Mechanism of Mutual Incorporation of Branched Chain Amino Acids and Isomorphic Amino Acids in Batch Crystallization

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We explored the mutual incorporation tendency of branched chain amino acids (L-leucine, L-isoleucine, L-valine) and isomorphic amino acids in cooling crystallization conducted in water solvent. In most cases, a guest amino acid (an impurity amino acid) whose side chain is longer than that of a host amino acid (a purified amino acid) was incorporated easily in a host amino acid. In this case, a solid solution was formed, and the c-axis of a host crystal structure was extended. Also, the crystal growth was inhibited. On the other hand, when a guest amino acid whose side chain is shorter than that of a host amino acid was incorporated in a host, the lattice length and the crystal morphology of a host amino acid was the same as that of control. From these results, a mutual incorporation model was proposed.

Introduction

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 by fermentation. Small quantities of other amino acids including BCAAs are also synthesized as impurities in processes where one BCAA is synthesized through fermentation. They are separated and purified by crystallization from fermentation broths containing impurities.

During the BCAA crystallization process, 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 (Torii and Iitaka, 1970, 1971; Harding and Howieson, 1976). Therefore, in many cases, it is difficult to separate a host amino acid and guest amino acids from one another using a simple crystallization method such as cooling or concentration crystallization conducted in a water solvent. For that reason, repetition of the recrystallization is necessary to purify BCAA.

To avoid this repetition and to obtain pure BCAA efficiently, purification utilizing crystallization of a complex with L-Leu and precipitants was developed on the L-Leu crystallization process (Bergmann and Stein, 1939; Stein et al., 1942; Chibata, 1976). Also, on L-Ile crystallization process, a combination of neutral cooling and acid addition was tried to minimize the level of impurities such as L-Leu and L-Val incorporated in the L-Ile products (Zumstein et al., 1990). In this way, various separation methods had been developed. However, these processes are complicated because de-hydrochloric acid or a de-precipitant process is needed after crystallization. Therefore, in order to make a process simpler, it is necessary to elucidate the incorporation mechanism of these amino acids at first, and then separation methods to be developed based on this mechanism. Koolman and Rousseau (1996) conducted research about the change of the crystal purity and structure of a host amino acid when guest amino acids were incorporated. Batch crystallization of L-Ile in the presence of small amounts of L-Leu and L-Val was chosen as a model system. This research is considered to be a clue to the incorporation mechanism elucidation. On the other hand, only a specific amino acid was examined in this research. Thus, there are still no models which expect a mutual incorporation and crystal morphology when changing variations of a host and a guest amino acid. Therefore, the purpose of this study is to propose a simple model of an incorporation
mechanism from the relationship between the mutual incorporation tendency and the change of the crystal structure in various combinations. In this research, L-Val is chosen as a host amino acid, and isomorphic amino acids such as L-Leu, L-Ile, L-alanine (L-Ala), L-α-amino butyric acid (L-α-Aba), L-norleucine (L-Nle), L-norvaline (L-Nva), L-homoleucine (L-Hol), are chosen as guest amino acids. Molecular formulas of these amino acids are shown in Figure 1.

In order to quantify the incorporation tendency, purification factors called for the distribution coefficient $P_i$ are defined with the following equation.

$$P_i = z_i/x_i$$

Here, $z_i$ 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 resulted in purification, when it equals 1, no purification has occurred, and when it is greater than 1, the crystalline product has been further contaminated (Zumstein et al., 1990).

In the following, at first, the distribution coefficient is measured in various combinations of L-Val (a host amino acid) and isomorphic amino acids (guest amino acids) by the cooling crystallization conducted in a water solvent. Then, the correlation between the crystal structure (such as crystal appearances and lattice changes) and the incorporation tendency (the distribution coefficient) is discovered. Finally, the model about the mutual incorporation is proposed from these results.

1. **Experimental**

1.1 **Materials**

L-Val and L-Ile and L-Leu L-Ala used in this experiment were obtained commercially (drag grade) from Ajinomoto Co. Inc. L-α-Aba, L-Nle, L-Nva were supplied by Sigma-Aldrich Co., Ltd. L-Hol was synthesized by Zieben Chemicals Co., Ltd.

1.2 **Crystallization method**

Crystallization experiments were conducted in a 1000 ml jacketed glass vessel at stirrer speeds of 300 rpm. Predetermined amounts of each guest amino acid were added to the crystallizing system of L-Val in water, respectively. This solution contained 0.5, 2.0, 4.0 wt% of a guest amino acid (on L-Val wt% basis). The initial mixture containing 2 g L-Val/100 g H$_2$O was heated to 80°C, and maintained at this temperature until the solution was dissolved completely. After dissolution, the solution was placed in a jacketed glass vessel and then the vessel was sealed. The temperature was controlled to 80 ± 0.1°C using circulating water in the jacket from a programmable water bath. Afterwards, 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 changes were observed in the liquid phase concentration. After
achieving steady state conditions, the contents of the vessel were filtered under vacuum to collect the solid phase. After filtration, the obtained crystals were washed with 10 times that weight of acetone in order to remove adhesive impurities on the crystal surface. Finally, crystals were dried at room temperature.

1.3 Distribution coefficient analysis

The contents of a host and a guest amino acid in the washed crystals (the crystals obtained finally) and in the saturated liquor at 20°C (mother liquor) were analyzed using an amino acid analyzer (L-8800, Hitachi Ltd.). Afterwards, the guest content of the washed L-Val crystals was plotted on the y-axis versus the guest content of the mother liquor on the x-axis. Also, this graph shows straight line relations. Finally, the distribution coefficient was obtained from the slope of this graph.

1.4 Crystal analysis

The remaining solid sample was subjected to powder X-ray diffraction analysis (X’Pert-Pro-MPD, Panalytical B.V.) with a Cu-Kα radiation source (wavelength = 1.54056 Å). Also, crystals were photographed through a microscope to observe the habit and size.

The experiment of L-Val single crystal growth was conducted in the presence of some guest amino acids as follows. L-Leu and L-α-Aba were chosen as guest amino acids. They were added to the L-Val saturated solution at 40°C, respectively (guest molar ratio, 5 × 10⁻²). Afterwards, the mixture was dissolved completely at 70°C, and then it was kept 40°C for 1 h. L-Val crystal, which was adhered to a wire made of stainless steel, was set in this solution. Then, the solution was cooled to 20°C for 24 h under no stirring conditions.

The surface morphology of the crystal was observed using atomic force microscopy (AFM) from Digital Instruments (Dimension 3000 scanning probe microscope) with Si₃N₄ tips in the contact mode. As shown in Figure 2, L-Val crystal was fixed so that the observed crystal face was parallel on a glass plate. An L-Val solution (saturated concentration at 25°C) was supplied between an AFM probe and a glass plate, and a liquid column was created so that a crystal was covered. In this liquid column, crystal surface growth was observed at 21 ± 1°C. The (001) and (100) faces were observed. At first, L-Val crystal was grown in a pure L-Val solution, and the growth behavior was confirmed. Afterwards, a guest amino acid (L-Leu) solution (guest molar ration, 1 × 10⁻²) was added to the liquid column using a syringe, and the change of the growth behavior was observed.

2. Results and Discussion

2.1 Distribution coefficient

The guest amino acid content of L-Val crystals as a function of the guest amino acid content of the mother liquor (ML) is shown in Figure 3. The distribution coefficients are obtained from the slope of these graphs and are summarized in Table 1. The side chains of L-Val 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. Thus, the content of these amino acids is enhanced when L-Val is crystallized from the aqueous solutions in the presence of these guest amino acids. On the other hand, the distribution coefficients of L-α-Aba, L-Ala, L-Nva, L-Ile are less than 1. Therefore, purification of L-Val occurs when L-Val is crystallized from the aqueous solutions in the presence of these guest amino acids.

Fig. 2 Experimental setup for AFM observation

Fig. 3 Guest amino acid content in L-Val crystals as a function of the guest amino acid content of the mother liquor when guest amino acids are L-Ala (*), L-α-Aba (■), L-Nva (△), L-Ile (□), L-Leu (▲), L-Nle (○), L-Hol (×), respectively

From the point of the side chain, the longer the side chain length is, the larger the distribution coefficient will be. The side chain structure is related to the hydrophobicity. The non-polar amino acids have a hydrophobic property, and the amino acids with the short side chain have low hydrophobicity. Also, the longer the side chain length is, the higher hydrophobicity will
Jonsson et al. (1989) reported the hydrophobicity of some of these amino acids, and it is high or in following order: L-Nle > L-Leu > L-Ile > L-Nva > L-Val > L-Ala.

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

2.2 The change of the lattice constant

L-Val is monoclinic and belongs to the P2₁ space group. Also, 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-Val and some guest amino acids are shown in Table 2 (Torii and Iitaka, 1970, 1971, 1973; Harding and Howieson, 1976). The unit cells of L-Val and guest amino acids are very similar except for the length of their c-axis. Therefore, the positions of the first peaks in their XRD patterns can be used to identify these amino acids. So in this case, the position of the first peak in the XRD pattern was used to confirm the lattice change when L-Val incorporates guest amino acids.

As a result, when L-Ile, L-Leu, L-Nle, L-Hol are incorporated in L-Val crystals, the peak position of the (001) face of L-Val shifts to a low angle and broadens with the composition of these guest amino acids. However, the own peak of the guest amino acid is not seen. Therefore, there is a possibility that a solid solution is formed in the crystal. As an example representation, powder XRD patterns of the (001) face of L-Val which incorporates L-Leu are shown in Figure 4(a).

On the other hand, when L-Ala, L-α-Aba, and L-Nva are incorporated in L-Val crystals, the peak position of the (001) face of L-Val does not significantly change when the contents of these guest amino acids increase. Thus, there is a possibility that a solid solution is formed in the crystal. As an example representation, powder XRD patterns of the (001) face of L-Val, which incorporates L-α-Aba, are shown in Figure 4(b). The lattice constant a, b, c are obtained from each XRD pattern and are shown in Figures 5–8, respectively. The length of the a-, b-axis of every guest amino acid does not significantly change when the contents of these guest amino acids increase (Figures 5 and 6). As for the c-axis, the length of the c-axis also does not significantly change when L-Ala, L-α-Aba, and L-Nva are incorporated in L-Val crystals (Figure 7). Thus, the incorporation of these guests does not affect the lattice length of L-Val.

On the other hand, when L-Ile, L-Leu, L-Nle, and L-Hol are incorporated in L-Val crystals, the c-axis is extended with the increase of the content of these guest amino acids (Figure 8). The length of the a-, b-axis of these guest amino acids are similar to that of L-Val, but the length of the c-axis of these guest amino acids are longer than that of L-Val shown in Table 2. Thus, it is considered that lattice changes of the solid solution are caused by the substitution of the L-Val molecule and a guest amino acid molecule in the unit lattice. Therefore, only the c-axis is extended in these cases.

In order to explain the substitution of the L-Val molecule and a guest amino acid molecule in the unit
The length of the $c$-axis of the solid solutions is calculated according to the Vegard’s law, and the calculated value and observed value are compared with each other. The Vegard’s law shows that the lattice length of a solid solutions is proportion to the content of substitution substances (Katoh, 1999). In this case, the length of the $c$-axis ($C_{\text{calc}}$) is shown in the following equation.

$$C_{\text{calc}} = C_{\text{host}} \times (1 - A) + C_{\text{guest}} \times A$$  \hspace{1cm} (2)

Here, $C_{\text{host}}$ is the length of the $c$-axis of L-Val, $C_{\text{guest}}$ is the length of the $c$-axis of a guest amino acid, and $A$ is a guest mole fraction in the crystal. As a result, the observed values are in good agreement with the calculated values (Figure 9). Thus, it is considered that the change of the $c$-axis is caused by the substitution of the L-Val molecule and a guest amino acid molecule in the unit lattice.

### 2.3 The change of the crystal appearance of L-Val

When guest amino acids are L-Ala, L-$\alpha$-Aba and L-Nva, even if the contents of guest amino acids increase, the crystal appearance of L-Val does not significantly change. On the other hand, when guest amino acids are L-Ile, L-Leu, L-Nle, and L-Hol, the crystal size of L-Val becomes fine as the contents of guest

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### Table 2 Unit cell constants from single crystal XRD data (Torii and Iitaka, 1970, 1971, 1973; Harding and Howieson, 1976)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$a$ [Å]</th>
<th>$b$ [Å]</th>
<th>$c$ [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Val</td>
<td>9.71</td>
<td>5.27</td>
<td>12.06</td>
</tr>
<tr>
<td>L-Ile</td>
<td>9.75</td>
<td>5.32</td>
<td>14.12</td>
</tr>
<tr>
<td>L-Leu</td>
<td>9.61</td>
<td>5.31</td>
<td>14.72</td>
</tr>
<tr>
<td>L-Nle</td>
<td>9.55</td>
<td>5.26</td>
<td>15.38</td>
</tr>
</tbody>
</table>
amino acids increase. As an example representation, the crystal appearance of L-Val when L-Val incorporates each guest amino acid is shown in Figure 10. Even if L-Val incorporates more than 2 mol% of L-α-Aba, and L-Nva, the crystal appearance of L-Val does not significantly change. On the other hand, the crystal size of L-Val becomes fine with a small amount of L-Nle or L-Hol.

L-Val crystal morphology is shown in Figure 11. From the crystal structure, the L-Val crystal grows in the direction of the a-, b-axis with the hydrogen bond. It also grows in the direction of the c-axis with a hydrophobic bond. Because the growth rate along the c-axis is slow relatively, the L-Val becomes a disk like crystal. From this point, these results shows that the crystal growth of L-Val is inhibited in all directions when guest amino acids are L-Ile, L-Leu, L-Nle, and L-Hol.

### 2.4 Single crystal growth

In order to confirm the growth inhibition in the a and b directions, the experiment of L-Val single crystal growth was conducted in the presence of typical guest amino acids. L-Leu was chosen as a guest amino acid whose side chain is longer than that of L-Val. L-α-Aba was chosen as a guest amino acid whose side chain is shorter than that of L-Val. L-Val crystals grown in the L-Leu mixed solution are shown in Figures 12(a) and (b). After 24 h, L-Val did not grow in each direction.

On the other hand, L-Val crystals grown in the L-α-Aba mixed solution are shown in Figures 12(c) and (d). In this case, L-Val could grow in each direction after 24 h. Thus, it is confirmed that L-Val crystals grown in the L-Leu mixed solution is inhibited in the a and b directions.

### 2.5 Crystal surface growth of L-Val

In order to clarify the cause of the crystal growth inhibition, surface growth of (001) and (100) faces of L-Val in the presence of a guest amino acid were observed by AFM. In this experiment, L-Leu was chosen...
as a guest amino acid. AFM images of the (001) face in the pure L-Val solution are shown in Figures 13(a)–(c). There were some steps, and the (001) face grew by forwarding of these steps. The AFM images of the (001) face in the L-Leu mixed solution are shown in Figures 13(d)–(f). In this case, there were also steps, but they stopped when L-Leu was added into the L-Val solution. As for the (100) face, the same phenomenon were observed. The AFM images of the (100) face in the pure L-Val solution and in the L-Leu mixed solution are shown in Figures 14(a)–(f). There were some steps and the (100) crystal face grew by forwarding of these steps. They also stopped when L-Leu was added into the L-Val solution. Thus, it is considered that the cause of the crystal growth inhibition is the stopping of the steps by adding L-Leu. The stopping of the steps is caused by the steric hindrance of the L-Leu side chain. The side chain length of L-Leu is longer than that of L-Val. Thus, as shown in Figure 15, when L-Leu is incorporated in the L-Val lattice, the side chain of
L-Leu causes steric hindrance, and the repulsion of the regular deposition of oncoming L-Val occurs. In this case, crystal growth of the c-axis direction is inhibited. Also, as shown in Figure 16(a), it is considered that steps cannot move forward for the a-axis direction. As for the b-axis direction, the same behavior of steps is considered.

On the other hand, when a guest amino acid whose side chain is shorter than that of a host amino acid is incorporated in a host amino acid, the lattice length of L-Val does not change, and no steric hindrance occurs. Thus, the regular deposition of oncoming L-Val is not be affected, and as for the a-, b-axis, steps can move forward as shown in Figure 16(b).

Fig. 13  (a)(b)(c) Change of the AFM images of L-Val (001) face in the pure L-Val solution: (a) initial, (b) after 14 min, and (c) after 22 min; (d)(e)(f) Change of the AFM images of L-Val (001) face in the L-Leu mixed solution: (d) initial, (e) after 17 min, and (f) after 51 min

Fig. 14  (a)(b)(c) The AFM images of L-Val (100) face in the pure L-Val solution: (a) initial, (b) after 11, and (c) after 25 min; (d)(e)(f) the AFM images of L-Val (100) face in the L-Leu mixed solution: (d) initial, (e) after 8 min, and (f) after 21 min
Fig. 15  L-Val structure with L-Leu substituted into lattice (viewed along $b$-axis); this model is displayed in consideration of the van der waals radius; and the side chain of L-Leu is surrounded by a dashed line circle.

Fig. 16  Incorporation model which expects a mutual incorporation and crystal morphology change: (a) When the length of the side chain of guest amino acid is longer than that of host amino acid; in this figure, L-Val is shown as the host amino acid and L-Leu is shown as the guest amino acid as a representative case; and (b) When the length of the side chain of guest amino acid is shorter than that of a host amino acid; in this figure, L-Val is shown as the host amino acid and L-α-Aba is shown as the guest amino acid as a representative case.

The crystal growth of the host amino acid is inhibited by the incorporated guest amino acid whose side chain is longer than that of the host amino acid. As a result, 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.

Conclusions

From above results, the following model which expects a mutual incorporation and crystal morphology change, is proposed.

The incorporation mechanism is divided into three stages. The first stage is the desolvation of 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 host amino acid. In the second stage, host and guest amino acids are connected by hydrogen bonds along the $a$, $b$-axis, and in this case, the side chain of guest amino acid is surrounded by a dashed line circle.
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 length of the side chain. When a guest amino acid whose side chain is longer than that of a host amino acid is incorporated in a host amino acid, the c-axis of a host amino acid increases. Increasing of the c-axis causes steric hindrance, and the repulsion of the regular deposition of oncoming host amino acids occurs. Also, in this case, crystal growth in the c-axis direction is inhibited, and steps cannot move forward for a-, b-axis direction. Thus, the crystal shape becomes fine.

On the other hand, when a guest amino acid whose side chain is shorter than that of a host amino acid is incorporated in a host amino acid, the lattice length of a host amino acid does not change, and no steric hindrance occurs. Therefore, the regular deposition of oncoming host amino acids is not be affected. Also, as for the a-, b-axis, steps can move forward and crystal appearance does not change.

Nomenclature

\[ \begin{align*}
A &= \text{a guest mole fraction in the crystal} \\
C_{\text{calc}} &= \text{the length of c-axis of a solid solution} \quad [\text{Å}] \\
C_{\text{guest}} &= \text{the length of c-axis of a guest amino acid} \quad [\text{Å}] \\
C_{\text{host}} &= \text{the length of c-axis of L-Val} \quad [\text{Å}] \\
P_i &= \text{the distribution coefficient} \quad [\text{—}] \\
x_i &= \text{the weight (or mole) ratio of a guest to a host in the mother liquor (ML)} \quad [\text{—}] \\
z_i &= \text{the weight (or mole) ratio of a guest to a host in the crystal} \quad [\text{—}] 
\end{align*} \]

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