Original Paper

Determination of Two-Steps Acid Dissociation Constants of L-Ascorbic Acid by Capillary Zone Electrophoresis

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Abstract

Two-steps acid dissociation constants of L-ascorbic acid (AA) were determined through the changes in the effective electrophoretic mobility in capillary zone electrophoresis. Although ascorbic acid is oxidatively degradable in an aqueous solution, especially in alkaline conditions, the effective electrophoretic mobility of AA was successfully measured in the pH range between 1.87 and 11.97. In the analysis of the first-step acid dissociation constant (pK_{a1}) at weakly acidic pH conditions, a coated capillary with 1,3-propanesultone and a pressure-assist were utilized to detect anionic AA. In the analysis of the second-step acid dissociation constant (pK_{a2}) at weakly alkaline pH conditions, AA was successfully detected at the pH range up to 11.97 with the help of Cu catalyst to remove the dissolved oxygen in the separation buffer. Acid dissociation constants were independently determined as pK_{a1} = 4.15±0.01 and pK_{a2} = 12.07±0.04 by non-linear least-squares analyses. AA did not fully dissociate at the weakly alkaline pH range, and the effective electrophoretic mobility of the dianion form of AA was extrapolated for the analysis.

Keywords: L-Ascorbic acid; Acid dissociation constants; Capillary zone electrophoresis

1. Introduction

L-Ascorbic acid (AA), also known as vitamin C, exhibits antioxidant properties, and it is widely used in medicines, foods, and environmental field [1-2]. Investigations on redox reactions of AA are indispensable to understand its antioxidant properties [3]. Acid dissociation constants (pK_a) are closely related with redox reactions each other, and determining the pK_a values of AA experimentally and precisely is helpful for the advanced understanding of its redox properties. However, AA is degradable under heat [4], alkaline pH [5], UV light [6], O_2 [7], and O_2 with Cu(II) [8]. AA is generally oxidized and hydrolyzed to dehydroascorbic acid. The degradability of AA makes it difficult to determine the pK_a values of AA.

Acid dissociation constants have traditionally been determined by potentiometric and spectrophotometric titrations. However, traditional methods are principally operated in homogeneous solutions, and the methods are difficult to apply them to degradable or impure substances. Acid dissociation constants of AA have been determined by potentiometry [9,10] and spectrophotometric titrations [10,11]. However, the determined pK_a values would be questionable because of the degradability of AA.

Capillary zone electrophoresis (CZE) is a useful separation technique for ionic species. Effective electrophoretic mobility of a subject analyte, acids or bases, is based on the protonation/deprotonation degree of the analyte, and pK_a value(s) of an analyte can be analyzed through the measurements of the effective electrophoretic mobility at several pH conditions. Since CZE includes electrophoretic resolution, pK_a values of degradable substances have been determined through the measurements

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of the effective electrophoretic mobility of a target substance [12]. By using the separation characteristics of CZE, acid dissociation constants were successfully determined with alkaline-degradable phenolphthalein [13] and catecholamines [14], acid-degradable tetrabromophenolphthalein ethyl ester [15], pravastatin [16] and hexamethylenetetramine [17], labile drugs [18], photo-degradable haloperidol [19], heat-degradable bupropion [20] and hydrochlorothiazide [21].

In this study, separation characteristics of CZE have been applied to AA for the determination of its pK_a values. Inner wall of a capillary was coated with 1,3-propanesultone to give anionic charge and fast electroosmotic flow (EOF) at acidic pH conditions [22] for the determination of the first-step pK_a value of AA. The second-step pK_a value of AA has successfully been determined at weakly alkaline pH conditions, where AA is degradable.

2. Experimental

2.1. Reagents

AA and L-ascorbic acid 2-phosphate (AAP, trisodium salt) were purchased from Kanto Chemical (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Structures of AA and AAP are shown in Fig. 1. Good's buffer components were from Dojindo Laboratories (Kumamoto, Japan). 1,3-Propanesultone was from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analytical grade. Water used was purified by Milli-Q Gradient A10 (Merck Millipore Japan, Tokyo, Japan).

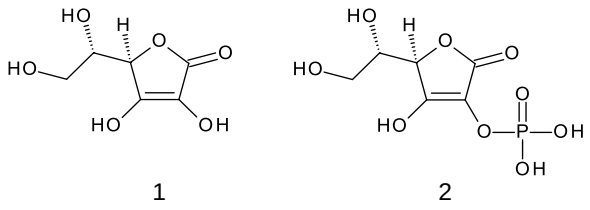

Fig. 1. Structures of AA (1) and AAP (2).

2.2. Apparatus

An Agilent Technologies (Waldbronn, Germany) ^{3D}CE was used as a CZE system, equipped with a photodiode array detector. A fused-silica capillary purchased from GL Sciences (Tokyo, Japan) was cut to a required length, and it was used as a separation capillary, after a detection window was made by burning a small portion of the polyimide coating. The capillary was held in a cassette cartridge, and the cartridge was installed in the CZE system. The dimensions of the capillary were 48.5 cm in the total length, 40 cm in the effective length from the injection end to the detection point, 75 μm inner diameter, and 375 μm outer diameter.

A Horiba (Kyoto, Japan) F-71 pH meter was used for the pH measurements of the separation buffers, after being calibrated daily with standard pH solutions.

2.3. Preparation of separation buffers at an identical ionic strength

PeakMaster (Ver. 5.3) [23-25] was utilized for the preparation of separation buffers. Compositions of the separation buffers used in this study are summarized in Table 1. The main buffer component was set at from 0.010 mol L^{-1} to 0.040 mol L^{-1}, and the pH was controlled with the amount of NaOH added. Ionic strength of the buffers was controlled at I = 0.015 mol L^{-1} by adding adequate amount of NaCl. Dissolved oxygen (DO) in the separation buffers of pH > 9 was substituted to N_2 by adding 50 ppm hydrazine and 1 ppm Cu^{2+} and standing for 6 h [26].

Table 1. Compositions of the separation buffers and the CZE voltages used in the pK_a determinations.

pH (Measured)	Buffer components	CZE voltage, kV	Electric power, W
1.86 – 2.91	H_3PO_4 − NaOH	7 − 11	0.2
3.16 – 4.01	HCOOH − NaOH	11	0.2
4.22 – 5.87	CH_3COOH − NaOH	11 − 12	0.2
9.31	CHES − NaOH	12	0.2
10.12 – 10.59	CAPS − NaOH	13	0.2
11.04 –11.80	H_3PO_4 − NaOH	10 − 13	0.2
11.97	NaOH	9	0.2

2.4. Preparation of the sample solutions

Stock solutions of AA and AAP were prepared at 10 mmol L^{-1}. Concentrations of AA and/or AAP in the sample solution were set at 5×10^{-4} mol L^{-1}. Dimethyl sulfoxide (DMSO) as an EOF marker and naphthalene-1-sulfonate (1-NS, sodium salt) as an internal standard of the electrophoretic mobility were added in the sample solution at the concentrations of 5 mmol L^{-1} and 5×10^{-5} mol L^{-1} on the CZE measurements at pH < 6. Similarly, DMSO as an EOF marker and methyl orange (MO) as an internal standard of the electrophoretic mobility were added in the sample solution at the concentrations of 5 mmol L^{-1} and 1×10^{-4} mol L^{-1} on the CZE measurements at pH > 9.

2.5. Preparation of a coated capillary

A coated capillary was prepared for the CZE measurement at the weakly acidic pH conditions to maintain fast EOF [22]. The inner wall of a fused silica capillary was activated by flushing 0.1 mol L^{-1} NaOH at 960 mbar for 10 min. Then a toluene solution containing 2 mol L^{-1} 1,3-propanesultone was filled in the capillary by flushing at 960 mbar. Both ends of the filled capillary was capped with silicone gum chip, and the capillary was held in a constant temperature oven thermostated at 100 °C for 24 h. After the reaction, the capillary was washed with a 20 mmol L^{-1} phosphoric acid solution (pH 2) by flushing at 960 mbar for 10 min.

2.6. Procedure for the CZE measurements

The coated capillary was used in the CZE measurements at weakly acidic pH conditions. When a separation buffer was changed with a different one, the capillary was equilibrated by flushing it with the new separation buffer for 10 min. After the separation capillary being filled and equilibrated with a separation buffer, a sample solution containing AA and AAP mentioned above was hydrodynamically injected into the capillary at 50 mbar for 3 s. A constant DC voltage, as well as 5 mbar hydrodynamic pressure, was applied to the capillary for the pressure-assisted CZE (pCZE). AA and AAP were photometrically detected at both 220 nm and 250 nm.

An uncoated capillary was used in the CZE measurements at weakly alkaline pH conditions. The capillary was also equilibrated with a new separation buffer as in the case of the coated capillary. After the separation capillary being filled and equilibrated with a separation buffer, a sample solution containing AA mentioned above was hydrodynamically injected into the capillary at 50 mbar for 1 s. A constant DC voltage was applied to the capillary for the CZE. AA was photometrically detected at 270 nm.

Effective electrophoretic mobility, μ_{eff}, of AA or AAP was calculated in an ordinary manner. The μ_{eff} values of AA or AAP were standardized with that of 1-NS or MO.

2.7. Determination of acid dissociation constants

AA is a diprotic acid, and two-steps acid dissociation equilibria are involved as in reactions (1) and (2) with their equilibrium constants (3) and (4), where H_2A is a protonated form of AA; HA^- and A^{2-} are monoanion and dianion of AA, respectively.

$$H_2A \overset{K_{a1}}{\rightleftharpoons} HA^- + H^+ \quad (1)$$

$$HA^- \overset{K_{a2}}{\rightleftharpoons} A^{2-} + H^+ \quad (2)$$

$$K_{a1} = \frac{[H^+]\,[HA^-]}{[H_2A]} \quad (3)$$

$$K_{a2} = \frac{[H^+]\,[A^{2-}]}{[HA^-]} \quad (4)$$

Since two pK_a values of AA are at weakly acidic and weakly alkaline pH regions, the two equilibria can be analyzed independently. The effective electrophoretic mobility of AA, $\mu_{eff,AA}$, at weakly acidic and weakly alkaline pH regions are written as in Eqs. (5) and (6), respectively.

$$\mu_{eff,AA} = \frac{[H^+]}{[H^+] + K_{a1}}\,\mu_{ep,H2A} + \frac{K_{a1}}{[H^+] + K_{a1}}\,\mu_{ep,HA} \quad (5)$$

$$\mu_{eff,AA} = \frac{[H^+]}{[H^+] + K_{a2}}\,\mu_{ep,HA} + \frac{K_{a2}}{[H^+] + K_{a2}}\,\mu_{ep,A} \quad (6)$$

where $\mu_{ep,H2A}$, $\mu_{ep,HA}$, and $\mu_{ep,A}$ are the electrophoretic mobility of H_2A, HA^-, and A^{2-} species, respectively. A series of pairs of pH of the separation buffer and the $\mu_{eff,AA}$ value were input in Eq. (5) or (6), and values of the electrophoretic mobility and the acid dissociation constant were optimized by a non-linear least-squares analysis [12]. A software of R program (Ver. 3.6.2) [27] was used for the analysis.

3. Results and discussion

3.1. Determination of the first-step acid dissociation constants of AA at weakly acidic pH conditions

Slow EOF at weakly acidic pH region makes it difficult to detect the anionic analyte of AA, and a fast detection of AA was aimed by using a coated capillary and a pressure-assist. A coated capillary was prepared with 1,3-propanesultone [22] as mentioned above; the speed of the EOF was developed by the coating. Advantages on using 1,3-propanesultone as a coating reagent are: (1) the reaction is of one-step, (2) photometric absorption over the wavelengths greater than 220 nm is little, and (3) the coating layer would not interact with the analyte AA. Pressure-assist is also helpful for the determination of acid dissociation constants [28-30], and it was also utilized together with the coated capillary. However, high assist pressure (> 50 mbar) may change the effective electrophoretic mobility and induce a serious peak broadening [31], and an assist pressure was set at 5 mbar in this study.

The first-step acid dissociation constant of AA was determined through the measurements of its effective electrophoretic mobility at weakly acidic pH region. In this series of the measurements, a second-step acid dissociation equilibrium of AAP has also been examined, as is similarly written in equilibrium (2) with its acid dissociation constant (4). Typical electropherograms are shown in Fig. 2. The photometric detection was made at two wavelengths at 220 nm and 250 nm to detect both AA and AAP as well as DMSO as an EOF marker. The speed of EOF was sufficiently fast with the coated capillary and by the pressure assist at acidic pH conditions as low as pH = 1.86. It can be seen from Fig. 2 that AA and AAP are detected as regular peaks in this pH region without degradation. The effective electrophoretic mobility of AA and AAP are standardized with that of 1-NS, and the results are shown in Fig. 3. In the analysis of the pK_{a1} value of AA, the charge of

protonated AA, H_2A, is zero, and a value of $\mu_{ep,H2A}$ has been set at zero. Changes in the effective electrophoretic mobility were analyzed by the analysis, and the pK_a values, as well as the standardized electrophoretic mobility, were obtained; the results are summarized in Table 2. A pK_{a2} value of AAP determined in this study is close to a reported value of 3.59 [32].

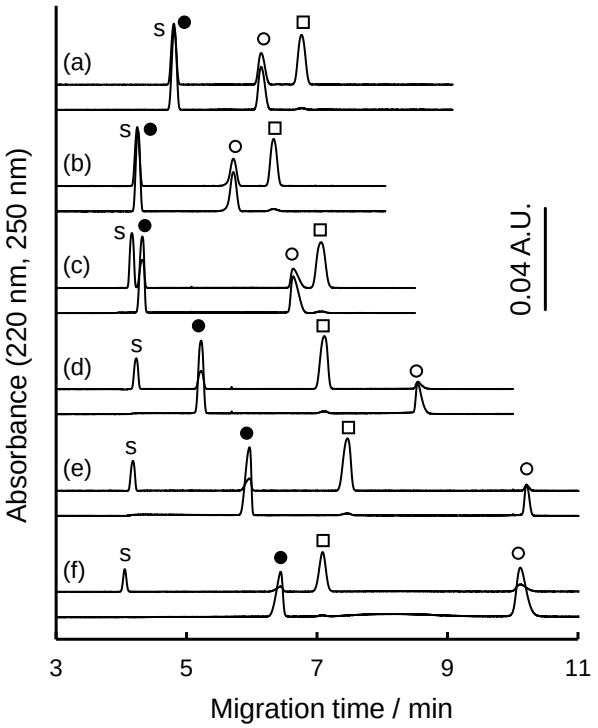

Fig. 2. Typical electropherograms of AA and AAP at weakly acidic pH conditions. pH of the separation buffer: (a) 1.86; (b), 2.33; (c), 3.16; (d), 4.22; (e), 4.74; (f), 5.87. Symbols: ○, AAP; ●, AA; □, 1-NS. S, solvent (EOF). Detection wavelength: upper, 220 nm; lower, 250 nm. Other pCZE conditions are written in the text.

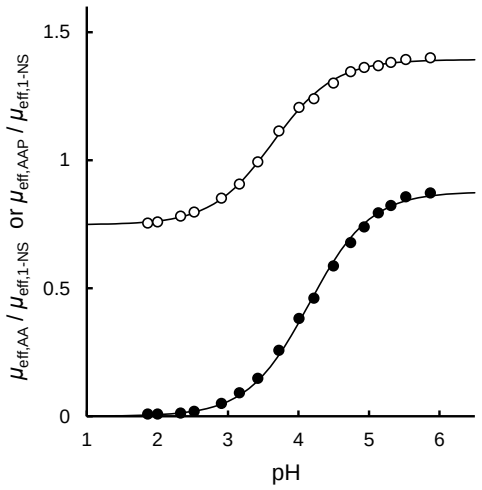

Fig. 3. Changes in the effective electrophoretic mobility of AA and AAP at weakly acidic pH conditions. The μ_{eff} values are standardized with 1-NS as an internal standard. Symbols: ○, AAP; ●, AA. pCZE conditions are the same as in Fig. 2.

Table 2. Acid dissociation constant of AA and AAP determined by pCZE.

Substance	pK_{a1} [a)]	pK_{a2} [a)]	$\mu_{ep,HA}$ [a) b)]	$\mu_{ep,A}$ [a) b)]
AA	4.15±0.01	–	0.88±0.01	–
AAP	–	3.64±0.02	0.75±0.01	1.39±0.01

a) Error: standard errors. b) Standardized values with 1-NS as an internal standard.

3.2. Determination of the second-step acid dissociation constant of AA at weakly alkaline pH conditions

The second-step acid dissociation constant of AA was examined by using ordinary uncoated silica capillary. Since AA is degradable in an alkaline aqueous solution in the presence of O_2 [5,7,8], and therefore, DO was replaced with N_2 [26]. In a series of the CZE measurements, an internal standard of MO was used instead of 1-NS, because the migration time of 1-NS overlapped with that of AA. MO is stably monoanionic at the weakly alkaline pH region. Electropherograms of AA at the alkaline pH regions are shown in Fig. 4. When the DO was not treated, or the separation buffers were bubbled with argon gas, AA was not detected at pH > 11 because of the degradation; a broad plateau signal attributed to the degradant(s) was detected instead. However, AA was successfully detected as a peak signal even at pH 11.97 by treating the DO with the Cu^{2+} catalyst. Although the peak height of AA decreased with increasing pH of the separation buffer, the peak area of AA

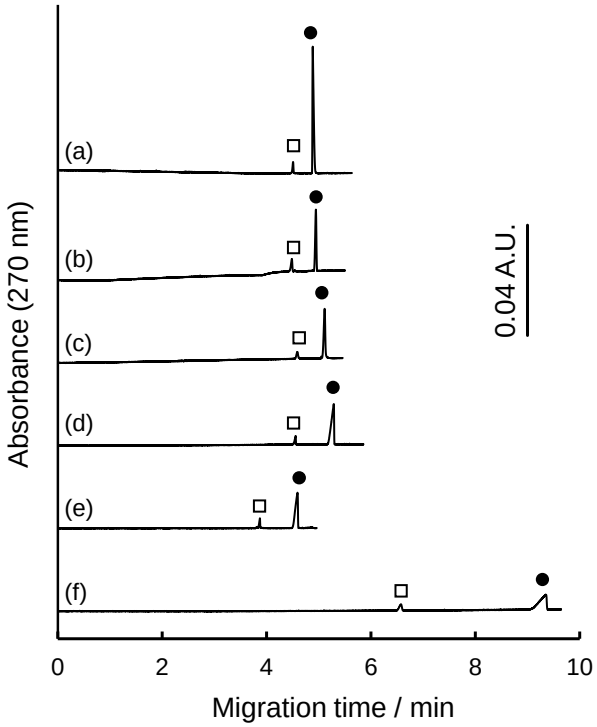

Fig. 4. Typical electropherograms of AA at weakly alkaline pH conditions. pH of the separation buffer: (a) 9.31; (b), 10.12; (c), 10.59; (d), 11.04; (e), 11.47; (f), 11.97. Symbols: ●, AA; □, MO. CZE conditions are written in the text. The EOF was simultaneously monitored at 220 nm with DMSO.

changed little, and the degradation of AA would be little in this pH range examined. The peak broadening or the fronting would be attributed to the continuous generation of the degradant during the electrophoretic migration. Anyway, the CZE signal of AA can be detected in the pH range examined, and we can analyze the second-step acid dissociation constant through the results.

Changes in the effective electrophoretic mobility of AA at weakly alkaline pH conditions are shown in Fig. 5. The standardized value of $\mu_{eff,AA}$ increased with increasing pH, suggesting that AA is getting more anionic at this pH range. Direct measurement of the $\mu_{ep,AA}$ was difficult at pH > 12 because of the increased ionic strength. Therefore, the value of $\mu_{ep,A}$ was estimated from the view point of the charge of AA. As is examined with AAP, the electrophoretic mobility of dianion form, A^{2-}, is 1.86 times to that of the monoanion form, HA^{-}. The result agrees with the fact that the electrophoretic mobility of ions is proportional to the charge. Therefore, the electrophoretic mobility of dianion form of AA is assumed to be twice to that of monoanion form, as examined with phenolphthalein [13] and catecholamines [14]. A simulated curve is drawn in Fig. 5 with the assumption, and a pK_{a2} value of 12.07±0.04 was determined. When a value of $\mu_{ep,A}$ was assumed to be 1.86 times to that of $\mu_{ep,HA}$, a pK_{a2} value of 11.96±0.05 was obtained; the two values are close with each other.

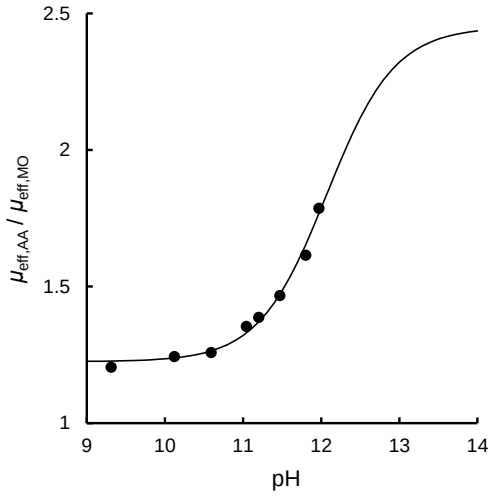

Fig. 5. Changes in the effective electrophoretic mobility of AA at weakly alkaline pH conditions. The $\mu_{eff,AA}$ values are standardized with MO as an internal standard. A simulated curve is drawn with $\mu_{ep,A}$ to be twice to $\mu_{ep,HA}$. CZE conditions are the same as in Fig. 4.

The pK_a values determined in this study are compared with some reported values; they are summarized in Table 3. The pK_{a1} vales agreed well with the reported values. AA does not degrade at the weakly acidic pH region, and the pK_{a1} value would precisely be determined by any determination methods. On the other hand, the pK_{a2} vale determined in this study is somewhat different from the reported values. Even though DO is removed by the N_2 bubbling [9], degradation of AA would be unavoidable, and the degraded species would interfere with the measurements in potentiometric and spectrophotometric titrations. CZE resolves the degraded species, and the equilibrium analysis of acid dissociation constant would be more precise than the traditional titrations.

Table 3. Acid dissociation constants of AA.

Method	pK_{a1}	pK_{a2}	Reference
pCZE	4.15±0.01 [a)]	–	This study
CZE	–	12.07±0.04 [a) b)]	This study
Potentiometry	4.14	11.43	[9]
Potentiometry	4.13	(None)	[10]
Spectrophotometry	4.13	(None)	[10]
Spectrophotometry	4.164	11.731	[11]

a) Values at I = 0.015 mol L^{-1}. Error: standard errors. b) Determined by an extrapolation assuming that the value of $\mu_{ep,A}$ is twice to that of $\mu_{ep,HA}$.

4. Conclusions

Two-steps acid dissociation constants of alkaline degradable AA were successfully determined by CZE. Pressure assist and a coating capillary was utilized in the measurement of the effective electrophoretic mobility of AA at weakly acidic pH region. Alkaline-degradable AA was also detected successfully at weakly alkaline pH region up to pH ≈ 12 with the help of DO removal. Since CZE resolves the degraded species from the equilibrium species of interest, and the pK_a values would be determined precisely in this study.

Conflict of interests

There is no conflict of interest.

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