence to DNA Data Bank of Japan (DDBJ) [24]. Peroxiredoxin is antioxidant and is involved in the removal of toxic oxygen radicals. Low temperature specific peroxiredoxin might be induced to prevent toxic effect against increased dissolved oxygen at low temperature environment but not at optimum and high growth temperatures.

The second and third spots were found between the acidic ranges 4.5-5.5. Interestingly both spots were expressed at normal growth temperature 85 °C, but at low growth temperature 70 °C one spot was appeared while the other at high growth temperature 95 °C (Fig. 1). For the complete separation of second and third spots, 2-D electrophoresis was performed using 18 cm IPG strip with pH gradient 4.5 – 5.5 (Fig. 2). Protein spots from the gel were transferred on PVDF membrane by blotting and N-terminal sequencing was performed. By submitting the sequence data to DDBJ, the second spot which was expressed at 70 °C was identified as molecular Chaperonins (CpkA) and the third spot as CpkB (Fig. 2). Chaperonins are a subset of the heat shock proteins involved in multi functions like correct assembly of polypeptides, translocation of proteins across the membranes and hindering proteins denaturation. Chaperonins have been classified into two distinct groups, known as group I and II. The first group arose from bacterial, mitochondrial and chloroplast chaperonins. The other group of chaperonins, group II, is found in archaea and cytosol of eukaryotes. There is diversity of subunit composition in group II chaperonin. Eukaryal cytosolic chaperonin consists of 8 different kinds of subunits. Archaeal chaperonins contain either only one or two kinds of subunits. For example, *Sulfolobus solfataricus* [25], *Sulfolobus shibatae* [26], *Pyrodictium occultum* [27] and *Thermoplasma acidophilum* [28] contain two subunits. However, chaperonin from *Methanopyrus kandleri* [29], *Methanococcus jannashii* [30] contain homo-oligomeric structure composed of one type of subunit. Previously we reported two kinds of chaperonin subunits, CpkA and CpkB in *Thermococcus kodakaraensis* KOD1 and their functions were analyzed *in vitro* [31, 32]. It has been shown by immunoblot analysis that Chaperonins expressed at 70 °C were mainly composed of CpkA and in the case of chaperonins at 95 °C, most components were CpkB [33]. It was also proved that chaperonin from KOD1 formed hetero-oligomeric complexes with CpkA and CpkB and the ratio of CpkA to CpkB in the complex changed in response to the growth temperature [33]. The present results by proteomics are quite an agreement with our previous results performed for the studies of chaperonins [31-33]. Fig. 1 indicates

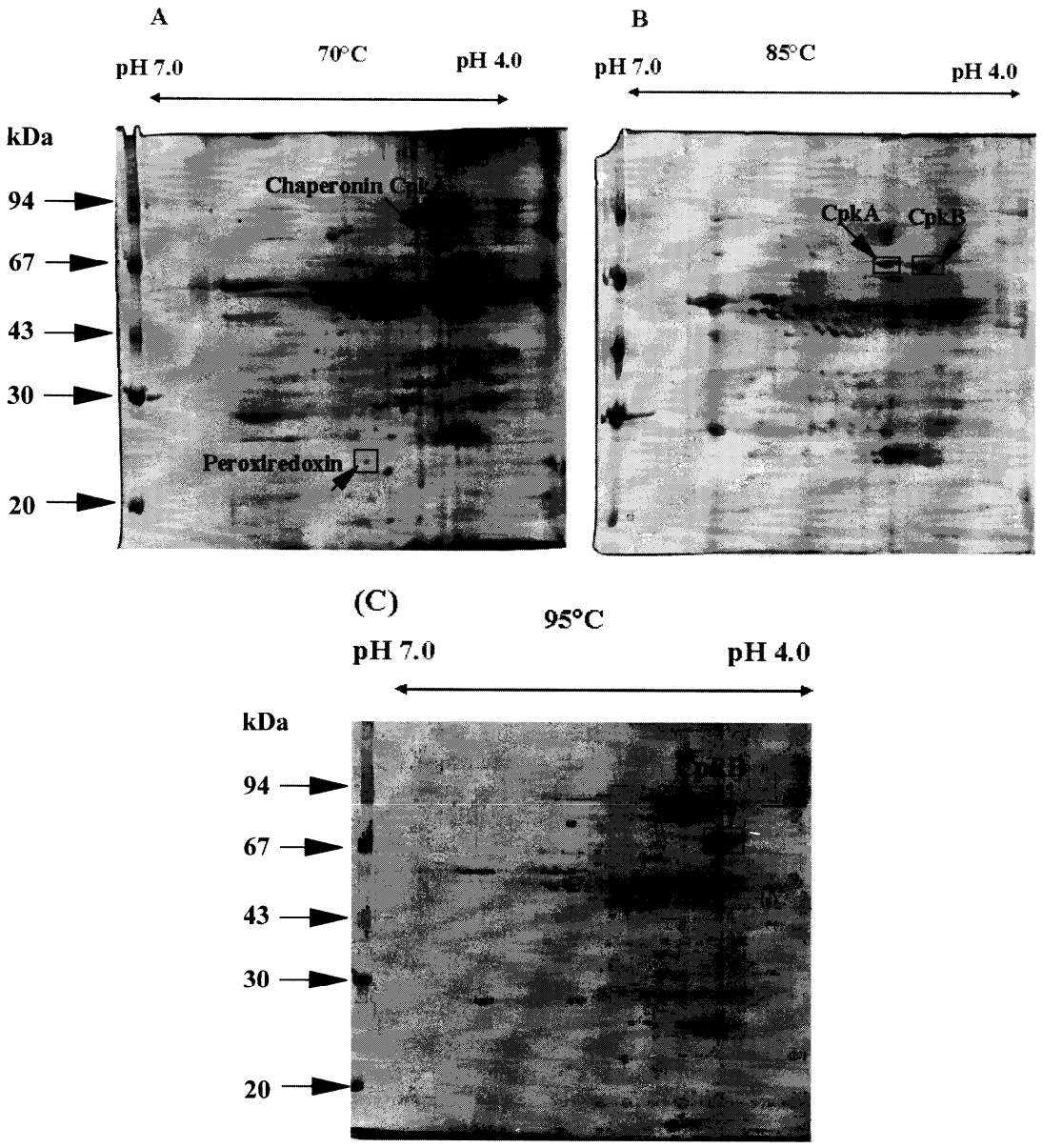

Fig. 1: 2-D gel electrophoresis of whole cell proteins from *T. kodakaraensis* grown at 70 °C (A), 85 °C (B) and 95 °C (C). Isoelectric focusing was performed on pH 4-7 IPG 13 cm strips using IPGphor. The 0.1 % SDS-12.5 polyacrylamide gel electrophoresis was performed on vertical gel. M: molecular weight markers (Phosphorylase b, 94,000; Albumin, 67,000; Ovalbumin, 43,000; Carbonic anhydrase 30,000; Trypsin inhibitor, 20,000.

that CpkA is mainly expressed at 70°C and CpkB at 95°C where as at optimum growth temperature both CpkA and CpkB are expressed. The expression of CpkB at 70 °C and CpkA, CpkB at 85 °C were confirmed on 18 cm IPG strip with the pH gradient 4.5 – 5.5 as shown in Fig. 2.

The result of 2-D electrophoresis suggests that proteome of *T. kodakaraensis* changes with different growth temperatures. These temperature dependent proteins can be identified by proteome analysis using the technique of 2-D electrophoresis. By studying the temperature dependant proteins the

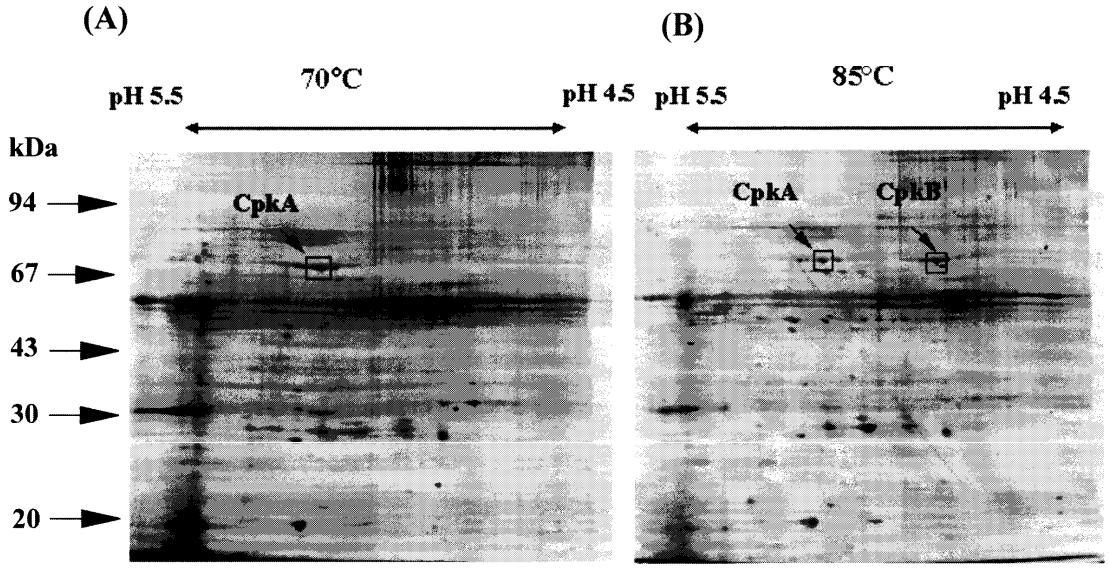

Fig. 2: 2-D gel electrophoresis of whole cell proteins from *T. kodakaraensis* grown at 70 °C (A) and 85 °C (B). Isoelectric focusing was performed on pH 4.5-5.5 IPG 18 cm strips using IPGphor. The 0.1 % SDS-12.5 polyacrylamide gel electrophoresis was performed on vertical gel.

process of evolution specially the low temperature adaptation of hyperthermophiles might be understand. For further studies of evolutionary aspects, characterization of several temperature dependant spots obtained by proteome analysis, microarray research is under process.

Experimental

Growth of Organism

T. kodakaraensis KOD1 was grown anaerobically in 800 mL of MA medium (4.8 g and 26.4 g of Marine Art SF agents A and B, respectively [Senju Seiyaku, Osaka, Japan], 5 g of yeast extract, 5 g of sodium pyruvate and 5 g of tryptone [Difco Laboratories] in 1 liter of deionized water) supplemented with 20 μL of polysulfide solution (20 % elemental sulfur in 3 M Na_2S). The cells were grown at high growth temperature 95 °C, optimum growth temperature 85 °C and at lowest growth temperature 70 °C.

Preparation of Total T. kodakaraensis Protein Samples

When the optical density (OD_{660}) reached to 1.0 the cells were harvested (5,000 X *g* for 30 min). The cells were dissolved in 1.0 mL culture medium and transferred to Eppendorf Centrifuge tube and centrifuge at 25 °C and 13,000 rpm for twenty minutes. The supernatant was discarded and cells were again centrifuged and dissolved in 0.1 mL buffer A [Sigma, St. Louis, Mo.] 7 M urea, 2 M thiourea, 2 % amidosulfobactine-14, 40 mM Tris-base). The cells were sonicated and centrifuged at 15,000 rpm for 30 minutes. The concentration of proteins was determined by PlusOne 2-D Quant kit (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the procedure in provided protocol. The protein concentration was maintained 6 μg/μL using PlusOne 2-D Clean-Up kit (Amersham Biosciences). The reduction of proteins was performed by using tributylphosphine (TBP) (Sigma) [20] in 1:40 TBP protein ratio and the reaction mixture was incubated at room temperature for one hour. In the next step the alkylation of proteins was performed with 0.5 M iodoacetamide (Sigma) stock solution (30 μL iodoacetamide/mL protein solution) [21]. The sample was incubated at room temperature for one and half hour and then centrifuged at 15,000 rpm for 30 minutes to remove insoluble materials.

First-dimension Isoelectric Focusing (IEF)

For first dimension isoelectric focusing (IEF) was performed by using ready made Immobiline Dry

Strip Gel (IPG) (Amersham Pharmacia Biotech) with the pH gradient 4 – 7 and 4.5 – 5.5 in IPG strip holder. Prior to IEF, the IPG strips were rehydrated with 250 μL of protein sample containing buffer A, TBP and iodoacetamide with trace amount of bromophenol blue (tracking die). The IPG strips were overlaid with 1.5 – 2 mL IPG cover fluid to minimize evaporation. Rehydration was performed for 16 hours at room temperature. The IEF was performed in IPGphor isoelectric focusing system using the following three-step program for 13 cm IPG strips (a) 0 – 500 V in one h (b) 1,000 – 3,000 V for 2 h (c) 6,000 V constant for 8 h with 50 μA/strip current and for 18 cm IPG strips (a) 0 – 500 V in one h (b) 1,000 – 3,000 V for 2 h (c) 6,000 V constant for 12 h with 50 μA/strip current.

Second-dimension SDS-PAGE

To perform the Second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), at first the IPG strips from first dimension isoelectric focusing were equilibrated with equilibrium buffer (urea 6 M, glycerol 87 %, SDS 2 %, bromophenol blue 0.005 M, TBP 2 vial/ 40 mL and 1.5 M Tris-HCl pH 8.8) for 30 minutes [34]. Later, each strip was loaded on top of a vertical SDS-polyacrylamide gel and sealed in place with 0.5 % low melting agarose dissolved in electrode buffer. The standard proteins markers (Bio-Rad) (Phosphorylase b, 94,000; albumin bovine serum, 67,000; ovalbumin, 43,000; carbonic anhydrase 30,000; and trypsin inhibitor, 20,000; were loaded in a side of the strip. Electrophoresis was performed according to previously reported method [35]. A constant current of 140 mA was applied until the indicator die (bromophenol blue) approached the bottom edge of the gel. Proteins were stained with Coomassie brilliant blue (CBB R-250). Images were acquired by an imaging densitometer (GS-800 Bio-Rad).

N-terminal Amino Acid Sequence Determination

From the gel proteins were electro transferred to polyvinylidene difluoride (PVDF) membrane (0.2 mm) (Bio-Rad, Hercules, CA) by Horiz blot (Atto. Tokyo, Japan). For blotting three buffers (A) containing per liter Tris 36.3 g, Methanol 200 mL and 5 mL 10 % SDS (B) Tris 03 g, Methanol 200 mL and 5 mL 10 % SDS (C) Tris 03 g, 6-aminohexanoic acid 05.2 g, Methanol 200 mL and 5 mL 10 % SDS were used. The constant voltage 100 mA for one hour was applied. The PVDF membrane was stained with CBB R-250 and destained with 30 % methanol till clear protein spots were appeared. The required spots of interest were excised and dissolved in 1 % trichloroacetic acid for *N*-terminal amino acid sequencing. The *N*-terminal amino acid sequences were determined by cyclic Edman degradation method [23]. Sequence determination was performed by automatic protein sequencer (Applied Biosystem Inc., Foster, Calif, USA).

Conclusions

Living organisms respond to the stress by inducing a class of conserved proteins. The temperature dependant specific proteins including chaperonins can easily be identified by proteomics. In the present study we cultivated cells of *T. kodakaraensis* at low growth limit 70 °C optimum 85 °C and high growth 95 °C temperatures. The conditions for 2-D electrophoresis for *T. kodakaraensis* proteome were optimized. After 2-D electrophoresis protein spots were transferred by traditional technique to PVDF membrane. The three specifically expressed spots at different growth temperatures were chosen for N-terminal sequencing and data base search was performed. The spots were identified as peroxiredoxin, CpkA and CpkB. The peroxiredoxin expressed only at low growth temperature, while high amount of CpkA was found at 70 °C and CpkB at 95 °C. With this method we can express, identify and characterized those proteins, which are not expressed at normal growth temperature environment. We can obtain primitive information of early stage of evolution by comparing the expressed proteins at different temperatures.

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