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Mechanism of Mutual Incorporation of L-Isoleucine and Isomorphic Amino Acids in Batch Crystallization

Toshimichi Kamei,*,† Kazuhiro Hasegawa,† Tatsuki Kashiwagi,‡ Eiichiro Suzuki,‡ Masaaki Yokota,§ Norihito Doki,§ and Kenji Shimizu§

Isolation and Purification Technology Laboratory, Fermentation and Biotechnology Laboratories, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi, 210-8681 Japan, Institute of Life Sciences, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi, 210-8681 Japan, and Department of Chemical Engineering, Iwate University, 4-3-5 Ueda, Morioka, 020-8551 Japan

Abstract:

We explored the mutual incorporation tendency of L-isoleucine and isomorphic amino acids in cooling crystallization conducted in water. In most cases, a guest amino acid (an impurity amino acid) with side chain longer than that of L-isoleucine was easily incorporated in L-isoleucine. In this case, a solid solution was formed and the *c*-axis of L-isoleucine structure was stretched. Furthermore, the crystal growth was inhibited. Using these results, a mechanism for mutual incorporation of these amino acids was proposed.

1. Introduction

Collectively, L-leucine (L-Leu), L-isoleucine (L-Ile), and L-valine (L-Val) are known as branched chain amino acids (BCAAs). They are essential amino acids used for intravenous feeding and as food additives. In most cases, they are commercially synthesized through fermentation. Small quantities of other amino acids, including other BCAAs, are also obtained as impurities in these fermentation processes. They are separated and purified by crystallization from fermentation broths containing impurities.

During BCAA crystallization processing, guest amino acids (impurity amino acids) are readily incorporated in a host amino acid (a purified amino acid) because host and guest amino acids have similar branched hydrophobic side chains and crystal structures.[1−3] Molecular structures of these amino acids are shown in Figure 1. Therefore, in many cases, it is difficult to separate host amino acids and guest amino acids from one another using a simple crystallization method such as cooling or concentration crystallization conducted in water. To obtain desired purity of BCAA recrystallization is often required. To avoid such repetition and to obtain pure BCAA efficiently, purification using crystallization of a complex with L-Leu and precipitants was developed on a L-Leu crystallization process.[4−6] Furthermore, on L-Ile crystallization process, the

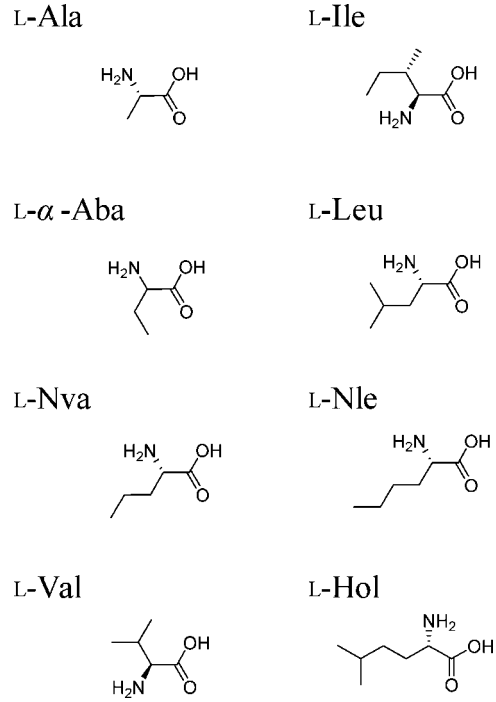

Figure 1. **Molecular structures of BCAA and isomorphic amino acids.**

combination of cooling and acid addition was attempted in order to minimize the level of impurities such as L-Leu and L-Val incorporated in the L-Ile products.[7] Thereby, a range of separation methods have been developed. However, these processes are complicated because dehydrochloric acid or deprecipitant processing is necessary after crystallization. Therefore, to simplify the process, it is first necessary to elucidate the incorporation mechanism of these amino acids. Then separation methods can be developed based on this mechanism.

This study is intended to propose a simple model of incorporation mechanism from the relationship between the mutual incorporation tendency and the change of the crystal structure in various combinations. For this research, L-Ile was chosen as the host amino acid, and isomorphic amino acids

* Corresponding author. E-mail: toshimichi_kamei@ajinomoto.com.
† Isolation and Purification Technology Laboratory, Fermentation and Biotechnology Laboratories, Ajinomoto Co. Inc.
‡ Institute of Life Sciences, Ajinomoto Co. Inc.
§ Department of Chemical Engineering, Iwate University.

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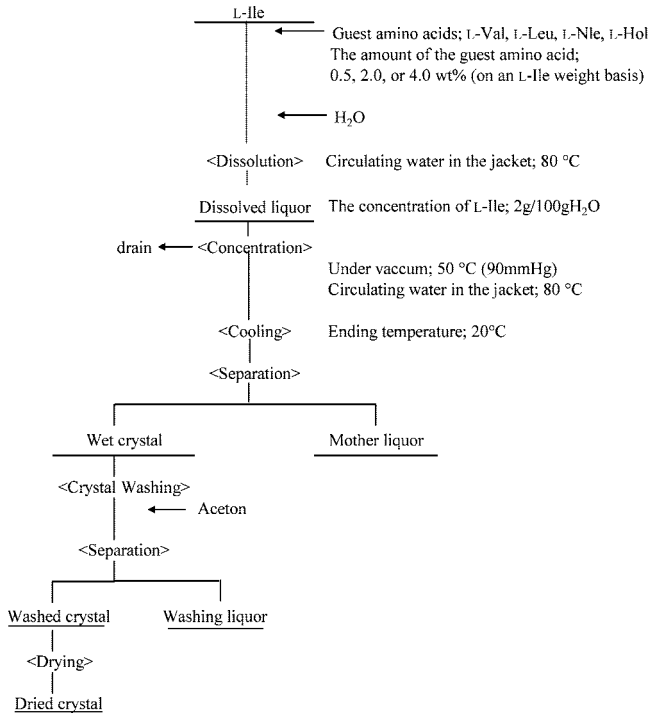


Figure 2. **Scheme of the crystallization method.**

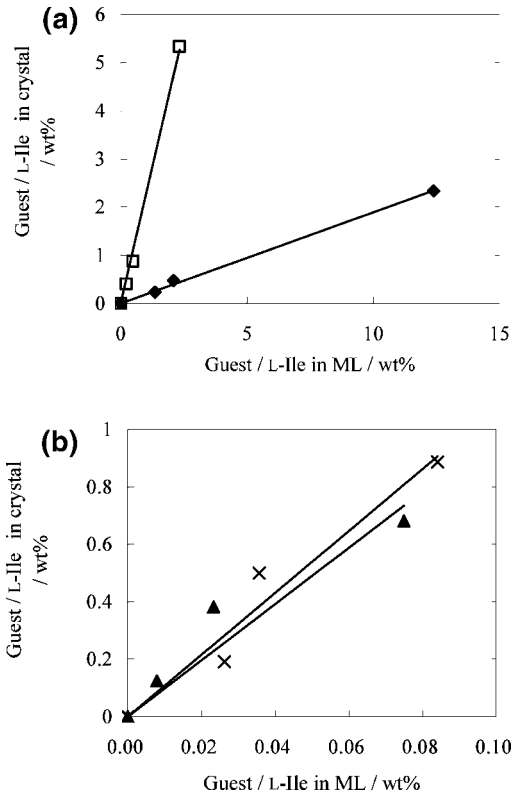


Figure 3. **Guest amino acid content in L-Ile crystals as a function of the guest amino acid content of the mother liquor (ML) when guest amino acids are L-Val (◆), L-Leu (□), L-Nle (▲), L-Hol (×).**

such as L-Leu, L-Val, L-norleucine (L-Nle), and L-homoleucine (L-Hol) were chosen as guest amino acids. To quantify the incorporation tendency, purification factors designated by the distribution coefficient P_i are defined using the following equation:

$$P_i = z_i/x_i$$

where z_i is the weight (or mole) ratio of a guest to a host in the crystal, and x_i is the weight (or mole) ratio of a guest to a host in the mother liquor (ML). When P_i is less than 1, the crystallization has caused purification. When it equals 1, no purification has occurred; when it is greater than 1, the crystalline product has been further contaminated.[7]

In the following, the distribution coefficient is measured in various combinations of L-Ile (a host amino acid) and isomorphic amino acids (guest amino acids) by concentration and cooling crystallization conducted in water. Then, as for a host amino acid, which incorporates a guest amino acid, the change in the lattice constant and the crystal growth are observed. Finally, the model related to mutual incorporation is proposed from these results.

2. Experimental Section

2.1. Materials. The L-Ile and L-Val and L-Leu used in this experiment were obtained commercially (pharmaceutical grade, ≥99.0%) from Ajinomoto Co. Inc. (Tokyo, Japan). The L-Nle was supplied by Sigma-Aldrich Corp (≥98% (TLC)). The L-Hol was synthesized by Zieben Chemicals Co., Ltd. (99.5% (LC)). Acetone used in this experiment was special grade chemicals (99.5% (GC)) from Junsei Chemical Co., Ltd. The water used in this experiment was made by Milli-Q (Millipore Corp.) (specific resistivity 18.2 MΩ, TOC 4 ppb).

2.2. Crystallization Method. Crystallization experiments were conducted in a 1000 mL jacketed glass vessel at agitation speeds of 300 rpm. The agitator used was a turbine stirrer, which was installed in the crystallizer just at its lower end to make the solution flow upwards in it. The predetermined amounts of each guest amino acid were added to the crystallizing system of L-Ile in water. This solution contained 0.5, 2.0, or 4.0 wt % of a guest amino acid (on an L-Ile weight basis). The initial mixture containing 2 g L-Ile/100 g H_2O was heated to 80 °C and kept at that temperature until the solution was dissolved completely. After dissolution, the solution was placed in a jacketed glass vessel, which was then sealed. The temperature was controlled to 80 ± 0.1 °C using circulating water in the jacket from a programmable water bath. Subsequently, the solution was concentrated in vacuum at 50 °C and crystallized. After concentration, the concentrated slurry in the jacketed vessel was cooled rapidly to 20 °C. The temperature was then kept constant at 20 °C until no change was observed in the liquid-phase concentration. After achievement of steady-state conditions, the vessel contents were filtered under vacuum to collect the solid phase. After filtration, the obtained crystals were washed with 10 times weight of acetone to remove adhesive impurities on the crystal surface. Finally, the wet crystals were dried at ordinary temperatures and pressures. The scheme of the crystallization method is shown in Figure 2.

2.3. Distribution Coefficient Analysis. The contents of the host and guest amino acid in the washed crystals (the resultant crystals) and in the saturated liquor at 20 °C (mother liquor, ML) were analyzed using an amino acid analyzer (L-8800; Hitachi Ltd.). Subsequently, the guest contents of the washed L-Ile crystals were plotted on the *y*-axis vs the guest content of

Table 1. **Distribution coefficient of each guest amino acid when host amino acid is L-Ile**

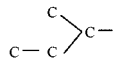
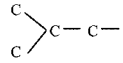
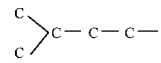
host	L-Ile			
side chain				
guest	L-Val	L-Leu	L-Nle	L-Hol
side chain				
distribution coefficient	0.19	2.27	9.80	10.79

Table 2. **Unit cell constants from single crystal XRD data**[1–3,9]

amino acid	lattice constant Å		
	a	*b*	*c*
L-Val	9.71	5.27	12.06
L-Ile	9.75	5.32	14.12
L-Leu	9.61	5.31	14.72
L-Nle	9.55	5.26	15.38

Table 3. **Compositions of starting solutions and corresponding crystals**

initial liquid phase (vs L-Ile wt %)		obtained crystal mole fraction	
run	L-Nle	L-Ile	L-Nle
1	pure L-Ile	1.00	0.00
2	3.30	0.97	0.03
3	6.70	0.93	0.07
4	13.30	0.84	0.16
5	pure L-Nle	0.00	1.00

the ML on the *x*-axis of a graph, which showed a linear correlation. Finally, the distribution coefficient was obtained from the slope of this graph.

2.4. Crystal Analysis. The remaining solid sample was subjected to powder X-ray diffraction analysis using the synchrotron radiation at the Pharmaceutical Industry Beamline BL32B2 of SPring-8. For measurements at SPring-8, powder samples were packed in glass capillaries, and their diffraction data were collected in the transmission geometry. The wavelength of the synchrotron radiation was set to 1.0000 Å. Diffraction images were recorded on the Imaging-Plate detector, R-AXIS V (Rigaku Corp.) and processed with a computer program (DisplayWin; Rigaku Corp.). Also, crystals were photographed through a microscope to observe the habit and size.

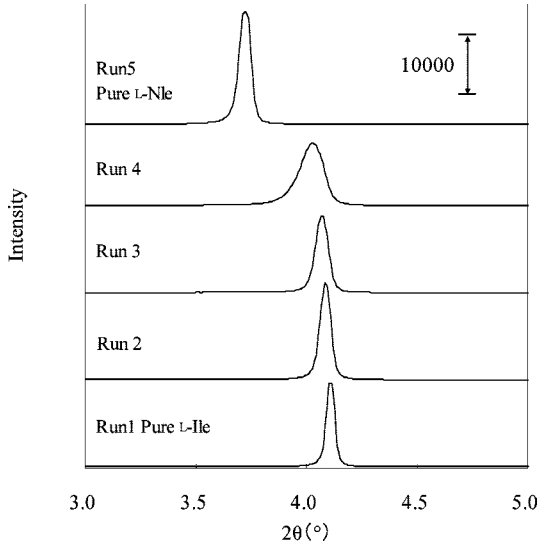


Figure 4. **Powder XRD patterns in runs 1−5 obtained in the experiments using synchrotron X-ray diffraction analysis (wavelength = 1.0000 Å).**

3. Results and Discussion

3.1. Distribution Coefficients. Figure 3 shows the guest amino acid content of L-Ile crystals as a function of the guest amino acid content of the ML. The distribution coefficients are obtained from the slopes of these graphs and are summarized in Table 1. The side chains of L-Ile and guest amino acids are also shown in this table.

As a result, the distribution coefficients of L-Leu, L-Nle, and L-Hol are greater than 1 (L-Leu: 2.27, L-Nle: 9.80, L-Hol: 10.79). Thus, the contents of these amino acids are enhanced when L-Ile is crystallized from the aqueous solutions in the presence of these guest amino acids. On the other hand, the distribution coefficients of L-Val are less than 1 (L-Val:0.19). Therefore, purification of L-Ile occurs when L-lle is crystallized from the aqueous solutions in the presence of L-Val.

From the point of the side chain, the longer the side chain, the larger the distribution coefficient is. The side chain structure is related to hydrophobicity. The less polar amino acids have a

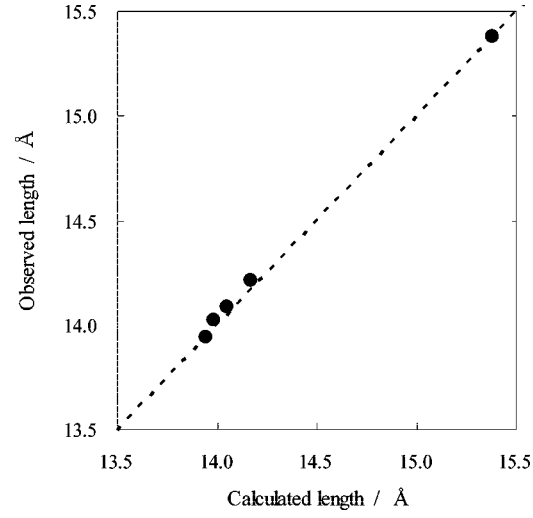


Figure 5. **Relations between the calculated length of the *c*-axis and observed length of the *c*-axis in runs 1−5. (●) observed value; (---) calculated value.**

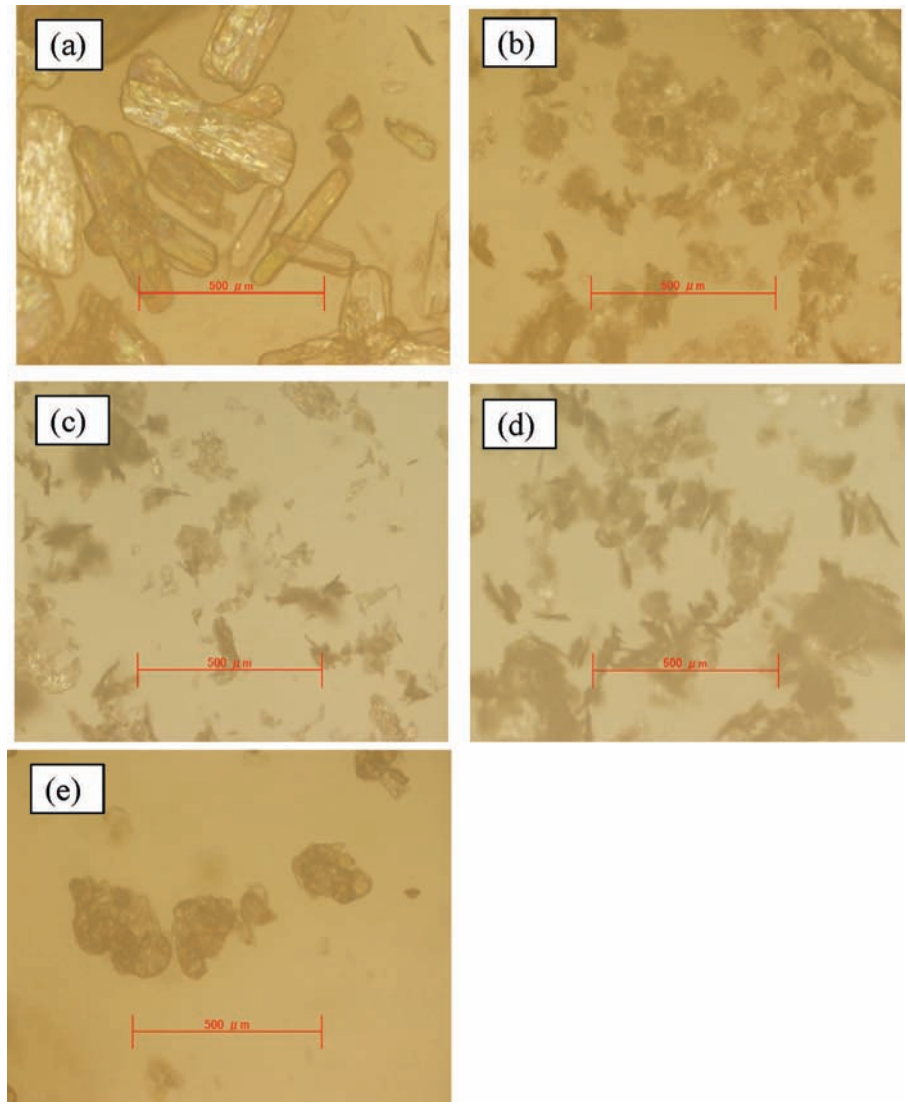


Figure 6. **Change of the crystal appearance: (a) L-Ile, pure, (b) L-Ile + L-Nle crystals obtained by crystallization (L-Nle mole fraction in crystals; 0.03), (c) L-Ile + L-Nle crystals obtained by crystallization (L-Nle mole fraction in crystals;0.07), (d) L-Ile + L-Nle crystals obtained by crystallization (L-Nle mole fraction in crystals;0.16), (e) L-Nle, pure (redline scale: 500 μm).**

hydrophobic property, and the amino acid with the short side chain has low hydrophobicity. Furthermore, the longer the side chain, the higher hydrophobicity is. Jonsson et al. (1989)[8] reported hydrophobicity of some of these amino acids; it is high in the following order.[8]

L-Nle>L-Leu>L-Ile >L-Val

This order resembles that of the incorporation tendency. Furthermore, it is considered that hydrophobicity is related to desolvation. Namely, guest amino acids which are more hydrophobic than L-Ile are desolvated easily from the water solvent. Therefore, it is considered that guest amino acids whose side chain is longer than that of L-Ile are incorporated easily in L-Ile crystals.

3.2. Change of the Lattice Constant. The L-Ile is monoclinic and belongs to the $P2_1$ space group. Furthermore, the first peak in the powder XRD pattern corresponds to the (001) face, or the *c*-axis, of the unit cell. The lattice constant of L-Ile and some guest amino acids are presented in Table 2.[1−3,9] The unit cells of L-Ile and guest amino acids are very similar except for the respective lengths of their *c*-axes. Therefore, the positions of the first peaks in their XRD patterns are useful to identify these amino acids.

To confirm the change of the lattice constant when L-Ile incorporates a guest amino acid whose side chain is longer than L-Ile, L-Nle was chosen as a representative guest amino acid. In fact, L-Nle was added to the crystallizing system of L-Ile in water. This solution contained 3.3, 6.7, or 13.3 wt % of a guest amino acid (on an L-Ile weight basis). Furthermore, crystallization was carried out in the same conditions as those described in subsection 2.2. The compositions of the initial and obtained crystals are shown in Table 3. The obtained crystals were subjected to powder X-ray diffraction analysis using the

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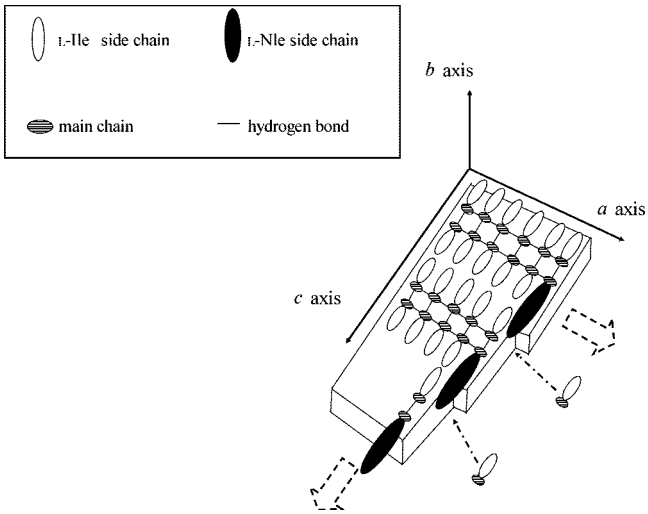

Figure 7. **Incorporation model which expects a mutual incorporation and crystal morphology change; When the length of the side chain of guest amino acid is longer than that of host amino acid. In this figure, L-Ile is shown as the host amino acid, and L-Nle is shown as the guest amino acid as a representative case.**

synchrotron radiation at the Pharmaceutical Industry Beamline BL32B2 of SPring-8. Furthermore, the position of the first peak in the XRD pattern was used to confirm the lattice change when L-Ile incorporates L-Nle. Figure 4 shows powder XRD patterns of the (001) face of L-Ile, which incorporates L-Nle.

The peak position of the (001) face of L-Ile shifts to a low angle and broadens with the composition of L-Nle. However, the own peak of L-Nle is not apparent. Therefore, it is not an overlap of the peak of L-Ile and L-Nle and the possibility exists that a solid solution is formed in the crystal.

The lengths of the *a*- and *b*-axes of these guest amino acids resemble that of L-Ile, but the length of the *c*-axis of L-Nle is longer than that of L-Ile, as shown in Table 2. Therefore, it is considered that lattice changes of solid solution result from the substitution of L-Ile molecule and L-Nle molecule in the unit lattice. Therefore, only the *c*-axis might be extended in these cases.

To explain the substitution of L-Ile molecule and a guest amino acid molecule in the unit lattice, the length of the *c*-axis of the solid solutions is calculated according to Vegard's law; then the calculated value and observed value are mutually compared. Vegard's law shows that the lattice length of solid solutions is proportional to the content of substitution substances.[10] Here, the *c*-axis length (C_{calc}) is shown in the following equation.

$$C_{calc} = C_{host} \times (1 - A) + C_{guest} \times A$$

In that equation, C_{host} is the length of the *c*-axis of L-Ile, C_{guest} is the length of the *c*-axis of L-Nle, and *A* is a guest (L-Nle) mole fraction in the crystal. Consequently, observed values show good agreement with calculated values (Figure 5). Therefore, it is considered that the change of the *c*-axis results from the substitution of L-Ile molecule and L-Nle molecule in the unit lattice.

3.3. Change of the Crystal Appearance of L-Ile. Figure 6 shows the crystal appearance. The crystal size of L-Ile becomes fine with L-Nle. From the crystal structure, the L-Ile crystal grows in the direction of the *a*- and *b*-axes with the hydrogen bond. Furthermore, it also grows in the direction of *c*-axis with hydrophobic affinity. Based on this point, the result shows that the crystal growth of L-Ile is inhibited in all directions when L-Ile incorporates L-Nle.

Incorporation model which expects a mutual incorporation and crystal morphology change is shown in Figure 7. From this model, it is considered that the crystal growth is affected by the side chain length. The *c*-axis of L-Ile increases when L-Nle whose side chain is longer than that of L-Ile is incorporated in L-Ile. Increasing of the *c*-axis causes steric hindrance, and repulsion of the regular deposition of oncoming L-Ile occurs. It is also considered that steps can not move forward for the *a*-axis direction. Regarding the *b*-axes direction, similar behavior of steps is considered. Therefore, the crystal size of L-Ile becomes fine with L-Nle.

4. Conclusion

Results described above demonstrate that the following model shows mutual incorporation and crystal morphology change, as proposed.

The incorporation mechanism is divided into three stages. The first stage is desolvation of the guest amino acids from the water solvent. The second stage is the adsorption of guest amino acids to a host amino acid. The third stage is the crystal growth after adsorption. In the first stage, guest amino acids, which are more hydrophobic than a host amino acid, are more easily desolvated than guest amino acids, which are less hydrophobic than the host amino acid. In the second stage, host and guest amino acids are connected by hydrogen bonds along the *a*- and *b*-axes; in this case, the side chain differences do not influence the adsorption of a host and guest amino acid. However, in the third stage, the crystal growth is affected by the side chain length. The *c*-axis of a host amino acid increases when a guest amino acid whose side chain is longer than that of a host amino acid is incorporated in a host amino acid. Increasing of the *c*-axis causes steric hindrance, and repulsion of the regular deposition of oncoming host amino acids occurs. Furthermore, in this case, crystal growth of the host amino acid is inhibited by the incorporated guest amino acid. Consequently, the ratio of the amount of the host amino acid and the guest amino acid which adsorb to the crystal changes. Therefore, the distribution coefficient also changes.

Nomenclature

z_i	weight (or mole) ratio of a guest to a host in the crystal [-]
x_i	weight (or mole) ratio of a guest to a host in the mother liquor (ML) [-]
P_i	distribution coefficient [-]
C_{calc}	length of *c*-axis of a solid solution [Å]
C_{host}	length of *c*-axis of L-Ile [Å]
C_{guest}	length of *c*-axis of L-Nle [Å]
A	guest mole fraction in the crystal [-]

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