

Purification of Carbonic Anhydrase from *Methanosarcina thermophila*

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Carbonic anhydrase (CA) is an ubiquitous zinc-containing enzyme catalyzing the reversible hydration of carbon dioxide ($\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$). Recently, two distinct classes of CAs have been recognized, which appear to have evolved independently: (1) a prokaryotic branch, represented by the chloroplast CAs of higher plants and two bacterial enzymes, and (2) a eukaryotic branch, including all seven isozymes from various higher vertebrates and two isozymes from the microalga *Chlamydomonas reinhardtii* (Fukuzawa et al. 1992). The primary structure of CA from *Methanosarcina thermophila* has been determined (Alber and Ferry 1994), but interestingly, it shows no significant sequence similarity to any of the CAs described so far.

CA activity from *M. thermophila* is elevated severalfold when the growth substrate is switched from methanol to acetate, suggesting an involvement of the enzyme in the conversion of acetate to methane (Karrasch et al. 1989; Jablonski et al. 1990). The physiological role of the enzyme in the pathway remains to be elucidated.

We used *Methanosarcina thermophila* strain TM-1, which utilizes acetate as well as methanol or trimethylamine as a sole energy and carbon source.

MATERIALS

Cells from harvested cultures (cell paste) of *Methanosarcina thermophila* (see Protocol 6)

Continuous flow centrifuge (Type LE, Cepa, New Brunswick Scientific)

pH meter with a fast-responding pH electrode (semimicro combination electrode, Corning 476541) connected to a chart-recorder

French pressure cell (40-ml capacity; SLM Aminco FA-073)

Spectra/Por 7 dialysis tubing (3500-m.w. cut-off) (Spectrum Medical Industries 132110 or equivalent)

FPLC (fast protein liquid chromatography) system (Pharmacia)

DEAE-cellulose column (2.6 x 15 cm; Whatman 4057W050)

Phenyl-Sepharose HiLoad 26/10 column (Pharmacia 17-1086-01)

Phenyl-Superose HR 5/5 column (Pharmacia 17-0519-01)

Mono Q HR 5/5 anion-exchange column (Pharmacia 17-0556-01)

Superose-12 gel filtration column (Pharmacia 17-0538-01)

p-aminomethylbenzenesulfonamide-agarose affinity column (0.5 x 2 cm; Sigma A 0796)
 Ammonium sulfate (solid; Sigma A 915)
 Sodium perchlorate (Aldrich 20,842-6)
 Polyethylene glycol (PEG 8000; Sigma P 2139)
 RNase A (Sigma R 5125)
 DNase I (Sigma D 4263)
 Assay buffer

veronal/H ₂ SO ₄ (pH 8.3)	20 mM
zinc sulfate	1 μM
Buffer A	
potassium phosphate (pH 6.8)	50 mM
zinc sulfate	1 μM
Buffer B	
potassium phosphate (pH 7.0)	100 mM
zinc sulfate	1 μM
ammonium sulfate	1.5 M
Buffer C	
potassium TES (pH 7.5)	50 mM
zinc sulfate	1 μM
Buffer D	
potassium phosphate (pH 7.1)	20 mM
zinc sulfate	1 μM

Caution: Sodium perchlorate is harmful if inhaled or swallowed. Material is irritating to mucous membranes, upper respiratory tract, and eyes. Wear gloves and safety glasses and work in a chemical fume hood.

METHODS

Cell Growth

1. CA activity is elevated in acetate-grown cells. Grow culture in a 10-liter pH auxostat in the presence of acetate as described by Sowers et al. (1984; see Protocol 6).
2. Harvest the cells at the end of exponential growth with a continuous flow centrifuge (Type LE) and store in liquid nitrogen until use.

Assay

1. Measure CA activity using a modification of the electrometrical method of Wilbur and Anderson (1948).
2. Start the reaction (mixture contains 1 ml of assay buffer and 100 μl of sample at 25°C) by adding 750 μl of CO₂-saturated H₂O (distilled H₂O gassed vigorously with CO₂ for ~1 hour). Record the time (*t*) required for a pH decrease from 7.85 to 7.0.
3. Calculate the units of activity using the equation $U = t_0 - t/t$, where t_0 is the time required for the pH change using a boiled sample of buffer without the enzyme.

Purification

All procedures are done aerobically and at room temperature unless otherwise indicated.

1. Suspend thawed cell paste (20 g wet weight) in 15–20 ml of cold Buffer A and pass twice through a chilled French pressure cell at 20,000 lb/in². After the first passage, add 0.1 mg of RNase A and 0.25 mg of DNase I and incubate the mixture for 10 minutes on ice. Centrifuge the crude extract twice at 20,000g for 15 minutes at 4°C.
2. Apply the supernatant to the DEAE-cellulose column equilibrated with Buffer A. Wash the column with 150 ml of Buffer A and develop with a 500-ml linear 0–1 M NaCl gradient, applied at 2.0 ml per minute. Discard the exclusion fraction (usually containing 10–20% of the total activity loaded), which is generally of low purity. CA elutes in 0.35–0.50 M NaCl. Pool active fractions and add solid ammonium sulfate to a final concentration of 1.5 M, stir slowly, and store at 4°C overnight.
3. Centrifuge the mixture at 12,000g for 15 minutes at 4°C and load the supernatant fraction onto the phenyl-Sepharose column preequilibrated with Buffer B. After a 120-ml wash, elute the enzyme with a 500-ml linear gradient of 1.5–0 M ammonium sulfate at 2.0 ml per minute. CA elutes in 0.9–0.65 M ammonium sulfate. Add additional solid ammonium sulfate to the pooled fractions to a final concentration of 1.5 M, stir slowly, and store at 4°C overnight.
4. After centrifugation at 12,000g for 15 minutes, concentrate the sample over the phenyl-Superose column as follows: (i) inject a portion of the sample (10 ml) onto the column previously equilibrated with Buffer B, (ii) wash the column with 5 ml of Buffer B, and (iii) repeat the first two steps until the entire sample has been applied. Elute the enzyme with 10 ml of Buffer B without ammonium sulfate and freeze at –20°C.
5. Repeat steps 1–4 three or four times. Pool all samples, dilute 1:5 with Buffer C, and equilibrate overnight at 4°C.
6. Load the diluted sample onto a Mono Q column equilibrated with Buffer C. After a 10-ml wash, elute the sample with a 60-ml linear gradient from 0 to 1 M KCl applied at 1.0 ml per minute. CA elutes in 0.4–0.6 M KCl. Concentrate pooled active fractions in dialysis tubing (3500-m.w. cut-off) imbedded in dry polyethylene glycol (PEG 8000) at 4°C. Frequently replace the PEG and monitor the sample constantly to avoid complete drying.
7. Load 0.2-ml aliquots onto the Superose-12 column equilibrated with Buffer A containing 150 mM NaCl. Wash the column at a flow rate of 0.4 ml per minute and combine the active fractions, concentrated as described in step 6, and dialyze against Buffer D at 4°C.
8. Load the sample onto the *p*-aminomethylbenzenesulfonamide-agarose column equilibrated with Buffer D at 4°C. Elute CA with 15 ml of Buffer D containing 0.5 M sodium perchlorate. Concentrate the enzyme as described

in step 6, dialyze extensively against Buffer D, and store at -20°C ; 80 g of cell paste typically yields 40 μg of purified enzyme (20% recovery).

COMMENTARY

- The assay used to monitor CA activity throughout the purification is rapid and sensitive; however, with a 10% margin of error, it is not very precise. In addition, the relationship between amount of enzyme and activity is only linear up to $U = 1.0$. The sample used in the assay must be diluted accordingly.
- It is not known if CA from *M. thermophila* contains zinc. Nevertheless, zinc sulfate is included in all buffers. In addition, compounds of the sulfonamide type are strong inhibitors for most CAs. This fact is taken advantage of by designing affinity resins used in purifications of many CAs (Chegwidden 1991). CA from *M. thermophila* has a rather low affinity to those type of inhibitors (Alber and Ferry 1994). The last step of the purification scheme presented here, using such an affinity resin, is only successful under very low-salt and low-temperature conditions.
- On the basis of a purification factor of $>10,000$, it can be estimated that there are only a few hundred molecules of CA in the cell. It is therefore very important to achieve maximum recovery of CA activity at each purification step.
- We have some evidence that CA from *M. thermophila* might be located outside the cell. If this holds true, the purification scheme may be simplified by including an initial step that would release CA without disruption of the cell membrane.

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