An Electrochemical Approach Coupled with Sb Microelectrode to Determine the Activities of Carbonic Anhydrase in the Plant Leaves

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Abstract. The electrochemical approach coupled with Sb microelectrode was developed to determine the carbonic anhydrase activity in a wide range. The sensing response of the microelectrode had a good linear relationship between potential and pH value in barbital buffer. The temperature would not affect the linear relationship. During the determination, the open-circuit potential method was taken to monitor the whole course of the reversible conversion catalyzed by carbonic anhydrase, and then the initial part of uniform velocity from the reaction curve was chosen to calculate the reaction velocity (the time to change one unit of pH). This technique, in comparison with the conventional method was used to determine the activities of bovine red blood cells carbonic anhydrase, foliar carbonic anhydrase and extracellular carbonic anhydrase in some plants. The result showed that the electrochemical approach coupled with Sb microelectrode would obtain more credible, accurate data than the conventional method.

Keywords: pH change; Carbonic anhydrase; Sb microelectrodes; Open-circuit potential (OCP).

1 Introduction

Carbonic anhydrase（CA; carbonate hydrolyase, EC 4.2.1.1）is a zinc-containing metalloenzyme that catalyses the reversible hydration of carbon dioxide. It has an important role in photosynthesis that reduces the diffusion resistance in the mesophyll cell for CO_2 by its catalysis, and provides reaction substrate for the carboxylation. Meanwhile, it has an exceptionally high CO_2 hydration turnover rate about 10^6 s^{-1} [1]. Extracellular CA, which is distributed on cytoplasm membrane, can facilitate the availability of CO_2 for photosynthesis [2].

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The conventional method to assay CA, described by Wilbur and Anderson [3], has been used by most works so far. This electrometric determination has some disadvantages. Firstly, the electrodes, which were used to determine the pH change in the reversible conversion of CO_2 to bicarbonate by the catalysis of CA, were commercial glass electrodes. The size of glass electrode is too large to be miniaturized because of its brittleness of the glass membrane, and its sluggish response will restrict its determination range of CA. When the CA activity of the sample is too low, it will be hard to measure owing to great measurement error. While the CA activity of the sample is too high, it will also affect the result of determination because the meter changes too fast to be caught by human eyes. Secondly, the data recorded by different operators would be diverse. Finally, only the data from two points were recorded by stopwatch at the interval of the pH drop in one unit, and the calculation of the CA activity was based only on the data. The measurement error was very great because the reaction process between the two points was incompletely uniform. Because of the above disadvantages, the CA activity, which is too low or too high, cannot be measured with the method. In addition, no documents have recorded the accurate data of extracellular CA activity in the leaves. Therefore, a new method aimed at determining the CA activity in a wide range should be developed.

In recent years, many metal oxide microelectrodes have been studied for pH measurement. Metal oxides such as PtO_2, IrO_2, RuO_2, TiO_2, Sb_2O_3, WO_3, Co_3O_4, PbO and Bi_2O_3 were used as pH sensitive film [4-6]. By far, of the various metal oxides that have been applied for pH measurement, antimony oxide is the most widely used. Compared with other metals of the platinum family such as iridium, platinum, and palladium, which are also frequently used as pH sensing materials, antimony oxide is much cheaper and easier [7-8].

In this research, several species of plants were chosen to assay their CA and extracellular CA with an electrochemical approach (open-circuit potential, OCP) coupled with Sb microelectrode. The whole course of the reaction of CO_2 to bicarbonate catalyzed by CA was monitored, and the pH value at any time can be recorded. This technique improves the determination accuracy, especially that of the activity of CA which is either too high or too low.

2 Materials and Methods

2.1 Fabrication of Sb Microelectrode

In this method, copper wires with a diameter of 0.6 mm and a length of about 5 cm were used as the substrate, and Sb_2O_3 solution as the electroplating solution. When polished by metallographic sandpaper, a copper wire was electroplated as the anode (platinum wire as the cathode) by i-t (Amperometric i-t Curve, CHI660C, Shanghai Chenhua Instrument Company). A bright and smooth grayer Sb metal was grown on the surface of the copper wire. The fabricated Sb microelectrodes were placed in oven at 120℃ overnight.

The Sb microelectrode was characterized in barbital buffer with the composition of 20 mmol L^{-1} sulfuric acid and 20 mmol L^{-1} barbital sodium.

2.2 Carbonic Anhydrase Activities Assay

1.5 mg pure carbonic anhydrase (SIGMA Company, 4860 WA units mg^{-1}) was solved in 100 ml deionized water. The enzyme solution was used for producing the curve of the relative CA activity *vs.* volume.

All the leaves were picked at 9:00 a.m. in one same day. The activities of CA were measured in 0.3-0.5 g samples of leaves. The leaves were quickly frozen in liquid N_2, ground into powder with a mill, and then homogenized with 3 ml of extraction buffer (10 mmol L^{-1} barbital buffer with 50 mmol L^{-1} 2-mercaptoethanol, pH 8.2). The homogenate was centrifuged at 10 000 g for 5 min. The supernatant was cold stored for determination.

Acetazolamide (acetazolamide, AZ), which did not go through the cell membrane, can inhibit CA activity [9]. Therefore, the extracellular CA was determined by adding AZ. The leaves were rinsed in AZ solution for two hours at 25℃, with light intensity of 200 μmol m^{-2} s^{-1}, the control experiment was deionied water instead of AZ. After that, the leaves were cleaned; the next steps were the same as above.

The conventional method of determination on CA activities is the electrometric one with glass electrode [3]. The samples were assayed at 0℃ by adding certain volume of the enzyme solution to 3 ml of 20 mmol L^{-1} barbital buffer, pH 8.2. The reaction was initiated by the addition of 2 ml ice-cold CO_2-saturated water. Units of activity were calculated with the equation t_0/t_1-1. The time (t_1) required for the drop in one unit pH from 8.2 to 7.2 was measured with glass electrode, while t_0 (the control) was the time required for the pH change (8.2-7.2) when buffer was substituted for the enzyme solution. Values are the means of three replicates.

Sb microelectrode, instead of the glass electrode, with a reference electrode was used to monitor the pH change during the whole course of the reaction by OCP (open-circuit potential, CHI660C, Shanghai Chenhua Instrument Company). The reaction system was the same as described above. The potential-t curves were saved. The data, where the potential changed uniform with time, were chosen to produce the linear equation. The slope of the linear equation can be obtained and recorded as k_1 referring to the reaction system with CA, so the value of t_1 was $41.7/k_1$ (41.7 is the sensitivity of microelectrode responding to the change of one pH unit, which is described in Characterization of the Sb microelectrode). Correspondingly, k_2 was the slope of the linear equation referring to the reaction system without CA, so the value of t_0 was $41.7/k_2$. Units of CA activity were also calculated with the equation t_0/t_1-1.

3 Results and Discussion

3.1 Characterization of the Sb Microelectrode

The microelectrode yielded a fast and stable response toward pH change of the test solution, with an average response time less than 1 s, and the measured pH range of 3-9. A good linear relationship was found in the calibration curves of the

potential response to the pH value of the solution (R^2=0.996, n=5, p<0.001). The average slope of the linear equation was 41.7 mV/pH, which showed that the present fabrication method for Sb microelectrodes had high sensitivity to pH change and good reproducibility.

The determination of CA activity was conducted under the reaction system of 0℃. Therefore, the effect of temperature on the reactivity was studied. The calibration curve of the microelectrodes' potential response to the pH value in the test solution under room temperature and 0℃ were shown in Fig.1. The temperature did not change the slopes of linear equations.

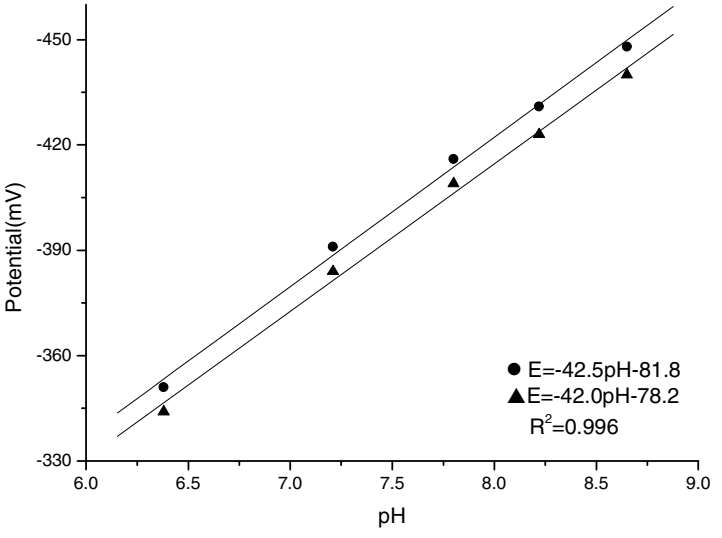

Fig. 1. Calibration curves of the potential *vs*. pH in barbital buffers responded by the Sb microelectrode at the room temperature and 0℃ (● 20℃, ▲ 0℃).

3.2 CA Activity of Standard Enzyme Solution *versus* Volume Curve

The CA activity of standard enzyme solution *versus* volume curve, measured with the glass electrode, was plotted as Fig.2. When CA activity was too low or too high, its value deviated from the curve. The pause duration of our human eyes, which saw a goal to get the visual impression, on the average took 172 ms [10]. Our vision may not catch the data on the pH meter owing to the timing by stopwatch when the CA activity was too high. In addition, when the CA activity was too low, the pH meter may not make an accurate record because of its sluggish response. However, the OCP method with microelectrode can overcome the above defects because of its continuous timing and fast transfer rate. The CA activity of standard enzyme solution *versus* volume curve, measured with Sb microelectrode, was plotted as Fig.3. A good linearity measured by the microelectrode can be seen from Fig.3.

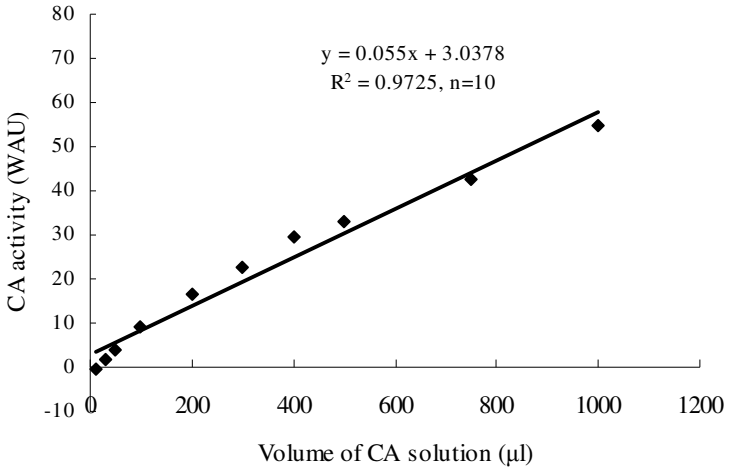

Fig. 2. The CA activity of standard enzyme solution *versus* volume curve measured with glass electrode in pure enzyme solution

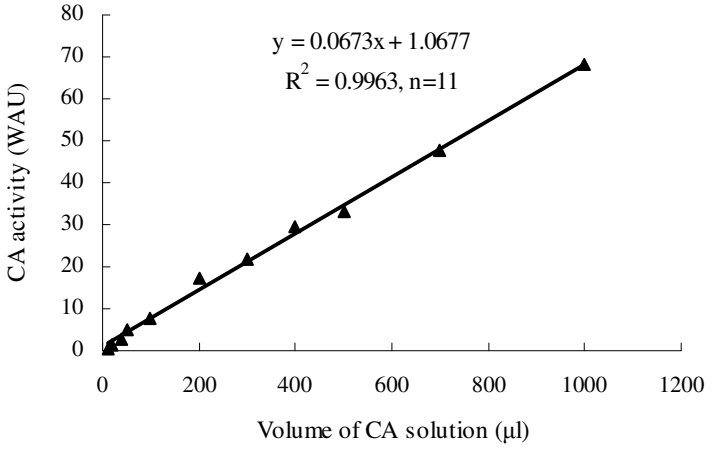

Fig. 3. The CA activity of standard enzyme solution *versus* volume curve measured with Sb microelectrode

3.3 Determination of Carbonic Anhydrase in the Leaves

Several different species of plants were chosen to determine their foliar CA activities. Table 1 gave the CA activities of *Morus alba*, *Ailanthus altissima*, *Brassica juncea* and *Parthenocissu tricuspidatas*, which were measured with the Sb microelectrode and the glass electrode, respectively. In addition, a series of volumes of 10 μl to 400 μl leaf extracts from *Morus alba* were also measured with the Sb microelectrode and the glass electrode, respectively. The CA activity in the leaves from *Parthenocissu tricuspidatas* or 10μl leaf extracts from *Morus alba* was too low to be measured with

the conventional method (glass electrode). The results of the two methods had no significant difference for the determination of the CA activities in the medium range. Therefore, the new method, the OCP approach coupled with Sb microelectrode, which measured the CA activity, had demonstrated the advantage for the determination of low CA activity.

Table 1. Comparison between the microelectrode and the conventional glass electrode in determining the CA relative activity (Unit: WAU) and the CA activity (Unit: WAU g^{-1}FW) in several plant leaves

Plant materials	Volume of leaves extracts (μl)	Sb microelectrode		Glass electrode	
		CA relative activity	CA activity	CA relative activity	CA activity
Morus alba	10	0.19	190	ND*	ND*
	30	0.36	120	0.32	107
	50	0.86	172	0.50	100
	100	1.06	106	1.87	187
	200	1.86	93	2.52	126
	300	3.37	112	3.06	102
	400	4.26	106	3.56	89
	Average ± standard error		127±15		119±15
Ailanthus altissima	Average ± SE		679±38		610±19
Brassica juncea	Average ±SE		490±9		547±22
Parthenocissus tricuspidata	Average ± SE		34.2±5		ND*

**ND stands for the CA activity not be determined*

At present, little information about the extracellular CA activity has been documented owing to the low accuracy of the conventional pH glass electrode. The results of the determination and calculation of extracellular CA by means of the new approach is highly credible. The activities of the extracellular CA in *Broussonetia papyrifera* and *Morus alba* leaves were determined by OCP approach coupled with Sb microelectrode and the conventional glass electrode, respectively; and the results were shown in Table 2. The variability of the extracellular CA activities, determined with the new method, was much smaller than that with the conventional method. A negative value was even observed in the results measured with the glass electrode. As a result, the OCP approach coupled with Sb microelectrode can be more successfully used for the determination of extracellular CA activities than the conventional method with glass electrode.

Table 2. Comparison between the microelectrode and the conventional glass electrode in determining the CA activity (Unit: WAU g^{-1}FW) and the extracellular CA activity (Unit: WAU g^{-1}FW) in *Broussonetia papyrifera* and *Morus alba* leaves

Determination	Plant materials	CA activity (without AZ)	CA activity (with AZ)	Extracellular CA activities and its proportion
Sb microelectrode	*Broussonetia papyrifera*	1478	919	559 (38%)
		1485	1163	322 (22%)
		1738	1140	598 (34%)
	Morus alba	150	111	49 (26%)
		212	138	74 (35%)
		159	108	51 (32%)
Conventional glass electrode	*Broussonetia papyrifera*	1041	854	187 (18%)
		1196	909	287 (24%)
		1586	983	603 (38%)
	Morus alba	187	136	51 (27%)
		143	152	-9 (-6%)
		132	108	24 (18%)

4 Conclusions

In summary, the electrochemical approach (open-circuit potential, OCP) coupled with a new calculation (the new method) has solved the long-standing deficiencies in the determination of CA activity by means of the electrometric method with the glass electrode (conventional method). Firstly, the new method obtains the time that is equivalent to one pH unit of potential changes in the reaction system through the statistics of multi-point data, so it is more credible compared with the conventional method to calculate two-point data responding to the change of a pH unit. Secondly, the new method is based on potential changes in the uniform part of the curve to calculate the CA activities; therefore, it is more precise compared with the conventional method. Thirdly, the reaction system of the new method took only little volume as the needle-like morphology of Sb microelectrode; therefore, it is possible to measure the foliar CA activity in a small range.

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