SOLUBILITY OF *N*-(BENZYLOXYCARBONYL)-L-ASPARTYL-L-PHENYLALANINE METHYL ESTER FORMING A COMPLEX WITH L-PHENYLALANINE METHYL ESTER IN AQUEOUS SYSTEM

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The solubility of *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-APM) was studied in an aqueous system with coexisting L-phenylalanine methyl ester (L-Phe-OMe). It was found that the Z-APM solubility shows very complicated behavior. Under acidic pH condition (pH < 3.5), pure Z-APM was precipitated and the solubility increased uniformly with increasing pH and L-Phe-OMe concentration. Under moderate pH condition (4.5 < pH < 8), Z-APM was precipitated with L-Phe-OMe, forming a complex of Z-APM·L-Phe-OMe. The solubility varied with the pH following a U-shaped curve and decreased with increasing L-Phe-OMe concentration at the same pH. The pH value at the transient point from pure Z-APM precipitation to Z-APM·L-Phe-OMe precipitation shifted toward acidic pH with increasing L-Phe-OMe concentration. From the experimental results it was assumed that the Z-APM·L-Phe-OMe complex was not only formed by ionic bond. An equilibrium model of the precipitation considering the formation of complex was proposed that well explains the complicated behavior of the solubility.

Introduction

The chemical equilibrium of an enzymatic synthesis of peptide in aqueous system is generally shifted in the unfavorable direction because the synthesis is accompanied by dehydration. To improve the equilibrium, simultaneous operation of enzymatic synthesis and product separation has been recently given much attention[3, 9, 18]. In the operation, the peptide produced in the reaction system is kept at low concentration by simultaneous separation, and the peptide can be synthesized in high yield.

The authors have studied the enzymatic synthesis of an aspartame precursor as an objective peptide, combined with precipitation and/or liquid extraction for simultaneous product separation[4-7]. The aspartame precursor, *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (abbreviated as Z-APM), is a precursor of the synthetic sweetener which has been produced commercially by enzymatic reaction as well as chemical reaction[8]. Z-APM can be enzymatically synthesized in an aqueous system from *N*-(benzyloxycarbonyl)-L-aspartic acid (abbreviated as Z-L-Asp) and L-phenylalanine methyl ester (abbreviated as L-Phe-OMe), forming a slightly soluble complex with Phe-OMe. In the case of the racemic Phe-OMe used, the product is obtained as a complex with the D-isomer of Phe-OMe[15-17]. Since the complex formation is useful for the optical resolution of D- and L-isomers, whether it may affect the yield and the productivity of Z-APM is a very important point to confirm. Harano, Ooshima *et al.*[1, 2] studied the synthesis of Z-APM accompanied by crystallization of the complex and found that the complex is comparatively hard to be nucleated and grown. Nakanishi, Matsuno *et al.*[14] simulated the time-course of Z-APM synthesis accompanied by liquid extraction in a biphasic system based on the data of partition in one-component systems, and considered that one possible reason for the deviation of the experimental results from the theoretical results at pH 6 is the partitioning of the complex into the organic phase.

In this study, first the Z-APM solubility was measured in an aqueous system at various pH values and L-Phe-OMe concentrations, and an equilibrium model was proposed to explain the solubility behavior, since the mechanism of this complex formation has been little studied. From these results, the complex formation of Z-APM was studied.

1. Experimental

1.1 Chemicals

L-Phe-OMe hydrochloride salt (abbreviated as L-Phe-OMe·HCl) was purchased from Kokusan Chemical Works. Z-APM was enzymatically synthesized from sodium *N*-(benzyloxycarbonyl)-L-aspartate and racemic Phe-OMe·HCl by thermolysin. The substrates and the enzyme were kindly supplied by Tosoh Co. Ltd. and Daiwa Kasei Co. Ltd. respectively. The Z-APM was

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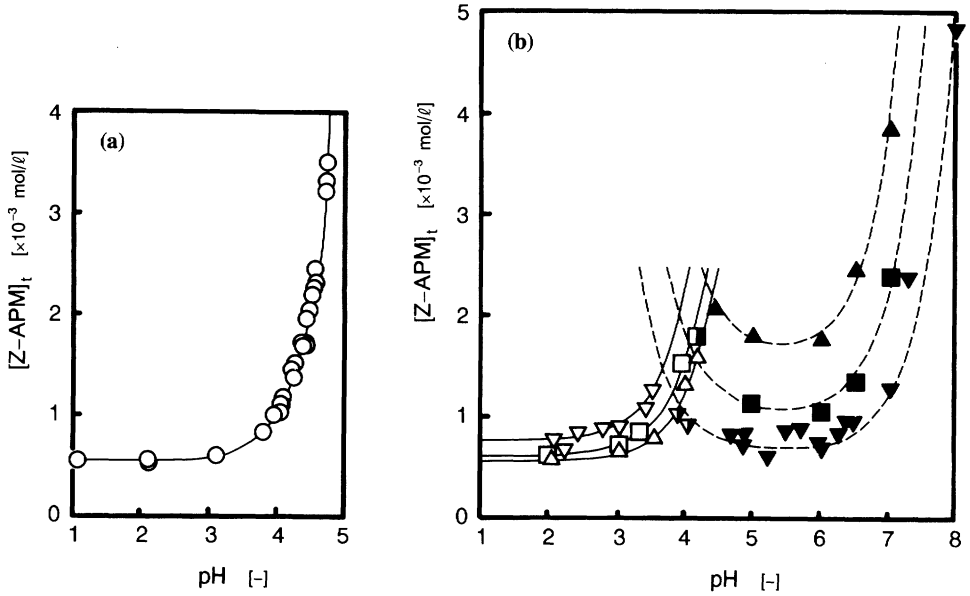

Fig. 1 Effects of pH on solubility of Z-APM at fixed L-Phe-OMe concentrations: (a), 0 mol/l (○); (b), 0.04 mol/l (△, ▲), 0.08 mol/l (□, ◨, ■) and 0.2 mol/l (▽, ▼, ▼). Compositions of the precipitation: pure Z-APM (open key), Z-APM : L-Phe-OMe = 1 : 1 (closed key) and the others (semi-solid key).

purified by repeated washings with 1N HCl, extraction into ethyl acetate and recrystallization from methanol. The other chemicals used were of analytical grades from Kokusan Chemical Works.

1.2 Chemical analyses

To measure the concentrations of Z-APM and L-Phe-OMe, HPLC systems, Tosoh UV-8000 detectors and CCPD solvent delivery systems were used. To measure the Z-APM concentration, a column of TSKgel ODS-80TM (Tosoh; 6.0 mm i.d. × 150 mm) and the eluent acetonitrile/0.12 mol/*l* sodium acetate solution (5:5, v/v; pH 4.8; 1 ml/min) were used, and Z-APM was detected at 258 nm. For L-Phe-OMe a column of TSKgel G2000SW (Tosoh; 7.5 mm i.d. × 300 mm) and the eluent acetonitrile/0.3 mol/*l* sodium acetate solution (7:3, v/v; pH 5.68; 1 ml/min) were used, and L-Phe-OMe was detected at 260 nm.

1.3 Measurement of Z-APM solubility

Z-APM was added to ion-exchanged water in slight excess of the solubility, and an appropriate amount of L-Phe-OMe·HCl (0-0.2 mol/*l*) was dissolved in the solution. After pH was adjusted to a settled value, the total ionic strength was adjusted to 0.1 by addition of NaCl. The mixture was stirred at 40 °C for 60 min. After the mixture was centrifuged at 40 °C, the concentrations of Z-APM and L-Phe-OMe in the supernatant were measured. To confirm that the mixture was in solid-liquid equilibrium, the same sample was stirred for an additional 30 min and the concentrations were measured in a similar manner.

1.4 Measurement of the composition of precipitate

The precipitate was dissolved in methanol after the mother liquor was completely removed by filter paper. The concentrations of Z-APM and L-Phe-OMe were determined.

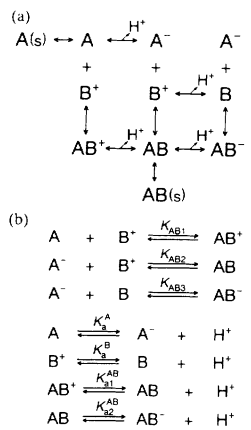

Fig. 2 Equilibrium model of precipitation of Z-APM considering the formation of the complex with L-Phe-OMe

2. Results and Discussion

2.1 Z-APM solubility

Figure 1 (a) and (b) show the effects of pH on the solubility of Z-APM in ion-exchanged water at fixed L-Phe-OMe concentrations of 0, 0.04, 0.08 and 0.2 mol/*l*. The behavior of Z-APM solubility in the aqueous system with coexisting L-Phe-OMe was very complicated, as shown in Fig. 1 (b). From Fig. 1 (a) and (b), three points are notable:

(i) Under acidic pH condition (pH < 3.5), pure Z-APM was precipitated and the solubility increased uniformly with increasing pH and L-Phe-OMe concentration.

(ii) Under moderate pH condition (4.5 < pH < 8),

Table 1. Values of acidic dissociation constants, formation constants and saturated concentrations of nonionized components (40°C)

K_a^A	8.93×10^{-5}	mol/l *1)
K_a^B	2.20×10^{-7}	mol/l *1)
K_{a1}^{AB}	4.57×10^{-4}	mol/l
K_{a2}^{AB}	3.01×10^{-10}	mol/l
K_{AB1}	2.17	l/mol
K_{AB2}	11.1	l/mol
K_{AB3}	1.52×10^{-2}	l/mol
$[A]_{sat}$	5.41×10^{-4}	mol/l
$[AB]_{sat}$	5.29×10^{-4}	mol/l

*1) Obtained by titration[10]

Z-APM was precipitated with L-Phe-OMe (Z-APM : L-Phe-OMe = 1:1) to form a complex of Z-APM·L-Phe-OMe, and the solubility varied with pH following a U-shaped curve and decreased with increasing L-Phe-OMe concentration at a given pH.

(iii) The pH value at the transient point from pure Z-APM precipitation to Z-APM·L-Phe-OMe precipitation shifted toward acidic pH with increasing L-Phe-OMe concentration.

To explain this complicated behavior, an equilibrium model was proposed as follows.

2.2 Equilibrium model

The proposed equilibrium model of the precipitation of Z-APM considering the formation of the complex with L-Phe-OMe was generalized schematically as shown in **Fig. 2** (a), and the complex formation constants and dissociation constants are defined as shown in Fig. 2 (b). In Fig. 2 (a) and (b), A means Z-APM, B, L-Phe-OMe and AB, Z-APM·L-Phe-OMe complex. Superscript + means protonated form and –, deprotonated form, and no superscript means nonionized form. Parenthesized s means solid (precipitate). In the model, it was assumed that only the nonionized forms of Z-APM and Z-APM·L-Phe-OMe are saturated and precipitated. Further, the complex of A^- with B^+ is considered to take two forms: salt or not salt; but in the model, it was assumed that all of the complex of A^- with B^+ is a salt and is bonded by an ionic bond in equilibrium.

The concentrations of Z-APM and L-Phe-OMe determined by the HPLC included all the forms (pure or complex, and ionized or nonionized) of Z-APM and L-Phe-OMe, respectively. Thus the Z-APM and L-Phe-OMe concentrations measured were represented as the total concentrations, $[A]_t$ and $[B]_t$, respectively. The solubility of Z-APM, $[A]_t$, was correlated with pH and $[B]_t$ as shown in Appendices 1 and 2.

For the precipitation of pure Z-APM under acidic pH conditions, we obtain

$$[A]_t = l[A]_{sat} + [B]_t / \{1 + m/(nK_{AB1}[A]_{sat})\} \quad (1)$$

For the precipitation of Z-APM·L-Phe-OMe under moderate pH conditions, we obtain

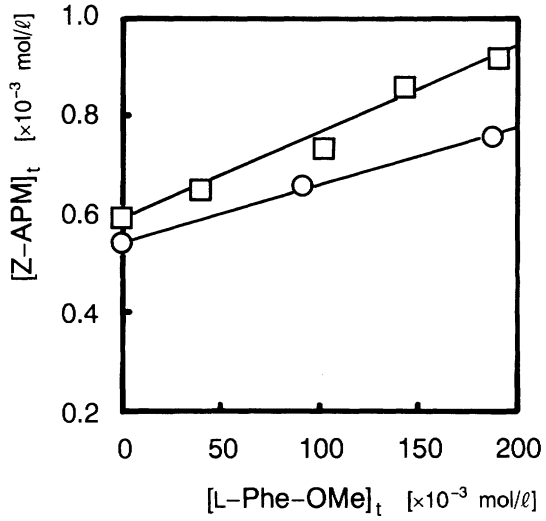

Fig. 3 Effect of L-Phe-OMe concentration on solubility of Z-APM at pH 1 (○) and pH 3 (□)

$$[A]_t = [AB]_{sat}\left\{p + \frac{mo}{K_{AB2}([B]_t - p[AB]_{sat})}\right\} \quad (2)$$

where

$$l = 1 + K_a^A/[H^+]$$
$$m = 1 + K_a^B/[H^+]$$
$$n = 1 + (1 + K_{a2}^{AB}/[H^+])K_{a1}^{AB}/[H^+]$$
$$o = 1 + [H^+]/K_a^A$$
$$p = 1 + [H^+]/K_{a1}^{AB} + K_{a2}^{AB}/[H^+]$$

When Z-APM is precipitated, [A] is constant at the saturated solubility of A, expressed as $[A]_{sat}$. When Z-APM·L-Phe-OMe is precipitated, [AB] is constant at the saturated solubilities of AB, expressed as $[AB]_{sat}$.

2.3 Analysis of the solubility of Z-APM

1) The precipitation of pure Z-APM Equation (1) was first simplified by omission of a term that can be considered to be negligibly small in each condition and the unknown parameters in Eq. (1) were estimated.

In the absence of L-Phe-OMe, the second term of Eq. (1) is zero, because $[B]_t$ is zero and $[A] = [A]_{sat}$ is a constant.

$$[A]_t = l[A]_{sat} \quad (3)$$

The saturated concentration of the nonionized form of Z-APM, $[A]_{sat}$, was estimated as shown in **Table 1** by a curve-fitting method on the data shown in Fig. 1 (a) using the least-square method, where the values of K_a^A and K_a^B were obtained as shown in Table 1 by the titration method[10]. In Fig. 1 (a), the keys are the experimental results and the solid line shows the calculated values, which well express the experimental values.

In the presence of L-Phe-OMe, at extremely low pH, pH ≦ 1, the value of $[H^+]$ is very large. Then $l = m = n \approx 1$ and $[A^-] = [B] = [AB] = [AB^-] \approx 0$, as seen from the definition of acidic dissociation constants shown in Fig. 2 (b). From these considerations, Eq. (1) is simpli-

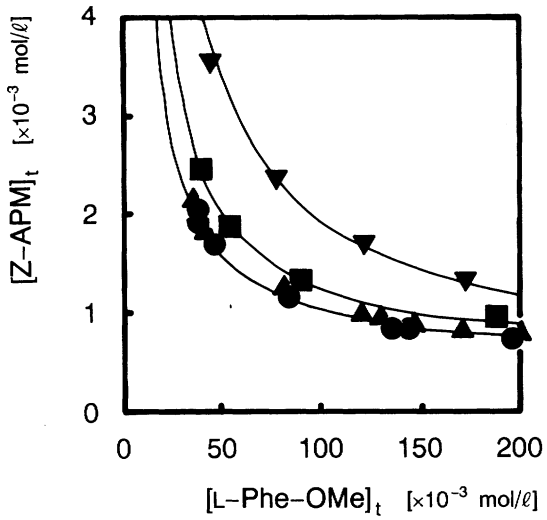

Fig. 4 Effect of L-Phe-OMe concentration on solubility of Z-APM at pH 5 (●), pH 6 (▲), pH 6.5 (■) and pH 7 (▼)

fied to Eq. (4) at extremely low pH.

$$[A]_t = [A]_{sat} + [B]_t / \{1 + 1/(K_{AB1}[A]_{sat})\} \quad (4)$$

Equation (4) means that $[A]_t$ is in proportion with $[B]_t$ and that the intercept of the straight line of $[A]_t$ vs. $[B]_t$ represents $[A]_{sat}$. The value of K_{AB1} can be obtained from the slope of the straight line. **Figure 3** shows the effects of L-Phe-OMe concentration on the solubility of Z-APM at pH 1 and 3. The keys show the experimental results. Equation (4) well expresses the experimental results at pH 1. In particular, the value of $[A]_{sat}$ determined for the intercept is the same as that obtained from Eq. (3) and Fig. 1 (a). The value of K_{AB1} was then obtained from the slope of the solid straight line as shown in Table 1.

At pH 3, it can be noticed that $[A]_t$ changes linearly with $[B]_t$, similarly as at pH 1, but both values of the intercept and the slope are a little larger than that at pH 1. This suggests that $[A^-]$ and [AB] should be included as the working species at pH 3. [B] and $[AB^-]$ are still negligibly small. From these considerations, Eq. (5) can be proposed in the case of pH 3.

$$[A]_t = l[A]_{sat} + [B]_t / \{1 + 1/(qK_{AB1}[A]_{sat})\} \quad (5)$$

where

$$q = 1 + K_{a1}^{AB}/[H^+]$$

The obtained values of K_a^A and $[A]_{sat}$ can be confirmed by comparison of the first term of Eq. (5) with the value of the intercept of the solid straight line at pH 3 in Fig. 3. The value of K_{a1}^{AB} was obtained from the slope of the line as shown in Table 1.

From the obtained values of K_a^A, K_{AB1} and K_{a1}^{AB}, the value of K_{AB2} can be easily obtained by Eq. (6) as shown in Table 1.

$$K_{AB2} = K_{AB1} K_{a1}^{AB} / K_a^A \quad (6)$$

2) The precipitation of Z-APM·L-Phe-OMe **Figure 4**

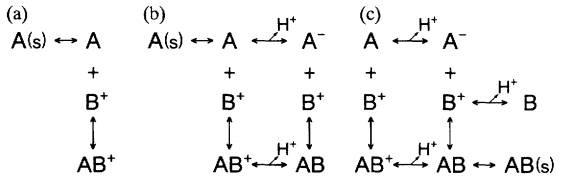

Fig. 5 Simplified equilibrium models of precipitation of Z-APM in various pH-ranges: (a), extremely low pH (pH ≦ 1); (b), acidic pH (2 < pH < 3.5); (c), moderate pH (4.5 < pH < 8)

shows the effects of L-Phe-OMe concentration on Z-APM solubility under the moderate conditions of pH 5, 6, 6.5 and 7, where Z-APM·L-Phe-OMe was precipitated as mentioned in 2.1. The remaining parameters, $[AB]_{sat}$ and K_{a2}^{AB}, were evaluated as shown in Table 1 by fitting Eq. (2) to the experimental results shown in Fig. 4 by the least-square method. K_{AB3} is obtained from the following equation.

$$K_{AB3} = K_{AB2} K_{a2}^{AB} / K_a^B \quad (7)$$

The obtained value of the formation constant of AB^-, K_{AB3}, was very small ($< 2 \times 10^{-2}$) compared to the formation constants of AB^+ and AB, K_{AB1} and K_{AB2}. Thus the formation of AB^- was regarded as likely to be negligible under the moderate pH conditions.

The calculated curves from Eqs. (1) and (2) with the values shown in Table 1 are shown in Figs. 1 (a), (b) and 4 by the solid and dotted lines respectively. As shown in the figures, the complicated behavior mentioned above can be explained very well by the equilibrium model proposed in Fig. 2, and it can be considered that the solubility of Z-APM in the aqueous system coexisting L-Phe-OMe can be predicted by the equations in the range of pH less than 8.

3) Transition from pure Z-APM precipitation to Z-APM·L-Phe-OMe The locus of the transient point from pure Z-APM precipitation to Z-APM·L-Phe-OMe is obtained by assuming $[A] = [A]_{sat}$, $[AB] = [AB]_{sat}$ and $[B] = [AB^-] \approx 0$ as shown by Eqs. (8) and (9).

$$[A]_t = [A]_{sat}(1 + K_a^A/[H^+]) + [AB]_{sat}(1 + [H^+]/K_{a1}^{AB}) \quad (8)$$

$$[B]_t = [AB]_{sat}\left\{1 + \frac{[H^+]}{K_{a1}^{AB}}\left(1 + \frac{1}{[A]_{sat}K_{AB1}}\right)\right\} \quad (9)$$

The experimental results for the transition can be explained by Eq. (9). That is, the transient point is shifted toward acidic pH with increasing L-Phe-OMe concentration.

4) Simplified equilibrium model The equilibrium model proposed in Fig. 1 (a) can be simplified in various pH ranges from the above discussion as shown in **Fig. 5** (a), (b) and (c). Z-APM is usually enzymatically synthesized from Z-L-Asp and L-Phe-OMe around pH 6. At that pH value, 65 % Z-APM is calculated to exist in the complex with L-Phe-OMe in the synthesis accompanied by precipitation at an L-Phe-OMe concentration of 0.2 mol/*l*.

This suggests that not only the product separation but also the enzymatic reaction may be affected by the complex formation.

Conclusion

Z-APM solubility was measured in an aqueous system at various pH values and L-Phe-OMe concentrations. The behavior of Z-APM solubility was complicated. Under acidic pH condition (pH < 3.5), pure Z-APM was precipitated and the solubility increased uniformly with increasing pH and L-Phe-OMe concentration. Under moderate pH condition (4.5 < pH < 8), Z-APM was precipitated as a complex with L-Phe-OMe (Z-APM·L-Phe-OMe) and the solubility varied with pH, following a U-shaped curve and decreasing with increasing L-Phe-OMe concentration at the same pH. The pH value at the transient point from pure Z-APM precipitation to Z-APM·L-Phe-OMe precipitation shifted toward acidic pH with increasing L-Phe-OMe concentration. The proposed equilibrium model well explained the complicated behavior of the solubility and can be effectively applied to the simultaneous operation of enzymatic synthesis and product separation.

Appendix 1

(1) Formation constants are defined as

$$K_{AB1} \equiv [AB^+] / ([A][B^+]) \quad \text{(A-1)}$$

$$K_{AB2} \equiv [AB] / ([A^-][B^+]) \quad \text{(A-2)}$$

$$K_{AB3} \equiv [AB^-] / ([A^-][B]) \quad \text{(A-3)}$$

(2) Acidic dissociation constants are defined as

$$K_a^A \equiv [A^-] / [H^+][A] \quad \text{(A-4)}$$

$$K_a^B \equiv [B][H^+] / [B^+] \quad \text{(A-5)}$$

$$K_{a1}^{AB} \equiv [AB][H^+] / [AB^+] \quad \text{(A-6)}$$

$$K_{a2}^{AB} \equiv [AB^-][H^+] / [AB] \quad \text{(A-7)}$$

(3) Material balance equations are as follows:

$$[AB]_t = [AB^+] + [AB] + [AB^-] \quad \text{(A-8)}$$

$$[A]_t = [A] + [A^-] + [AB]_t \quad \text{(A-9)}$$

$$[B]_t = [B^+] + [B] + [AB]_t \quad \text{(A-10)}$$

Appendix 2

• Derivation of Eq. (1)

From Eqs. (A-6), (A-7) and (A-8)

$$[AB]_t = n[AB^+] \quad \text{(A-11)}$$

From Eq. (A-4)

$$[A^-] = [A]_{sat} K_a^A / [H^+] \quad \text{(A-12)}$$

Substituting Eq. (A-12) in Eq. (A-9)

$$[A]_t = l[A]_{sat} + [AB]_t \quad \text{(A-13)}$$

From Eqs. (A-5) and (A-10)

$$[B]_t = m[B^+] + [AB]_t \quad \text{(A-14)}$$

where, from Eqs. (A-1), (A-11) and (A-14)

$$[B]_t = [AB]_t \{1 + m / (n[A] K_{AB1})\} \quad \text{(A-15)}$$

From Eqs. (A-13) and (A-15), Eq. (1) can be obtained.

• Derivation of Eq. (2)

From Eqs. (A-2), (A-4) and (A-9)

$$[A]_t = o[A^-] + [AB]_t$$
$$= o[AB]_{sat} / ([B^+] K_{AB2}) + [AB]_t \quad \text{(A-16)}$$

From Eqs. (A-5) and (A-10)

$$[B]_t = m[B^+] + [AB]_t \quad \text{(A-17)}$$

From Eqs. (A-6), (A-7) and (A-8)

$$[AB]_t = p[AB]_{sat} \quad \text{(A-18)}$$

From Eqs. (A-16), (A-17) and (A-18), Eq. (2) can be obtained.

Nomenclature

K_i	= formation constant	[l/mol]
K_a^j, K_{a1}^j, K_{a2}^j	= acidic dissociation constant	[mol/l]

<Subscripts>

i	= component (AB1 = AB^+, AB2 = AB, AB3 = AB^-)
sat	= saturated
t	= total

<Superscript>

j	= component

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