A.H. Taşkın Kafa et al. / Hacettepe J. Biol. & Chem., 2021, 49 (3), 219-231

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Cloning, Over-Expression, and Purification of β–Carbonic Anhydrase from an Extremophilic Bacterium: *Deinococcus radiodurans*

Ekstremofilik bir bakteri olan *Deinococcus radiodurans*'dan β–Karbonik Anhidraz'ın klonlanması, Aşırı İfadesi ve Saflaştırılması

Ayşe Hümeyra Taşkın Kafa[1*], Arzu Coleri Cihan[2], Mehmet Kuzucu[3], Murat Cankaya[3]

[1]Cumhuriyet University, Faculty of Medicine, Department of Medical Microbiology, Sivas, Turkey.

[2]Ankara University, Faculty of Science, Biology Department, Ankara, Turkey.

[3]Erzincan University Faculty of Arts and Sciences Department of Biology, Erzincan, Turkey.

ABSTRACT

In this study, the cloning, purification and initial characterization of carbonic anhydrase (DrCA) enzyme which we consider to be important in the resistance physiology from extremely radioresistant bacteria Deinococcus radiodurans is performed. In addition, the effect of increased gamma irradiation doses on pH-related DrCA enzyme activity was determined. DrCA activity after radiation treatment showed that the activity continuously increased by 6 fold, up to the first 800 Gy, which a decrease in activity was observed thereafter. The maximum CO_2 hydration activity for DrCA enzyme was observed at pH 7.0 and 40°C. DrCA enzyme, homo-dimer complex, is slightly thermostable. The activity of DrCA was significantly enhanced by several metal ions, especially Zn^{2+}, which resulted in 5-fold increases of CO_2 hydration activity. Also sulfonamide showed inhibitory effect on the pure enzyme. The apparent Km and Vmax for CO_2 as substrate were 8.4 mM and 637 WAU/mg for DrCA respectively. The CO_2 hydration assay demonstrated that the specific activity of purified recombinant enzymes (DrCA) was significantly high.

Key Words

Deinococcus radiodurans, carbonic anhydrase, ionizing radiation.

ÖZ

Bu çalışmada, radyasyona ekstrem dirençli bir bakteri olan Deinococcus radiodurans'dan direnç fizyolojisinde önemli olduğunu düşündüğümüz karbonik anhidrazın (DrCA) enziminin klonlanması, saflaştırılması ve başlangıç karakterizasyonu gerçekleştirilmiştir. Ayrıca, artan dozlarda gama radyasyonun pH ile ilişkili DrCA enzim aktivitesi üzerindeki etkisi belirlenmiştir. Radyasyon uygulamasından sonra DrCA aktivitesi, 800 Gy'a kadar sürekli artarak 6 kat, artmıştır, bu noktadan sonra aktivitede azalma olduğu gözlenmiştir. DrCA enzimi için maksimum CO_2 hidrasyon aktivitesi, pH 7.0 ve 40°C'de gözlenmiştir. Homo-dimer kompleks yapısında olan DrCA enzimi hafif termostabildir. DrCA'nın CO_2 hidrasyon aktivitesini, çeşitli metal iyonları, özellikle Zn^{2+} önemli ölçüde, 5 kat artırmıştır. Ayrıca sülfonamid saf enzim üzerinde inhibe edici etki göstermiştir. DrCA'nın substrat olarak CO_2 için belirlenen Km ve Vmax değerleri sırasıyla 8.4 mM ve 637 WAU/mg idi. CO_2 hidrasyon deneyi saflaştırılmış rekombinant enzimin (DrCA) spesifik aktivitesinin önemli ölçüde yüksek olduğunu göstermiştir.

Anahtar Kelimeler

Deinococcus radiodurans, karbonik anhidraz, iyonlaştırıcı radyasyon.

Article History: Received: Jul 20, 2020; Revised: Dec 21, 2020; Accepted: Dec 20, 2020; Available Online: May 5, 2021.

DOI: https://doi.org/10.15671/hjbc.775093

Correspondence to: A.H.T. Kafa, Cumhuriyet University, Faculty of Medicine, Department of Medical Microbiology, Sivas, Turkey.

E-Mail: ahtaskin@cumhuriyet.edu.tr

INTRODUCTION

Although radiation has detrimental effects on a large number of living groups, there are different types of microorganisms with special abilities to survive at high levels of radiation. One of these bacteria, *Deinococcus radiodurans* is an extremophilic bacterium with exceptional capacity to withstand severe environmental stress including dryness, oxidants, ultraviolet and ionizing radiation. This non-pathogenic and non-photosynthetic bacterium, with its ability to resist >10,000 Gy of ionizing radiation, has entered the Guinness records as the most resistant aerobic organism to radiation [1-5].

Carbonic anhydrases (carbonate hydrolysis, carbonate dehydratase EC 4.2.1.1) are set of common metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate: $CO_2+H_2O\leftrightarrow H^++HCO_3^-$. CO_2 hydration of carbonic anhydrase is known to be one of the fastest biocatalysis reaction in nature and has turnover numbers in million per second [6,7]. CAs have been existed in all domains of living organisms: Archaea, Eukarya and Bacteria. CAs are classified into six different groups [α, β, γ, δ, ζ, and η]. Of these six groups, α, β, and γ CAs are most common and have been extensively characterized. While β-carbonic anhydrases are widespread in plants and bacteria, γ-carbonic anhydrases are usually found in archaea [8-11]. All β-CAs have an α/β fold with the core being a five-stranded β-sheet [12]. β-CAs are the most widespread enzymes all over the phylogenetic tree in microorganisms and they are not found in mammals (including humans). Furthermore, β-CAs, which also plays an important role in urea and bicarbonate metabolism, is also suggested to be important in acid resistance [13,14]

Ionized rays decompose the water, which is the most abundant molecule in the cell. This event takes place in a very short time of 10-12 seconds, resulting in very active H^+ and OH^- radicals. Afterward, pH balance and metabolic reaction continuity disappear inside the cell. A concentration of H^+ ions other than physiological pH directly affects cell metabolism, causing changes in the structure and function of cellular proteins, enzymes. Cells try to restore pH homeostasis using enzymatic or non-enzymatic antioxidant and pH stabilizer systems [15-17]. Carbonic anhydrase [CA] plays an important role in acid-base homeostasis. It catalyzes carbonic buffering, enhances intracellular H^+ motility, and facilitates the activity of pHi regulatory transporters [18,19]. The carbonic anhydrase enzyme keeps the ambient pH at alkaline pH and protects the cells against the harmful effect of the radiation against pH irregularity occurring in the cell with the effect of radiation. By utilizing this feature of CA, the sensitivity of cancer cells to radiation and apoptosis can be increased during radiotherapy with the use of CA inhibitors [20]. Tumor cells expressing high levels of CA have been shown to respond less to radiation, but CA inhibitors increase radiosensitivity [21].

Although CAs are very well studied, most of the work has been performed on pathogenic and thermophilic microorganism CAs. To date, *D. radiodurans* carbonic anhydrase [DrCA], which is thought to be the relationship between radiation resistance and pH homeostasis doesn't have been characterized. Here, we report on the cloning, purification, and initial characterization of the β-CA from the extremely ionizing radiation-resistant bacterium, *D. radiodurans*.

MATERIALS and METHODS

Bacterial Strains, Culture Conditions, and Chemicals

D. radiodurans R1 [DSM 20539 GenBank Accession number NC_001263] and *Escherichia coli* DH5α was used for cloning were obtained from Leibniz-Institute DSMZ GmbH [DSM 6897]. The expression host ***E. coli*** BL21 [DE3] was purchased from Sigma [CM0014]. All chemicals from Sigma-Aldrich [St. Louis, MO, ABD]. *D. radiodurans* was grown at 32°C for 36 h in Tripton Glucose Yeast Agar [TGYA] [1% tripton, 0.5% glucose and 0.5% yeast extract, 1.5% agar] and at 32°C with shaking at 175 rpm for 10 h in Tripton Glucose Yeast Broth [TGYB] [1% tripton, 0.5% glucose] at pH 6.8. PCR was performed using a Bio-Rad C1000 Touch™ thermal cycler [Bio-Rad] and the Pfu polymerase used in PCR reactions was purchased from Termo. Flexi Enzyme Blend, SgfI & PmeI restriction enzymes were purchased from Promega. ***E. coli*** DH5α was grown on Luria Bertani [LB] medium at 37°C with shaking at 200 rpm. Agar plates containing 50 µg/ml, 1 mM isopropyl-b-DD-thiogalactopyranoside [IPTG] and 20 mg/ml 5-bromo-4-chloro-3-indolyl-b-DD-galactopyrano-side [Xgal] were used for the selection of clones including recombinant vector the pFN2K [GST] [Promega].

E. coli BL21 containing recombinant pFN2K [GST] plasmid [Promega] [used for expression vector with N terminal GST fusion tag] was grown in Magic Media [Invitro-

gen] used for protein induction. The protein purification was carried out using MagneGST™ Protein Purification System [Promega].

Radiation application to *D. radiodurans* followed by CO_2 hyration activity of DrCA

Radiation experiments were carried out in Turkey Atomic Energy Agency Saraykoy Nuclear Research and Training Centers. *D. radiodurans* R1 culture was incubated at 30°C, 175 rpm until OD600 was approximately 0.6 in the TGYB medium. The growing culture was kept in ice for 10 minutes to ensure that the cell reproductive phase remained stable. Gamma radiation doses up to 1000 Gy were applied to the tubes placed in the device three times each time, starting from 100 Gy respectively and increasing by 100 Gy. 1 ml lysis buffer [8 g / L NaCL, 0.2 g / L KCL, 1.44 g / L Na_2HPO_4, 0.24 g / L KH_2PO_4, 1 mM PMSF, 11.494 ml 87% glycerol, 0.0943 ml Triton-X100 and 0.2 mg / ml lysozyme] on pellet centrifuged at 20000 xg for 10 minutes after application of doses. Samples kept on ice for 20 minutes were sonicated with ultrasonic [Sonics Vibra Cell VCX750]. Sonication was performed in 35-45% amplitude as 20 seconds “on” 15 seconds “off” for a total of 5 minutes. After sonication, drCA activity was measured by centrifuging again and supernatant was taken and an activity curve was created.

PCR Amplification and Cloning of D. radiodurans β–CA

D. radiodurans R1’s genetic sequence [GenBank accessory number: AE000513.1], the gene coded DR_2238 belonging to DrCA, which is included in the β family, has been designed according to the base sequence in the database of NCBI [National Center for Biotechnology Information]. Gene-specific primers were designed previously and synthesized by *Flexi Vector Primer Design Tool* online program. Primers designed with this tool were investigated in the *D. radiodurans* R1 genome with BLAST [Basic Local Alignment Search Tool].The gene encoding for β–CA was amplified using primers FLEXY Forward [Dr_2238_F] 5’GCGTGCGATCGCCATGGGGCG3’ and FLEXY Reverse [Dr_2238_R] 5’CGAGGTTTAAACTTAGAGTGC3’ [Underlines indicate restriction enzyme sites]. The PCR reaction mix and cycle sequenceis detailed in table The resulting 795 bp product for β–CA. PCR

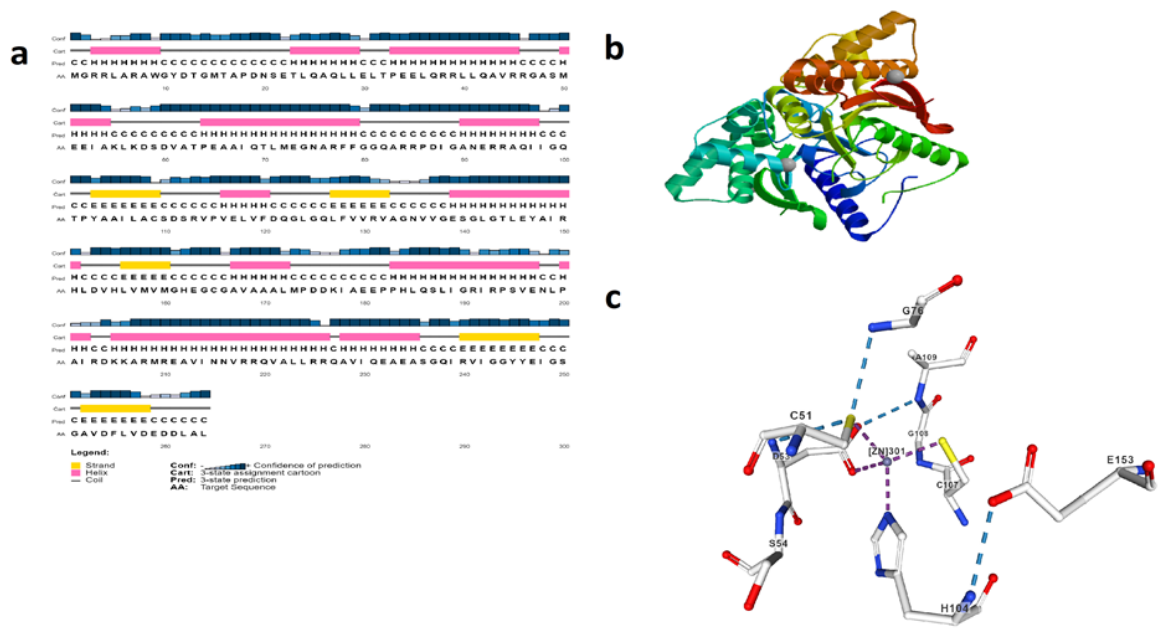

Figure 1. Predicted secondary and tertiary structure of D. radiodurans isolate carbonic anhydrase. [A] Predicted secondary structure of DrCA. Using PSIPRED, the secondary elements in the protein were predicted to consist of nine α-helices and five β-sheets [B] Ribbon structure and [C] stick of homology model predicted using Swiss-Model. Potential hydrogen bonds are represented as blue, halogen bonds are as turquoise, hydrophobic contacts are as grey, pi interactions are as orange and yellow. DrCA active site showing the zinc ion interactions are as purple.

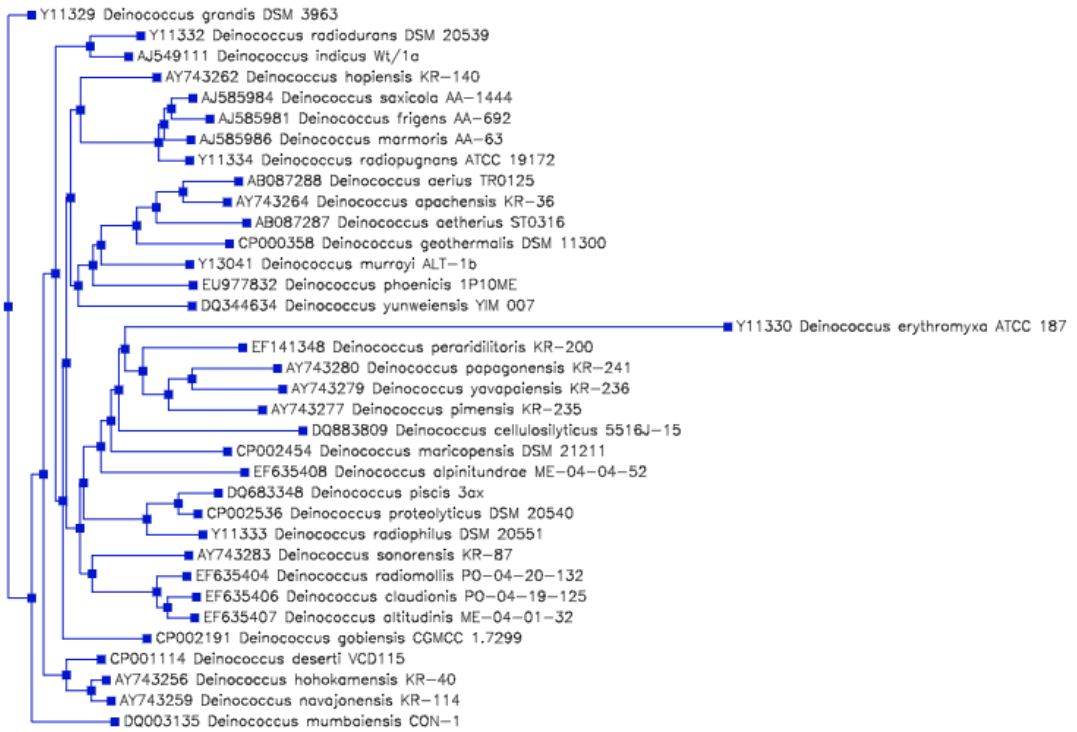

Figure 2. Phylogenetic tree of the genus Deinococcus [http://www.straininfo.net/]

products were digested using *SgfI* and *PmeI* restriction enzymes 1h at 37ºC. After restriction digest, the PCR products were ligated with pFN2K Flexi vector. Ligation was performed using T4 DNA ligase [New England Biolabs] 1 h at 25ºC. The ligations were then combined with calciumchloride- competent *E. coli* DH5α and ***E. coli*** BL21[DE3] cells, and transformations were done using the electroporation. Ligated plasmids were purified from the transformants using a Gen Elute HP Plasmid purification Kit and the presence of CA genes in the plasmids was confirmed by gene sequencing using cloning primers.

Over-Expression of DrCA and SDS-PAGE Analysis

Over-expression of the CAs was carried out by transferring the recombinant plasmid containing β–CA to the ***E. coli*** BL21[DE3] expression strainand the transformants were then inoculated in MagicMedia *E. coli* expression medium grown 18 h at 30 °C with shaking [200 rpm]. Simple protocol eliminates time-consuming OD monitoring and induction steps. The overnight cultures were used to inoculate 15 ml flasks containing 1 ml of Magic media, and the cultures were incubated 18 h at 30°C with shaking [200 rpm] following the manufacturer's instructions. After incubation, the cells were harvested by centrifugation [15.000 g, 5 min at 4°C], and the cell pellets were stored at -20°C until use. Frozen pellets containing CA proteins were thawed in an ice-bucket filled with ice and then suspended with MagneGST lysis buffer [This buffer supplemented with 10 mg / ml lysozyme solution prepared in 25 mM Tris HCl]. 100 µl lysis buffer for OD_{600}= 1 and 300 µl lysis buffer for OD_{600}= 3 were added. After 2 µl DNAse free RNAase was added. The resuspended pellets were lysed with shaking for 30 minutes at room temperature. Then pellets were distrupted by ultrasonication for 15 s with a 5 s break [pellets were kept on ice] at 35-45% amplitude [Sonics Vibra Cell VCX75020]. An aliquot of lysed cells was divided as total protein, and the remaining lysate was centrifuged for 60 minutes at 15.000 rpm at 0°C. the supernatant was reserved for SDS-PAGE. A small portion of the supernatant containing soluble proteins was transferred to a new Eppendorf tube, and the remaining supernatant was used for protein purification. All protein fractions were mixed with Laemmli loading buffer and separated in 12% SDS PAGE. Then gel was stained with commasie brilliant blue dye to make the proteins visible.

Purification of Over-expressed β–CA from *D. radiodurans*

Small-scale purification of β–CA was carried out using MagneGST™ Protein Purification System following manufacturer's instructions. MagneGST™ Glutathione Particles were added directly to the cleared or crude lysate. GST-fusion proteins bound to the particles during incubation at 4°C and then were three times washed to remove unbound and nonspecifically bound proteins.

GST-fusion protein is eluted from the particles with 10–50 mM reduced glutathione at pH 8. The presence of proteins in the elutions was confirmed by SDS-PAGE. After purification of GST-fusion protein, the undesirable GST tag was cleaved by Pro-TEV Plus protease. Pro-TEV protease specifically cleaves the sequence Glu-Asn-Leu-Tyr-Phe-Gln-↓-[Gly/Ser] [22].

ProTEV Plus has an HQ tag at the N-terminus. After cleaving the fusion protein, ProTEV Plus was removed from the reaction mix by incubating with MagneHis™ Ni-Particles. Protein concentration was determined by the Lowry method.

Assay of DrCA activity

Enzymatic activity of DrCA was measured using CO_2 hydration assay and presented as Wilbur–Anderson units [WAU]. As a result of CO_2 hydration activity bicarbonate ions and protons are formed, and the resulting pH change is monitored using bromothymol blue as an indicator. The indicator is yellow at pH less than 6.2 and blue when pH is higher than 8.3.

Briefly, reaction mixture was prepared by adding 10 µl CA enzyme, 1 mL of ice-cold 20 mM Tris-SO_4 pH 8.3 containing 0.2 g L^{-1} bromothymol blue was kept on ice. The reaction was initiated by adding 1 mL of ice-cold CO_2-saturated water. The bovine CA [BCA] was used as positive control. The DrCA activity was determined and expressed in Wilbur-Anderson Units per mg of the enzyme. WAU is identified with $[t_0 - tc]/tc$. The time interval [t_0 and tc] represented the time required for the pH value to drop from blue [pH 8.3] to yellow [pH 6.2] at 0°C in the absence and presence of CA, respectively. CO_2-saturated solution was prepared by treating CO_2 into pure water on ice bath for at least 30 minutes. All activity measurements were done in triplicates.

Increasing concentrations of CO_2 were used to define the kinetic properties of DrCA. Serial dilutions of ice-cold CO2 non-saturated solution from 10 to 60 mM were prepared using ice-cold CO_2 saturated solution [70 mM]. DrCA activities at different CO_2 concentrations were determined. The kinetic parameters of Km and Vmax were achieved with the use of Michaelis-Menten equation.

RESULTS and DISCUSSION

Many studies have been conducted in detail on carbonic anhydrase of pathogenic or non-pathogenic microorganisms until recently [23]. However, a study on carbonic anhydrase of a microorganism with high radiation resistance is not available in the literature. In a recent study investigating the effect of radiotherapy on cancer cells, it was seen that inhibition of the enzyme carbonic anhydrase IX (CAIX) in tumor cells caused a decrease in intracellular pH values and increased cell death. These results suggest that the active CAIX enzyme protects cells against radiation by maintaining the alkaline pH [24,25]. Because of investigation of enzymes and proteins that can resist pH irregularity occurring in the cell with the effect of radiation, especially in this type of extremophilic organisms, is important in clarifying the resistance physiology. The studies focused on CA, which is thought to have an important place in prokaryotic physiology, are important for determining the new functions of this enzyme and the role of CAs in extremophilic bacteria is thus beginning to be better understood.

Searching putative carbonic anhydrase of *D. radiodurans* R1

Physicochemical parameters of DrCA such as number of amino acid, molecular weight, theoretical isoelectric point, amino acid composition, instability index, aliphatic index, Grand Average of Hydropathicity [GRAVY] were described by Expasy ProtParam tool [https://web.expasy.org/protparam/] [26].

Locus NP_295960.1 from the DrCA genome consists of 795 nucleotides, encoding a 264-amino-acid protein with a theoretical molecular mass of 28.7 kDa and isoelectric point of 5.39. So it seemed strongly acidic protein.

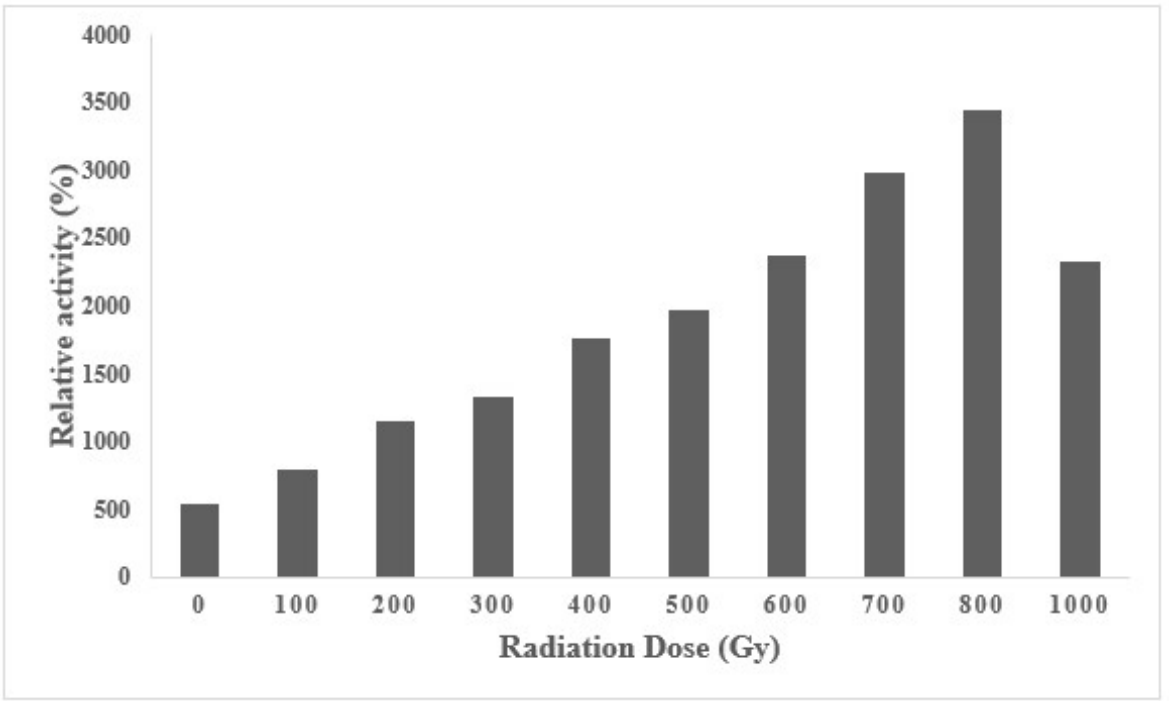

Figure 3. DrCA CO_2 hydration activity of *D. radiodurans* exposed to certain doses of gamma radiation [Gy].

The ***D. radiodurans*** sequence composed of 36 negatively charged residues [Asp + Glu] and 30 positively charged residues [Arg + Lys] and the comparatively high percentage [12.5%] of amino acid present is alanine. The instability index is computed to be 52.21. This classifies the protein as unstable. *D. radiodurans* had a CA protein with aliphatic index of 100.19 which positively indicates the thermostability of CA protein. Aliphatic index plays role in protein thermal stability. Proteins with a high aliphatic index which suggested that are thermostable. [27]. The GRAVY of CA protein is negative for *D. radiodurans* [-0.134] which indicates hydrophilicity of DrCA protein in nature. The low GRAVY range indicates the possibility better interaction of protein and water [28, 29].

The physiochemical properties of the DrCA were predicted using amino acid sequence comparable to previous studies [30-32].

While characterizing the CA of *Bacillus safensis*, as an Leu rich protein in which positively charged amino acid residues were dominant. Moreover, the enzyme was classified as unstable according to the instability index and aliphatic index was high which indicates the thermostability of CA protein as in this study [33].

Comparative amino acid sequence analyses showed that the predicted DrCA amino acid sequence identity and cluster with distinct sets of homologues. The CDD prediction placed DrCA in clade C [cd03378] of β-class CAs. A homology search performed using Basic Local Alignment Search Tool [BLAST, http://blast.stva.ncbi.nlm.nih.gov/Blast.cgi] revealed that the amino acid sequence of *D. radiodurans* CA shares 39.47 % identity with *Mycobacterium tuberculosis* variant bovis AF2122/97 CA protein [GenBank: YP_009360978.1], 38.31% identity with *Synechocystis sp.* [GenBank: WP_010873497.1], 37.19% identity with *Streptomyces coelicolor* A3[2] [GenBank: NP_627913.1], 35.29% identity with *Microcystis aeruginosa* [GenBank: WP_012264892.1], 33.95% identity with *Schizosaccharomyces pombe* [GenBank: NP_596512.2].

The CA secondary structure predicted using PSIPRED (Figure 1A). The CA secondary protein structure prediction result showed that the protein has 44% α helical [9 helices; 96 residues] 14% β sheet [5 strands; 32 residues]. The β-CAs common function as dimers or larger multi-oligomeric states. They have a compact structures with a β-sheet core composed of 5 anti-parallel strands with 4 or more αhelices and a shallow [34].

Homology model of the DrCA protein was predicted using SWISS-MODEL [http://swissmodel.expasy.org] server (Figure 1B**).** Based on the template search about 50 templates were recommended. About templates with higher identity were selected for model production. The property of the predicted model was analyzed using PROSA web server [https://prosa.services.came.sbg.ac.at/prosa.php]. The models predicted using the template 1ym3 with high fidelity regions of amino acid residues in Ramachandran Plot [http://eds.bmc.uu.se/ramachan.html] and Z-score was chosen. The molecular surface of the forecasting model was described in figure 1C. DrCA protein has a compact structure with a β-sheet core with anti-parallel strands, α-helices. The enzyme tertiary structure has been found to contain zinc ions at these active sites and the enzyme exists as homo-dimer structure.

Systematic Position and Phylogeny

As of 2019, there were 79 species of *Deinococcus* described according to LPSN database [http://www.bacterio.net/]. Phylogenetic tree constructed as a result of phylogenetic analysis of *Deinococcus* 16S rRNA gene sequences is shown in Figure 2.

CO_2 hyration activity of DrCA in *D. radiodurans* exposed to radiation

Exposure to ionizing radiation induces a dose-dependent CO_2 hydration activity increase in *D. radiodurans* cells. DrCA activity is constantly increasing up to 800 Gy. After 800 Gy, It is observed that the activity decreased. As shown in figure 3, gamma radiation doses could activate and enhance CO_2 hydration activity of DrCA, especially 800 Gy, which significantly increased CO_2 hydration activity to 3440 % when compared to that of control. CO_2 hydration activity was observed as 540 % in the non-radiation control group.

These results indicate that the DrCA enzyme can protect the cell against the metabolism regularity due to radiation and that *D. radiodurans* bacteria has an important place in radiation resistance.

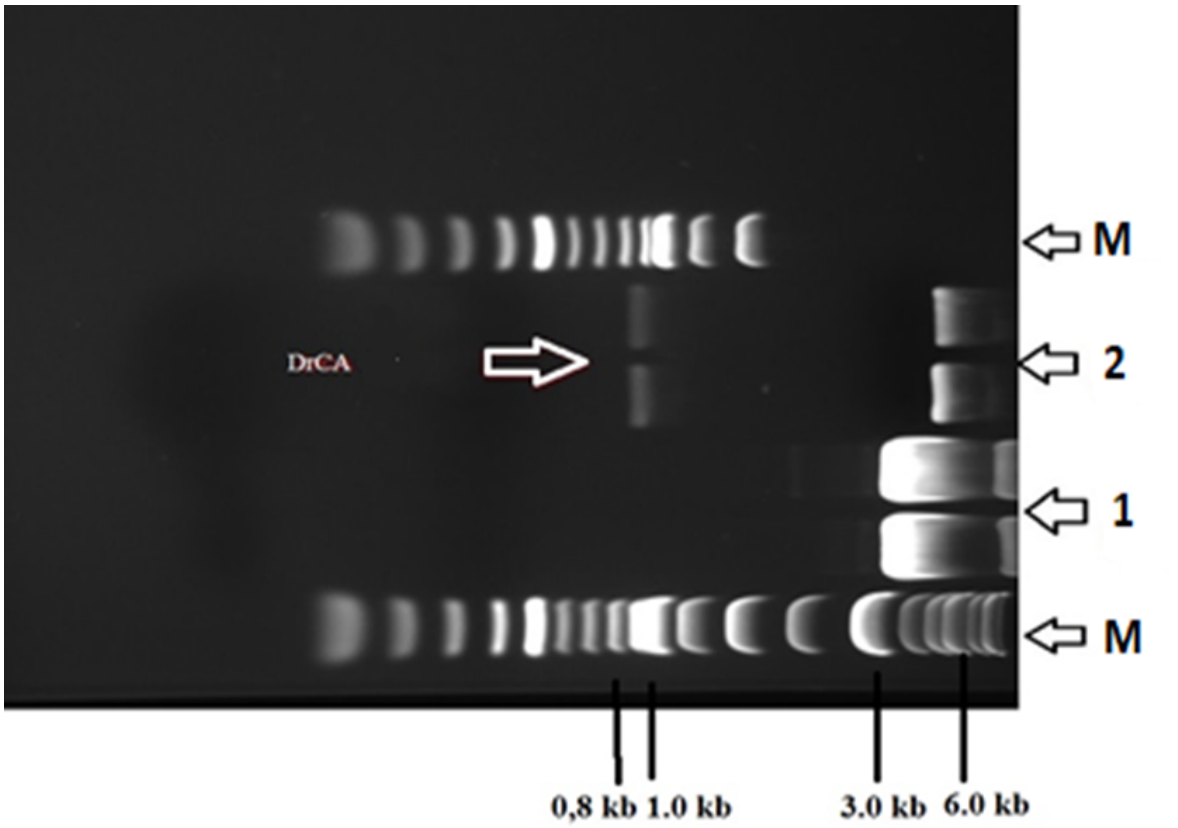

Figure 4. The electrophoresis diagram of pFN2K [GST]/DrCA and digestion with SgfI and PmeI. M: Marker, DNA ladder. Lane 1: pFN2K [GST]/DrCA; Lane 2: pFN2K [GST]/DrCA digested by NdeI and XhoI.

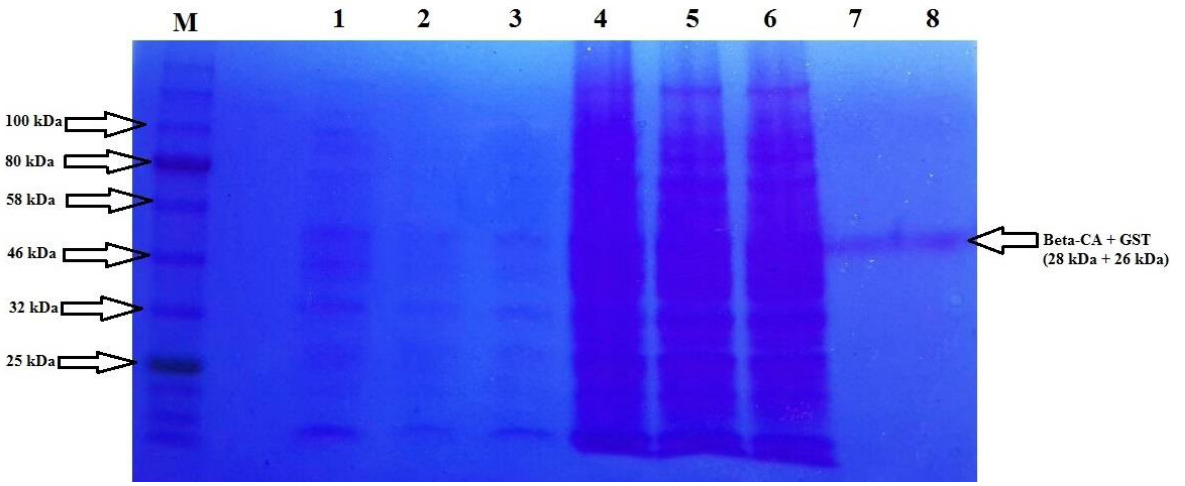

Figure 5. 12% SDS-PAGE analysis of DrCA expression. The molecular weight of fusion protein is between 54 kDa. Lanes: M: Marker [New England Biolabs, P7712S], 1-3 Elutions, 4-6: Lysate, 7-8: Purified GST-tagged fusion protein by Magne-GST

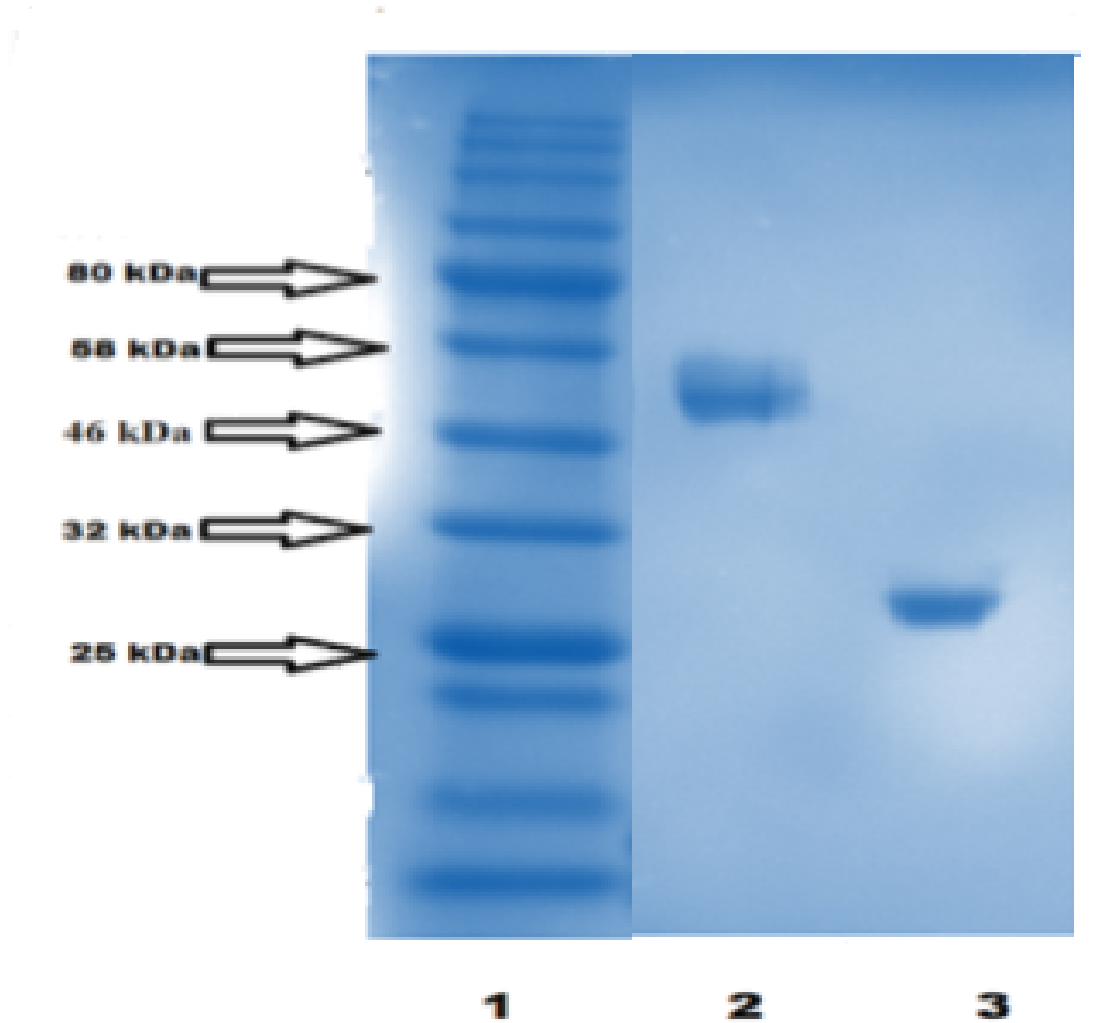

Figure 6. SDS-PAGE [12%] analysis of GST-DrCA fusion protein after cleavage by TEVp. Lane 1: Marker, Protein Ladder; Lane 2: The purified GST-DrCA fusion protein; Lane 3: The purified DrCA peptide by MagneHis™ Ni-Particles.

Construction of recombinant pFN2K [GST]/DrCA plasmid for protein expression

In order to construct expression plasmid of pFN2K [GST] Flexi® vector according to DrCA gene sequence, the DrCA gene was amplified by PCR and sequenced with the expected length of 820-bp DrCA sequence [including restriction enzyme cutting sites] and then cloned into the pFN2K [GST] Flexi® vector digested by SgfI and PmeI to obtain recombinant pFN2K/DrCA plasmid for protein expression (Figure 4).

Expression of recombinant DrCA protein

The recombinant pFN2K [GST]/DrCA plasmid was transformed into E. coli BL-21 [DE3] strain grown on MagicMedia™ *E. coli* expression medium was grown 18 h at 30 °C with shaking [200 rpm]. The DrCA was produced as a fusion protein with a glutathione tag at its N-terminus. A band with an approximately 54 kDa from the cell culture grown MagicMedia in 12% SDS-PAGE was observed, which is consistent with the expected molecular mass of DrCA fusion protein (Figure 5).

TEV cleavage and purification of DrCA peptide

To produce recombinant DrCA peptide, the purified fusion protein GST-DrCA was treated with Pro-TEVp. Quite efficient cleavage was performed in standard buffer at 20 µg fusion protein four unit TEVp at 30˚C overnight. TEV cleavage of the DrCA peptide from the GST fusion protein resulted in release of DrCA with a native N-terminal Methionine. A band, total protein 54 kDa [GST-tag 26 kDa+DrCA 28 kDa] is clearly confirmed in the SDS-PAGE. After the TEV cleavage, the DrCA peptide was successfully separated from the reaction product by MagneHis™ Ni-Particles. Purified DrCA protein concentration was determined by Lowry protein assay as 165 µg/mL. To verify the purity of DrCA protein, SDS-PAGE was applied and displayed single band close to 28 kDa in good agreement with the determined molecular weight of 28 kDa (Figure 6).

Effects of Temperature and pH on the Activities of DrCA

The effects of temperature on the activities of DrCA was measured in the range of 0-70 °C. The results presented in figure 7A showed that the optimal temperature for CO_2 hydration activities of DrCA was 40°C. A rapid decrease in CO_2 hydration activities of DrCA was observed in the range of 40°C-70°C. The pH effects on CO_2 hydration activities of DrCA was determined in the range of 6.25-8.25 at 40°C using HEPES-NaOH buffer. As shown in figure 7B, the optimum pH for DrCA activity was found to be 7.0, which was similar to the optimal pH values of other CA varieties [35-38] Many other factors, including buffer and ion concentration, affect activity, which making it difficult to compare activity measurements of CAs [12].

More than 40% of DrCA residual activity could be retained in the pH 8.0. The Bovine erythrocyte CA exhibited activity in the pH range of 6.5-8.0 and temperature range of 30-40 °C.

Thermostability Test

For the determined of stability, the purified DrCA was preincubated at 7 h in 0.025 M Hepes buffer [pH 7.0] at 10-70°C. The purified protein solution was prepared to be approximately 1 mg/ml. Enzyme activity was measured under the standard condition and that is without any treatment was taken as 100%.

Rapidly decreasing CO_2 hydration activities of DrCA was observed in the range of 40°C-70°C. Less than 40% of the maximum DrCA activity was retained beyond 55°C. DrCA protein is slightly thermostable and was completely inactivated in 10 s at 70°C.

Radiation-resistant organisms need proteins with high thermostability that can withstand the significant temperature fluctuations occurring in the cell under the influence of ionizing radiation. Therefore, it is an expected feature of DrCA to be a thermostable enzyme.

Effects of Metal Ions and Sulfonamide on the Activities of DrCA

The effects of metal ions including Na^+, Mn^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+} and Sulfonamide on CO_2 hydration activities of DrCA were investigated at the concentration of 0,5 mM. As shown in figure 8, CO_2 hydration activity of DrCA was activated and enhanced by all tested metal ions, especially Zn^{2+}, which significantly increased CO_2 hydration activity to 500% when compared to control. Sulfonamide which significantly decreased CO_2 hydration activity to 20% when compared to that of no ion control. These results showed that Zn^{2+} was the most effective cation for DrCA activity. Because zinc is needed for catalytic activity in β-CAs [39]. Although sulfanamide and its derivatives have been shown to cause inhibition of CAs belonging to pathogenic microorganisms [40-43], it has been demonstrated in this study that *D. radiodurans* β-CA could also be targeted by sulfonamide inhibitors.

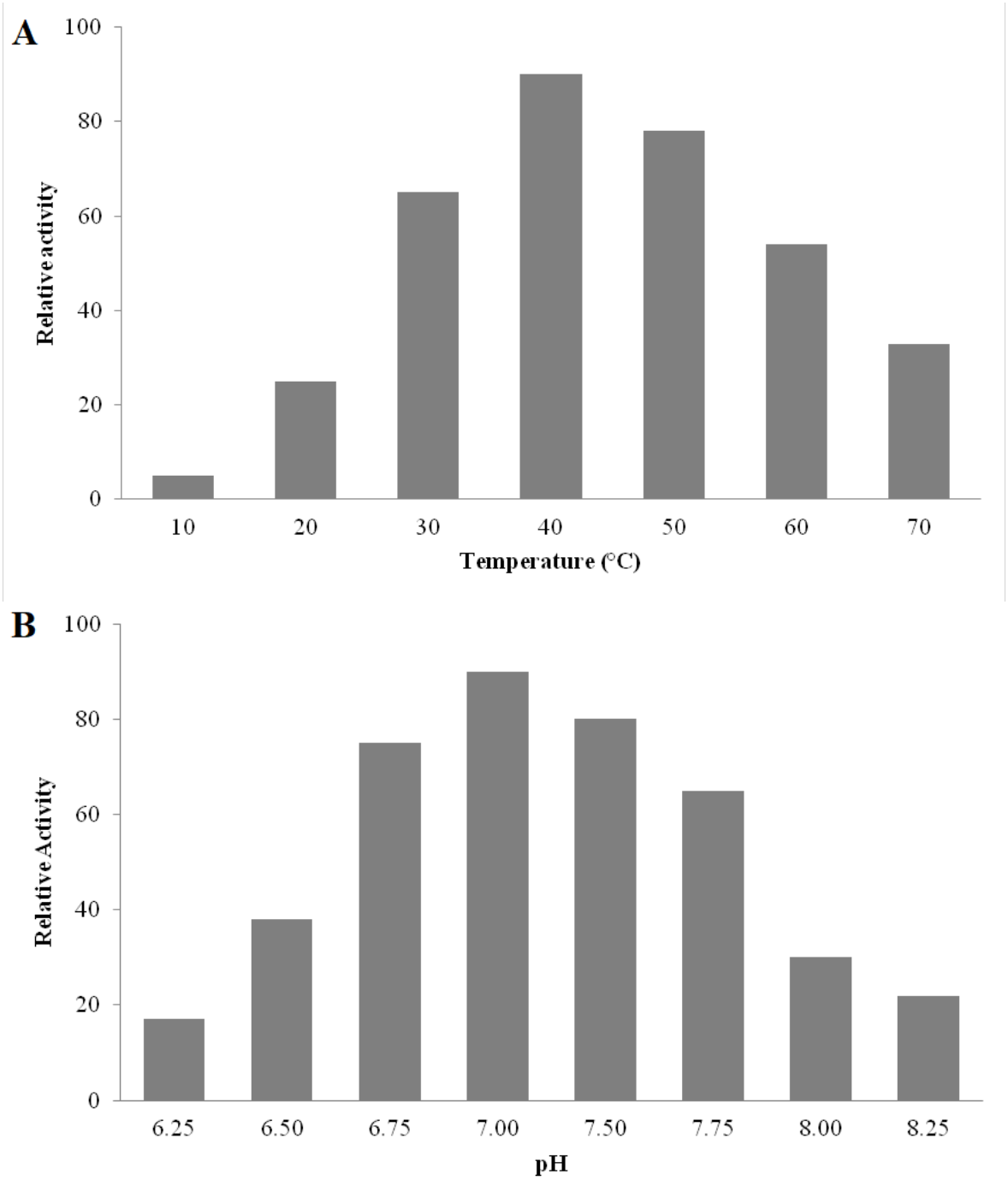

Figure 7. Effects of temperature and pH on the hydration activities of DrCA [a, b]. [A] Relative activity at different temperatures; [B] Relative activity at different pH conditions.

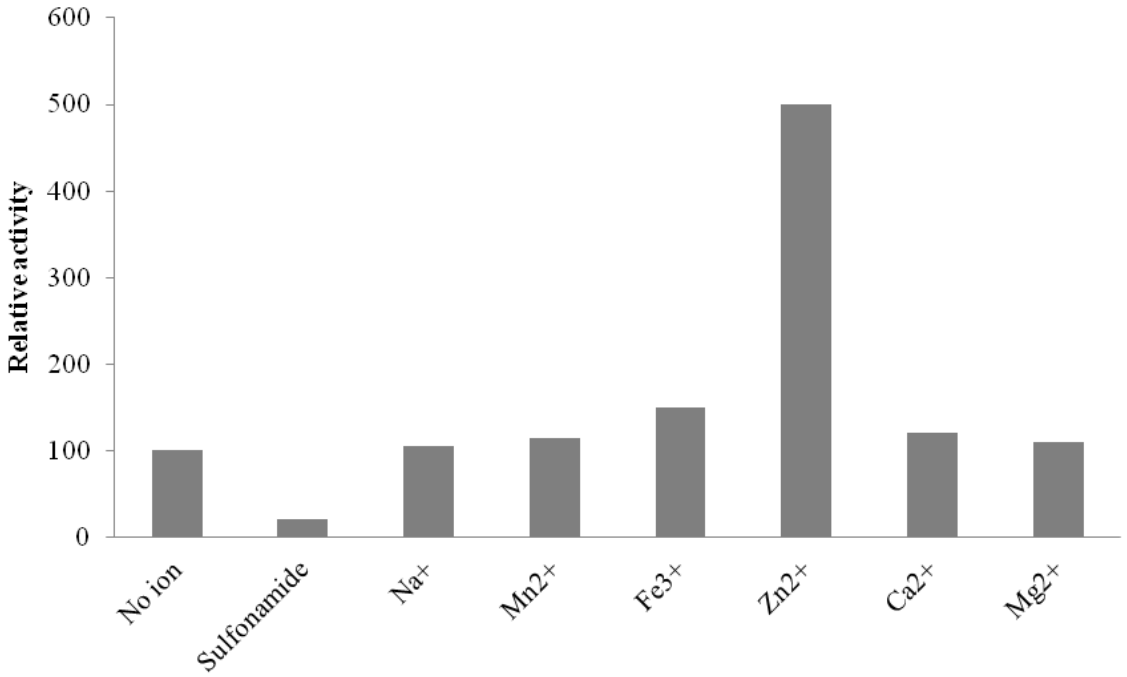

Figure 8. Effects of metal ions on CO_2 hydration activities of DrCA.

Table 1. Comparison of β-carbonic anhydrase [β-CA] hydration activities between DrCA and several published CA enzymes.

Species	CO_2 hydration activity [WAU/mg]	References
Chlamydomonas reinhardtii [CAH7]	3.1	39
Chlamydomonas reinhardtii [CAH8]	4.2	39
Serratia marcescens Wy064 [CA1]	165	40
Serratia marcescens Wy064 [CA3]	234	40
Pseudomonas aeruginosa [CA1]	401	41
Pseudomonas aeruginosa [CA2]	21	41
Bacillus subtilis	714	42
Deinococcus radiodurans R1	637	This Study

Kinetics Parameter Assays

To measure the kinetic parameters of enzyme reaction mixtures were prepared with the substrate concentration of CO_2 varied from 10 to 60 mM. The Michaelis-Menten equation for hydration of CO_2 to HCO^{3-} was determined by using the nonlinear regression method and then Km and Vmax values were calculated as 8.4 mM and 637 WAU / mg for DrCA respectively. DrCA showed extracellular high activity. Comparison of several CAs reported in previous studies and DrCA hydration activities were presented in Table 1. These results meant there could be some variation in the structure of DrCA [44-47].

CONCLUSIONS

In this study, the cloning, purification and initial characterization of β-CA enzyme from extremely ionizing radiation resistant bacteria *D. radiodurans* was performed. Km and Vmax values of DrCA enzyme were determined as 8.4 mM and 637 WAU / mg, respectively. And enzyme is slightly thermostable.

As a result, with this study, it has been shown that CA enzyme has an prospective role in the resistance against extramophilic *D.radiodurans* bacteria against high dose radiation. Any study investigating the purification of new CA isozymes and classes, their catalytic activities and mechanisms will contribute to closing the information gap in this regard.

Acknowledgement

The work was completed with the financial support of Erzincan University Scientific Research Projects Management Unit. (project no. Science-A-080715-0153).

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