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Characterization and Properties of a New Thermoactive and Thermostable Carbonic Anhydrase

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A new carbonic anhydrase was isolated and characterized from the thermophilic bacterium *Sulfurihydrogenibium* sp. YO3AOP1. The encoding gene was cloned and expressed in *Escherichia coli* and the recombinant protein purified to homogeneity. This enzyme (SspCA) belongs to the α class of the carbonic anhydrase family, is a monomer of 26.1 kDa and shows esterase activity. The kinetic parameters were determined by using CO_2 and *p*-nitrophenylacetate (p-NpA) as substrates. Thermoactivity and thermostability studies showed that SspCA is active in the temperature range from 0 to 100 °C and retains full activity after 2 h incubation at 100°C. SspCA was immobilized within a polyurethane foam and was found to be unalterably active and stable up to 50 h at 100 °C.

1. Introduction

Carbonic anhydrases (CAs) are zinc-containing enzymes that catalyze the reversible conversion of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$) with very high efficiency, namely they are among the most efficient enzymes. In eukaryotes, the enzyme participates in various physiological functions including respiration, pH homeostasis, ion transports and photosynthetic CO_2 fixation (Tashian, 1989). All CAs are divided into five evolutionary distinct classes α, β, γ, δ and ε. Members of three classes have been described for prokaryotic organisms (Smith and Ferry, 2000): two enzymes from the unicellular green alga *Chlamydomonas reinhhardtii* (Fukuzawa et al., 1990) belong to the α class similarly to all carbonic anhydrases from mammals so far characterized. Within the Bacteria domain, the enzymes purified from *Neisseria gonorrhoeae* and *E. coli* belong to the α and β classes (Guilloton et al., 1992, Chirica et al., 1997), respectively. In the Achaea domain, a γ CA from *Methanosarcina thermophila* and a β CA from the thermophilic *Methanobacterium thermoautotrophicum* have been identified. The enzyme from *M. thermoautotrophicum* was stable at temperatures up to 75 °C (Smith and Ferry, 1999). Here we report the characterization of an innovative carbonic anhydrase (SspCA) identified by translated genome inspection of a novel thermophilic bacterium, the species YO3AOP1 of *Sulfurihydrogenibium*, isolated in the Yellowstone National Park, USA. With the aim of structural assignment and enzyme characterization, the SspCA gene was overexpressed in *E. coli* and the recombinant protein purified in large amounts. The recombinant SspCA was confirmed to belong to the α class and was shown to be endowed with exceptional thermostability, with unaltered residual activity after prolonged exposure to heat up to 100 °C.

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2. Materials and Methods

2.1 Gene identification, construct preparation, protein expression and purification

The search of CAs was performed with the "FASTA" program and sequences selected for significant similarity scores. As similarity probe, the sequence of the carbonic anhydrase from archeon *Methanosarcina thermophila (accession number:* GI: 511682) was used. Among others, the CA sequence from *Sulfurihydrogenibium sp.* YO3AOP1 (SspCA) was screened. Competent *E. coli* BL21 (DE3) cells were transformed with the expression vector pET15-b/SspCA, grown at 37 °C and gene expression induced with 1 mM IPTG. After additional growth for 5 h, the cells were harvested and disrupted by sonication at 4°C. Following centrifugation, the cleared extract was heated at 90 °C for 30 min and centrifuged. The supernatant was loaded onto His-select HF Nickel affinity gel and the protein was eluted with 250 mM imidazole. At this stage of purification the enzyme was at least 95% pure.

2.2 Assay for carbonic anhydrase with CO_2 as substrate

CA activity assay was a modification of the procedure described by Chirica (Chirica et al., 1997). The assay was based on the monitoring of pH variation due to the catalyzed conversion of CO_2 to bicarbonate. Bromothymol blue was used as the indicator of pH variation. The assay was performed at 0 °C adding 1.0 mL ice-cold CO_2-saturated water to 1.0 mL mixtures of Tris-SO_4 buffer 25 mM containing different amounts of the enzyme. One Wilbur-Anderson unit (WAU) of activity is defined as $(T_0 - T)/T$, where T_0 (uncatalyzed reaction) and T (catalyzed reaction) are recorded as the time (sec.) required for the pH to drop from 8.3 to the transition point of the dye in a buffer control and in presence of enzyme, respectively.

2.3 Esterase activity.

Activity for *p*-nitrophenylacetate (p-NpA) hydrolysis was determined at 0 °C using a modification of the method proposed by Armstrong et al. (1996). The reaction mixture contained 0.3 mL of freshly prepared 3 mM p-NpA in aqueous 3% and 0.7 mL of 15 mM Tris Sulphate buffer, pH 7.6. 10 µL of enzyme solution was added, and the catalyzed reaction was monitored reading the increase in A_{348nm} for 5 minutes. One enzyme unit was defined as the amount capable of producing an OD_{348nm}= 0.03 in 5 min.

2.4 Determination of kinetic constants using CO_2 as the substrate

Kinetic measures with the substrate CO_2 were performed at 0 °C. In order to determine Michaelis-Menten constants, increasing concentrations of the CO_2 substrate were used. An ice-cold CO_2-saturated water (70 mM) was prepared and concentrations from 12 to 62,5 mM were made using ice-cold water. Reactions were started by adding 30 ng of carbonic anhydrase.

2.5 Determination of kinetic constants using p-NpA as substrate

Enzyme activity at ten different p-NpA concentration (0.125-5 mM) was determined at 0 °C by measuring the increase of absorbance at 348 nm. The Michaelis-Menten constants (Km and Vmax) were calculated at pH 7.6. The enzyme concentration in the assay was 300 ng.

2.6 Temperature studies

2.6.1 *Temperature dependence activity*

The temperature dependence activity of SspCA was measured using the p-NpA as substrate. Protein concentration used in the assay was of 300 ng. The activity was measured in the temperature range from 25 to 100 °C.

2.6.2 *Effect of temperature on the SspCA and BovCA stability*

To compare the stability of SspCA and BovCA at different temperatures, enzymes at the concentration of 3 µg/mL in 10 mM Tris/HCl, pH 8.3 were incubated at 25, 40, 60, 80 and 100 °C for 60 and 180 min. Enzyme aliquots (30 ng) were withdrawn at appropriate times and the residual activity was measured at 0 °C using CO_2 as the substrate.

2.7 Immobilization of carbonic anhydrases within polyurethane (PU) foam

A 10 mg of CA enzyme was dissolved in 3 mL of distilled water and poured onto about 3 g of viscous HYPOL2060 prepolymer in a 50 mL falcon tube (Kanbar and Ozdemir, 2010). After completion of the

enzyme immobilization, a piece of foam was cut from the middle of the whole product and assayed for immobilized CA activity. For CA activity determination, a piece of CA-immobilized PU foam (3 mg of foam containing 10 µg of enzyme) was cut out. The reaction was started by addition of the foam to the substrate-containing mixture under stirring. All the experiments were carried out in parallel with the mammalian enzyme. For studies on stability, foam slices containing the immobilized SspCA or BovCa were incubated at 90 °C. The immobilized enzyme activity was determined every 12 h over a total 48 h range. The assay was carried out using 3mg of foam containing 10 µg of carbonic anhydrase (SspCA or BovCA) and CO_2 as substrate.

3. Results

3.1 Enzyme purification

The recombinant SspCA was isolated and purified to homogeneity at room temperature from *Escherichia coli* (DE3) cell extract (Table 1)

Table 1: Purification of recombinant SspCA produced in E. coli.

STEP	Proteins (mg)	Total activity (U^a)	Specific activity (U^a/mg)	Recovery (%)	Purification fold
Sonication supernatant	728	318000	463	100	1
Thermoprecipitation at 90 °C	150	212000	1413	66	3.1
Affinity column	12	87058	7254	27	16

[a]WAU (Wilbur Anderson Units) see description in the paragraph 2.2.

Most of the carbonic anhydrase activity was recovered in the soluble fraction of *E. coli* cell extract after sonication and centrifugation as described in “Materials and Methods”. Table 1 shows that the heterologously expressed SspCA enzyme was purified 3 fold with the thermoprecipitation step. Using the affinity column (His-select HF Nickel affinity gel), the SspCA was purified 16 fold to apparent homogeneity, as indicated by a single protein band after SDS-PAGE (Figure 1, lane 4). Figure 1 shows the SDS-PAGE of the recombinant SspCA at various steps during purification from *E. coli*.

3.2 Biochemical characterization

3.2.1 Determination of molecular weight

The native molecular weight by SDS-PAGE was estimated to be 26.0 kDa. A subunit molecular mass of 26.4 kDa was calculated on the basis of the amino acid sequence translated from the gene. By gel filtration chromatography of recombinant SspCA a molecular mass of 26.1 kDa was determined (data not shown). These results clearly indicate that SspCA has a monomeric structure as expected for an enzyme belonging to the α class of carbonic anhydrases.

3.2.2 Esterase activity

Carbonic anhydrases (α-type) from mammalian sources catalyze the reversible hydrolysis of esters. With p-nitrophenyl acetate as the substrate SspCA showed esterase activity as reported in Table 2. In the table are indicated also the value of the esterase activity of the commercially available bovine α carbonic anhydrase (BovCA). The excellent specificity of the bacterial enzyme towards p-NpA, as suggested by the Km and Vmax values (Table 2) univocally assigns to SspCA to the α class of mainly mammalian carbonic anhydrases.

3.2.3 Kinetic analysis

Kinetic parameters were determined for the purified recombinant SspCA and the commercially BovCA (Table 2). The kinetic constants (Km and Vmax) were calculated for either CO_2 or p-NpA as substrates using the program Prism ver. 5.0. Using CO_2 as substrate, the Km of SspCA was approximately 2.7 times lower than Km calculated for the bovine enzyme suggesting a higher affinity of the bacterial enzyme for the substrate. The values for the reaction rates (Vmax) of the bacterial and bovine enzymes differed of about 1.5 fold. As opposite to this, the esterase activity of the two enzymes

showed approximately the same Km. Nevertheless the Vmax calculated for the bovine carbonic anhydrase was about 3.6 times higher than the Vmax relative to the bacterial enzyme (Table 2).

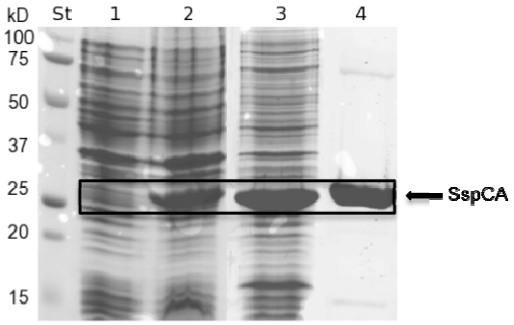

Figure 1: SDS-PAGE of the recombinant SspCA purified from E. coli. Lane 1, cell extract protein from E. coli before induction with IPTG; Lane 2, cell extract protein after induction with IPTG; Lane 3, cell extract protein after thermoprecipitation at 90 °C and centrifugation; Lane 4, purified SspCA from affinity column.

Table 2: Comparison of the biochemical properties for SspCA and BovCA. Assay units using CO_2 or p-NpA as substrate are defined in paragraph 2.2 and 2.3, respectively.

Biochemical properties	**Carbonic anhydrase**	
	SspCA	BovCA
SDS-Page Molecular Weight (kDa)	26.0	26.5
Native Molecular Weight (kDa)	26.1	27.0
Specific Activity (Units/ mg protein; substrate CO_2)	6670	4750
Km (mM; substrate CO_2)	26	70
Vmax (Units/ mg protein; substrate CO_2)	7100	11100
Specific Activity (Units/ mg protein; substrate p-NpA)	322	1282
Km (mM; substrate p-NpA)	2.8	3.4
Vmax (Units/ mg protein; substrate p-NpA)	1414	5155
Specific Activity (Units/ mg protein; Immobilized enzyme)	1900	1352
Specific Activity (Immobilized enzyme after 3 h at 100 °C)	1934	25
Specific Activity (Immobilized enzyme after 48 h at 100 °C)	1889	No activity

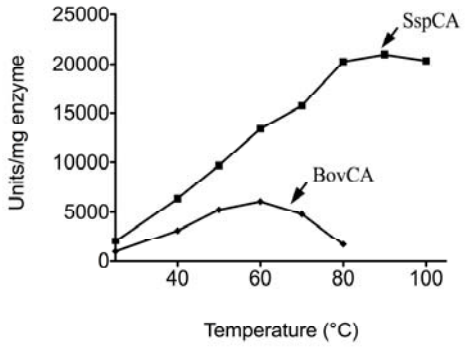

Figure 2: The temperature dependence activity of SspCA and BovCA. The enzyme activity was measured at the indicated temperature and using p-NpA as substrate.

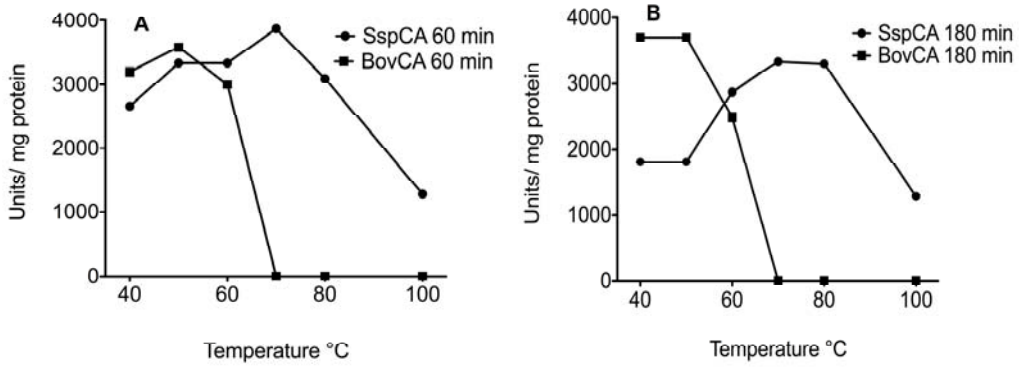

Figure 3:Thermostability of SspCA and BovCa. The enzymes were incubated for 60 min (panel A) and 180 min (panel B) at the indicated temperature.

3.2.4 Stability studies

3.2.4.1 Temperature dependence of the activity

The temperature dependent activity of SspCA was assayed using the p-NpA as substrate, and measurements were performed in parallel with BovCA using 300 ng of each enzyme (Figure 2). The optimum temperatures for SspCA and BovCA were determined by incubating enzymes at 25, 40, 60, 70, 80 and 100°C. The reaction was monitored following the absorbance at 348nm for 5 min. The optimum temperature for bovine enzyme was found to be 60 °C, while the SspCA (optimum at 95°C) was still active at 100 °C (Figure 2).

3.2.4.2 Temperature effect on the SspCA and BovCA stability

The stability of SspCA and BovCA was compared at the temperatures above mentioned (Figure 3A and B). After 60 and 180 minutes of incubation, the activity of SspCA was optimal at 70 °C (Figure 3A and 3B) while the bovine enzyme was already inactivated at temperature higher than 60 °C (Figure 3A) and 50 °C (Figure 3B) for both incubation times. Noticeably, the bacterial enzyme retained its activity at temperature of 100 °C. These studies indicate that the bacterial enzyme was more stable at high temperature and retained its activity for a longer time (e.g. 180 min) when compared to the mammalian enzyme.

3.3 Immobilization of SspCA and BovCA within the PU foam

Immobilization was performed as described in paragraph 2.7. The specific activity of the immobilized enzymes is listed in Table 2. The bacterial enzyme was 1.4 fold more active than the bovine and maintains its activity after several reaction cycles. The polyurethane foam containing the immobilized SspCA or BovCa was incubated at 100 °C. The results are reported in Table 2. The activity of the immobilized BovCA showed a 50-fold decrease of the initial activity, while the activity of the SspCA remains constant up to 48 hours. The immobilized SspCA after 50 h retained full activity.

4. Discussion and Conclusions

Carbonic anhydrases, isolated from mammals or prokaryotes and other mesophilic sources, are active at physiological temperature (37°C) and are, like many enzymes, quite unstable under operative conditions. Studies on the enzymes from thermophiles, microorganisms living at temperatures from 70°C to 110°C, demonstrated that they are thermostable, thermoactive, generally stable to the common enzymes denaturants (Rossi, 1995, Pouwels et al., 2000). With the aim of the recombinant DNA technology, a novel carbonic anhydrase, SspCA, identified in the genome of the thermophilic bacterium, *Sulfurihydrogenibium sp*, was overexpressed in *E. coli* and biochemically characterized in comparison with bovine carbonic anhydrase. SspCA is a monomer of 26.1 KDa and shares significant similarity with the α class CAs, previously believed present only in animal organisms. The dependence

of the SspCA activity on temperature was determined in comparison with the bovine counterpart. Starting from 25 °C, temperature at which the activity of both enzymes was comparable, the SspCA activity increased constantly up to 95 °C whereas the bovine enzyme had a maximal activity at 60°C. However the activity of the bovine enzyme at the optimum temperature was still about one half of the bacterial enzyme activity. The results of the studies on thermostability showed that SspCA was stable for 180 min at 100°C whereas the bovine enzyme was fully inactivated at 70°C. The first documented carbonic anhydrase from a thermophile was purified from the thermophilic methanoarcheon *M. thermoautotrophicum,* but the enzyme was thermostable at temperatures up to 75 °C and belonged to the β class CAs (Smith and Ferry, 1999, Smith and Ferry, 2000). Comparative kinetic studies on SspCA and BovCA revealed that, at 0°C, the bacterial enzyme showed lower values of Km namely a higher affinity toward both CO_2 and p-NpA whereas Vmax was lower. SspCA or BovCA were also demonstrated to be efficiently immobilized within PU foam at 100 °C, with the immobilized SspCA maintaining 100% activity over 50 h and the mammalian enzyme being fully inactivated already after 3 h. Carbonic anhydrases has received much attention due to their potential use in carbon dioxide sequestration. The use of carbonic anhydrases, either free or immobilized, has been reported in several technical approaches for CO_2 capture, from combustion and other gases mixtures, and its utilization or disposal. This utilization of CA requires stable enzymes, even at high temperature, and the possibility re-using the enzyme in many subsequent cycles for an efficient sequestration process. The results obtained in this work strongly suggest that the thermostable SspCA is an almost ideal candidate for hydration of CO_2 in biomimetic sequestration.

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