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Working

Carbonic Anhydrase Activity Assay

Forked from Carbonic Anhydrase Activity Assay

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Weis Lab Oregon State | Anthopleura-Microbe Model System

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ABSTRACT

Carbonic anhydrase (CA) is an enzyme which catalyzes the interconversion of bicarbonate ions (HCO_3^-) and carbon dioxide (CO_2). The activity levels of CA can be measured by taking advantage of the chemical reaction that it catalyzes. When CO_2 is added to a tissue homogenate, the CA present converts the CO_2 into HCO_3^- and a hydrogen ion (H^+), which results in a decrease in pH. The rate at which the pH drops can be measured and converted into a CA activity mesaure. If there is no CA activity, the pH level decreases slowly and if there is a high CA activity, the pH decreases quickly.

GUIDELINES

Keep tissue samples on ice as often as possible! Enzymes denature with heat!

BEFORE STARTING

VERY IMPORTANT: Keep **any biological samples** on ice as much as possible. Get two coolers full of ice. You need to adjust your pH probe so that it records a measurement every second (or quicker if possible); the CA reaction happens very quickly.

Mixing Carbonic Anhydrase (CA) Buffers

1 **Stock 50mM barbital buffer**
- Mix 1 vial of barbital buffer (Sigma B5934-1VL) with 800 mL of deionized water. Once all powder is dissolved, add deionized water to 1L.

2 **25 mM Veronal buffer (Buffer A)**
- Mix 250mL of 50mM barbital buffer with 250mL of deionized water
- Adjust pH of Buffer A to 8.2 with 1M HCl

3 **Extraction buffer**
- Mix 250mL 50mM barbital buffer with 250mL of deionized water
- Add 0.93g EDTA
- Add 1.23g $MgSO_4$
- Stir solution until EDTA and $MgSO_4$ are completely dissolved
- Adjust pH of Extraction buffer to 8.2 with 1M NaOH

Preparing your workspace

4 First, take out three new 1 ml pipette tips. At the top edge of the tip, mark one with an “A” to use with Buffer A only, one with “E” to use with Extraction Buffer only, and the third with “C” to use with CO_2-saturated seawater only. Reuse these pipette tips on every sample you run, and be very careful that they don’t get contaminated by accidentally using them on other solutions. You will need to use new pipette tips for each anemone native and denatured samples.

5 Calibrate the pH probe daily according to manufacturers specifications.

Prepare CO2-Saturated DI Water

6 Prepare two flasks of CO_2 saturated DI water in case the pH in one is not low enough when you are ready to begin assays. The pH of your water needs to be below 4.00. (**go to steps 7-11 or 12-15**)

Prepare CO2-Satured DI Water with CO2 Tank (Preferred Method)

7 Bubble CO2 using an air stone into a 500 mL flask of 300 mL of chilled DI water for 15 minutes

8 Twist in the rubber stopper into the flask and let the CO2 equilibrate for 15 minutes.

9 Measure and record the pH of the water.

10 Keep your CO2 flask on ice (or the CO2 will start coming out of solution).

11 Once the necessary pH has been reached, keep the stopper tight on the flask to reduce CO2 offgassing.

Prepare CO2-Satured DI Water with Dry Ice

12 If a CO2 tank is unavailable, you can add dry ice to flask. Drop 4 or 5 half-dollar sized chunks of dry ice into the flask and place the rubber stopper on top loosely to allow excess pressure to escape the flask.

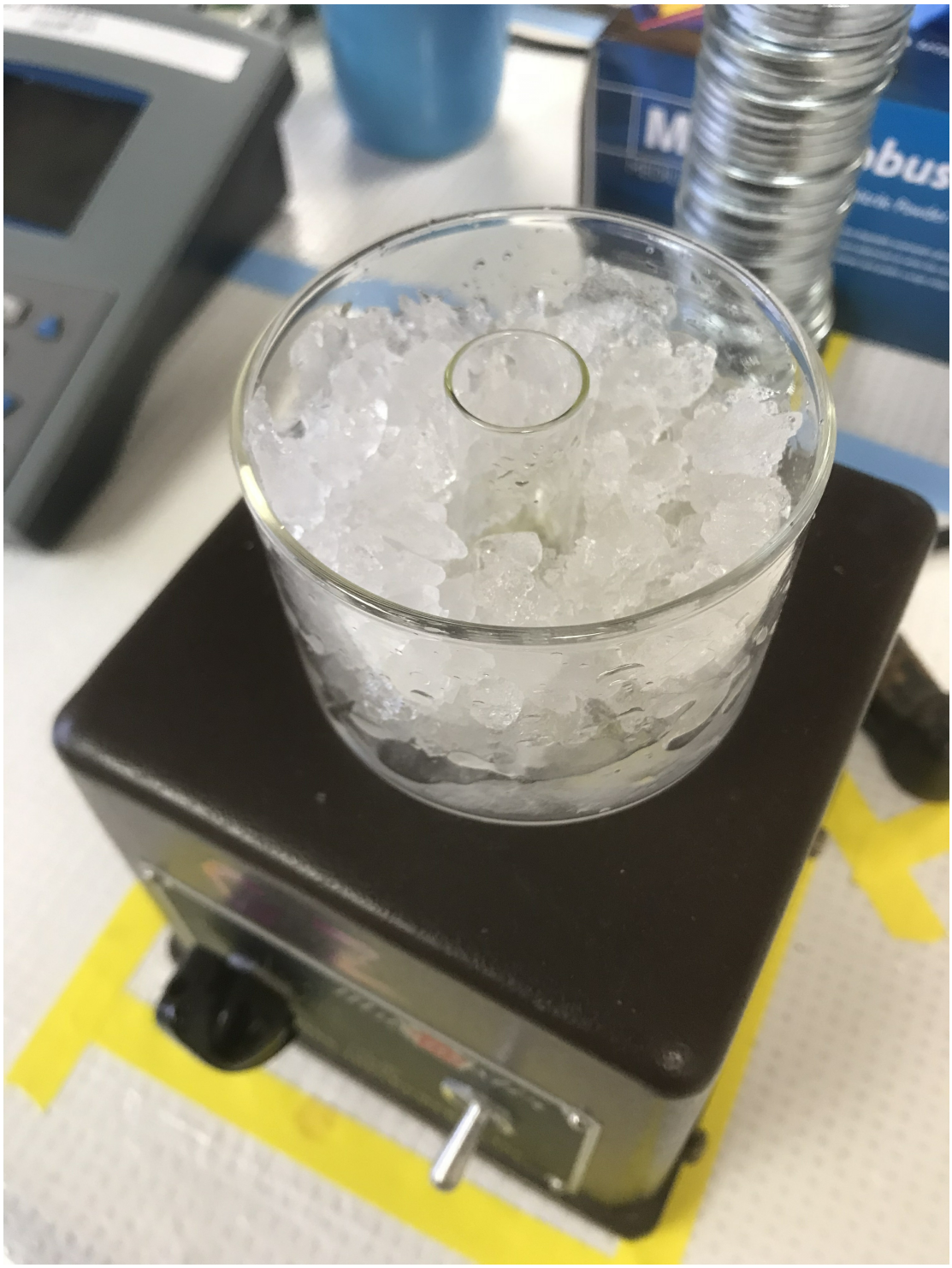
13 Once the dry ice has dissolved completely, measure the pH of the water.

14 If the pH is not low enough, add more dry ice and repeat.

15 Once the necessary pH has been reached, keep the stopper tight on the flask to reduce CO2 offgassing.

Run a Negative Control Daily, Before You Run Your Samples

16 Run the assays in a 6-12 mL sample vial/shell vial (Thomas Scientific 60975L-3, 224744, 224745, 60965D-1, or 60965D-3) (referred to as "beaker" below) equipped with little stir bars (either custom made from pasteur pipet and piece of staple or tiny bars from science supplier). The beaker should be in ice during measurement to keep the reaction cold.



17 Rinse out the beaker with 1 mL “Buffer A.” Dump into waste flask and do not lose the stir bar! If you lose the stir bar, wash it with DI water and repeat the Buffer A wash step.

18 Add 2 mL “vernol Buffer A” to the beaker (using the “A” pipette tip).

19 Place the beaker on the stir plate. Insert the pH probe. Ensure that the probe is submerged and that the stir bar is not hitting the probe.

20 Hit “read/log” then quickly add 1 mL CO2-saturated seawater to the beaker, and watch the pH slowly drop as the CO2 offgasses.

21 Wait at least 120 seconds before stopping the reading to ensure that the probe is reading ~-0.01 pH/second (normal CO2 offgassing).

22 Rinse the beaker with DI water and empty into the waste beaker. Be carfeul not to lose the stir bar.

23 Transfer the data onto flash drive or onto the computer log.

EXTRACT ANEMONE CA FROM ANEMONES (This can be applied to other organisms as well).

24 Label two 15 mL tubes (“Anemone ID” + native or denatured) and two 1.5 mL tube (PROT or ALGAE + “Anemone ID”)

25 For A. elegantissima remove between a half and an eighth of the anemone for the assay (a half for very small anemones and an eighth for very large anemones). The minimum amount of material used for this assay should be about 0.2 grams frozen material. If you are concerned that you do not have enough material, try a few practice samples using the amount of material you plan to use for real samples.

26 From the piece of anemone that will not be used for the assay, make a diagonal cut from the middle of the oral cavity to the outer edge of the anemone and place the slice into the algae 1.5 ml snap tube. Place this tube into the freezer for analysis later.

27 Place the rest of the anemone back into the freezer.

28 Finely mince anemone with a razor blade, then place the anemone in a glass-teflon tissue homogenizer with 3 mL of “extraction buffer.”

29 Keeping the grinder in ice, grind the anemone for 1-1.5 minutes (there should be no large tissue chunks). With the pestle still in the homogenizer, pour the homogenized anemone into the Anemone ID labeled 15 mL tube. Keep this tube on ice.

30 Rinse the tissue grinder with 4 mL “extraction buffer”, swish, grind a few more times, and add to the anemone homogenate. You should have 7 mL of homogenate.

31 Vortex the homogenate briefly. Centrifuge the homogenate at 4˚C at 4300 RCF for 10 minutes to pellet algae and large cellular debris. Don’t forget to balance the centrifuge.

32 Carefully pour the supernatant into the Native 15 mL tube. You may want to freeze the cellular debris/algae pellet for future analysis.

33 Use a P1000 pipettor to add 3 mL of the supernatant to the 15 mL Denatured tube.

34 You need 3 mL of homogenate in the Native tube. Remove between 200 µL and 1 mL of the homogenate and add to the “PROT” labeled 1.5 mL tube. Place the 1.5 mL tube in the designated box in the freezer for later protein analysis.

PREPARE DENATURED SAMPLES

35 Fill a 1000 mL beaker with ~900 mL of DI water and place on a hot plate. Let the water reach a boil. The high temperature denatures

proteins, and as a result, inactivates enzymes (including CA).

36 Take the tubes labeled “denatured” and loosen the cap very slightly to allow the pressure to equilibrate.

37 Insert the tube into a hole on a float, and let the float sit on top of the beaker of boiling water. Make sure the homogenate is submerged in the water.

38 Keep the denatured tube in the boiling water for five minutes. **00:05:00**

39 Remove the tube from the water and allow the tubes to cool on ice for five minutes. **00:05:00**

RUN NATIVE SAMPLES

40 Rinse out the beaker with 1 mL “Buffer A.” Dump into waste flask and do not lose the stir bar!

41 Add 1 mL “vernol Buffer A” to the beaker (using the “A” pipette tip).

42 Briefly vortex/pipette mix your native sample homogenate. Add 1 mL of your native sample homogenate to the beaker.

43 Place the beaker on the stir plate. Insert the pH probe. Ensure that the probe is submerged and that the stir bar is not hitting the probe.

44 Hit “read” then quickly add 1 mL CO2-saturated seawater to the beaker, and watch the pH slowly drop as the CO2 is converted to HCO3- and H+ ions.

45 Rinse the beaker with DI water and empty into the waste beaker. Be carfeul not to lose the stir bar. Repeat this three times.

46 Repeat steps 40-45 two more times for the same sample so that you end up with three replicates from the native sample.

go to step #40

RUN DENATURED SAMPLES

47 Rinse out the beaker with 1 mL “Buffer A.” Dump into waste flask and do not lose the stir bar!

48 Add 1 mL “vernol Buffer A” to the beaker (using the “A” pipette tip).

49 Briefly vortex/pipette mix your denatured sample homogenate. Add 1 mL of your denatured sample homogenate to the beaker.

50 Place the beaker on the stir plate. Insert the pH probe. Ensure that the probe is submerged and that the stir bar is not hitting the probe.

51 Hit “read” then quickly add 1 mL CO2-saturated seawater to the beaker, and watch the pH slowly drop as the CO2 is converted to HCO3- and H+ ions.

52 Rinse the beaker with DI water and empty into the waste beaker. Be carfeul not to lose the stir bar. Repeat this three times.

53 Repeat steps 47-52 two more times for the same sample so that you end up with three replicates from the native sample.

go to step #47

DATA ANALYSIS

54 The data you will produce will be numerous pH values and a time stamp.

55 You will be calculating the slope of each run and taking the steepest slope between two data points. Then you will average the slopes of your triplicate replicates. See my example below:

RAW DATA

Time (s)	Native 1	Native 2	Native 3	Denatured 1	Denatured 2	Denatured 3
0	8.00	8.1	8.00	8.00	8.1	8.0
1	7.5	7.5	7.5	7.9	7.9	7.9
2	7.2	7.0	7.2	7.8	7.8	7.8
3	7.1	6.9	7.1	7.7	7.6	7.7
4	7.0	6.8	7.0	7.6	7.5	7.6
5	6.9	6.7	6.9	7.5	7.4	7.5
6	6.8	6.6	6.8	7.4	7.3	7.4

Raw Data

56 Slope = (y2-y1)/(x2-x1)

The x variable is time (in seconds)

The y variable is pH

57 Calculate the slope and find the largest change (min value since it it negative)

Time (s)	Native 1	Slope 1	Native 2	Slope 2	Native 3	Denatured 1	Denatured 2	Denatured 3
0	8.00	-0.5	8.1	-0.6	8.00	8.00	8.1	8.0
1	7.5	-0.3	7.5	-0.5	7.5	7.9	7.9	7.9
2	7.2	-0.1	7.0	-0.1	7.2	7.8	7.8	7.8
3	7.1	-0.1	6.9	-0.1	7.1	7.7	7.6	7.7
4	7.0	-0.1	6.8	-0.1	7.0	7.6	7.5	7.6
5	6.9	-0.1	6.7	-0.1	6.9	7.5	7.4	7.5
6	6.8	-NA	6.6	NA	6.8	7.4	7.3	7.4
	MIN SLOPE	-0.5		-0.6				

Example of slope calculations from raw data and min values (not performed for all columns)

58 Average your replicates, calculate raw CA/second and convert to minutes. We convert to minutes since most literature reports CA activity in minutes; so, we can compare our values to other literature.

	Native 1	**Native 2**	**Native 3**	**Denatured 1**	**Denatured 2**	**Denatured 3**
MIN SLOPE	-0.5	-0.6	-0.5	-0.1	-0.2	-0.1
AVERAGE SLOPE	Native	-0.53		Denatured	-0.13	
		RAW CA/SECOND	Native - Denature	-0.4*-1 =	0.4	I convert to positive because it is more logical when graphing.
		RAW CA/MINUTE	0.4 ΔpH/s * 60	**24 ΔpH/minute**		

59 LASTLY, MEASURE PROTEIN FROM YOUR "PROT" SAMPLE. We use a Bradford Protein Assay This should be converted to mg/mL. Then, divide RAW CA/MINUTE by PROTEIN (mg/mL).

So in the example above, lets say that the protein was measured to be 10 mg/mL.
24/10 = **2.4 ΔpH/minute/mg animal protein = CA Activity**