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The Artificial Synthesis of Raphide

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ABSTRACT

Have you ever had an itchy mouth after eating yam? This is because the calcium oxalate needle shaped crystals, known as raphide, is contained in the plant that irritate the skin. raphide has been studied in various fields such as medicine and agriculture because of their characteristic effects. Although previous studies have reported useful effects, no successful artificial synthesis of raphide crystals has yet been reported. Therefore, I researched for factors that affect the synthesis of raphide. First, I conducted targeted experiments on drop rate, rotation speed, and concentration. As a result, it was found that the drop rate and rotation speed are factors that change the crystal structure. When these crystals were analyzed by X-ray crystallography, it was confirmed that they were calcium oxalate crystals. However, they were not raphide, so I attempted to synthesize them using a catalyst. For the catalyst, I decided to use the cell fluid part of aloe vera and an aqueous solution of amino acids. When the synthesized crystals were observed under a microscope, there were few raphides could be seen. Furthermore, since the extracted crystals may be the catalytic amino acid, ninhydrin reaction was performed on the filtrate and crystals, and no reaction was observed in both. Since it reacted in the solution before the experiment, I can say that it is calcium oxalate crystal. Therefore, it can be said that the synthesis of raphide was successful and the purpose of the research was achieved.

Introduction

Background

Many people have experienced itching and soreness around their mouths when they eat yam or kiwi fruit. I got curious about this inflammation and, I found that the symptoms were caused by calcium oxalate needle shaped crystals known as raphide. At the same time, I was relieved that I was not allergic, I became interested in these crystals and began this research, thinking that their specific effects could be used as biotechnology for real-life applications, such as in medicine.

Purpose

Raphide hold many possibilities. According to the Konno (2016), raphide has its characteristic behavior called "needle effect" which enhances the effects of substances. The research proved an increase in insecticidal efficacy against pests when it is combined with cysteine protease. Also, "needle effect" has the potential to be used in pharmaceuticals in terms of increasing efficacy of combined substances, including anticancer drugs that target cancer cells. Furthermore, the research by Cha-um et al. (2019) presented a method to remove raphides from plants to eat the plants safely. These reported researches suggest that the technology has already been established to use raphides as pesticides, while removing the combined crystals. However, artificial synthesis of raphide is not yet been reported. For example, research done by Grases et al. (1990) reported the method to synthesis calcium oxalate crystal artificially but in undefined shape. While research papers have been published on the subject of synthesis, there are not any research which has successfully synthesized raphide crystal artificially.

On the other hand, raphide can be extracted from plants. In the research done by Konno (2016) used raphide that was extracted from kiwifruit. Study done by Ishii (1991) also extracted raphide from several plants. However, it is known that only a small number of crystals can be extracted from plants. Therefore, the artificial synthesis of raphide has a high degree of novelty that has not been done by any researcher. If mass synthesis becomes possible, the characteristic reported in previous studies could be applied in terms of agriculture, medicine, and other fields of science. This year I aimed to discover the elements that are necessary for artificial synthesis of raphide. To judge whether the crystal is raphide or not, I used the definition that is written in Konno (2016) paper which is "crystals with a length of about 0.1mm and extremely spiky ends".

Methods

I referred to a paper by Grases et al. (1990) for a method of synthesizing raphide. According to the study, they succeeded in artificial synthesize of calcium oxalate crystal monohydrate. Crystallizer was used to drop reagents into 7 L of water in a previous study, but due to the limitations of school facilities, two burettes were prepared and used to drop the raw reagents, sodium oxalate solution and calcium chloride solution Figures 1A and B. Concentrations of both reagents were set for each experiment. Furthermore, calcium oxalate crystals are known to have low water solubility. Thus, filtration is the best way to extract the crystals produced. Also, other substances that are produced by the reaction are highly water soluble (Figure 1C). Therefore, I thought that by drying the crystals that remained on the filter paper, only the target crystals could be extracted through our process.

Controlled experiment to discover an important factor

I hypothesized that three of the factors necessary for the synthesis of raphide are important: drop rate, concentration of material solution, and rotation of the magnetic stirrer. Therefore, I conducted a controlled experiment to see if differences appeared in the crystals under each of these conditions. The experimental method is shown in the flowchart in Figure 1A below, and the conditions for each experiment are shown in Table 1. Only the drop rate was changed in Experiments 1-1~3, the concentration in Experiments 1-4~6, and the rotation speed in Experiments 1-7~9.
In addition, I used X-ray crystallography in order to compare the extracted crystals and the reagent calcium oxalate in order to identify the structural substance. Furthermore, if the target material was produced, I could evaluate the validity of the experimental method considered in this study.

Table 1. List of environments in controlled experiment

Experiment	Dropping Speed (mL/h)	Concentration (mol/L)	Rotation Speed (RPM)
1-1	215 mL/h	7.5×10^{-3} mol/L	250 RPM
1-2	135 mL/h	7.5×10^{-3} mol/L	250 RPM
1-3	83 mL/h	7.5×10^{-3} mol/L	250 RPM
1-4	83 mL/h	7.5×10^{-2} mol/L	250 RPM
1-5	83 mL/h	7.5×10^{-3} mol/L	250 RPM
1-6	83 mL/h	7.5×10^{-4}mol/L	250 RPM
1-7	83 mL/h	7.5×10^{-3} mol/L	600 RPM
1-8	83 mL/h	7.5×10^{-3} mol/L	800 RPM
1-9	83 mL/h	7.5×10^{-3} mol/L	1000 RPM

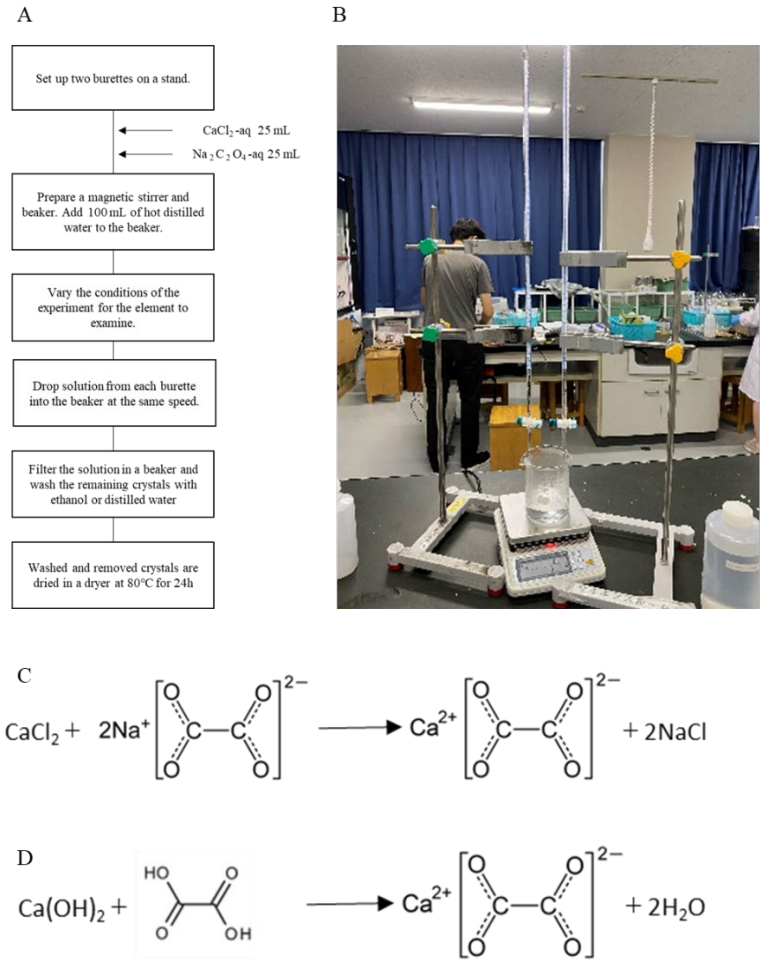

Figure 1. 1A Flowchart of procedure to synthesis. 1B Experiment and instrument. 1C Chemical equation for basic synthesis. 1D Chemical equation for experiment that used Calcium hydroxide and Oxalic acid

Using a different reaction route to synthesis

In a previous experiment it was conducted by reacting aqueous sodium oxalate solution with aqueous calcium chloride solution. I decided to use an aqueous oxalic acid solution and calcium hydroxide to evaluate whether there are any differences to its structure by a different route (Figure 1D). The experimental procedure was the same as the flow chart in Figure 1A, except that the raw reagents were changed to 7.5 x 10^{-3} mol/L aqueous oxalic acid solution and 7.5 x 10^{-3} mol/L aqueous calcium hydroxide solution. Synthesis conditions are shown in Table 2, and heated distilled water was kept at 80°C throughout the experiment.

Table 2. List of environments used in the experiment to observe the different passage

Experiment	Dropping Speed (mL/h)	Concentration (mol/L)	Rotation Speed (RPM)
2-1	83 mL/h	7.5×10^{-4} mol/L	800RPM
2-2	83 mL/h	7.5×10^{-3} mol/L	1000RPM

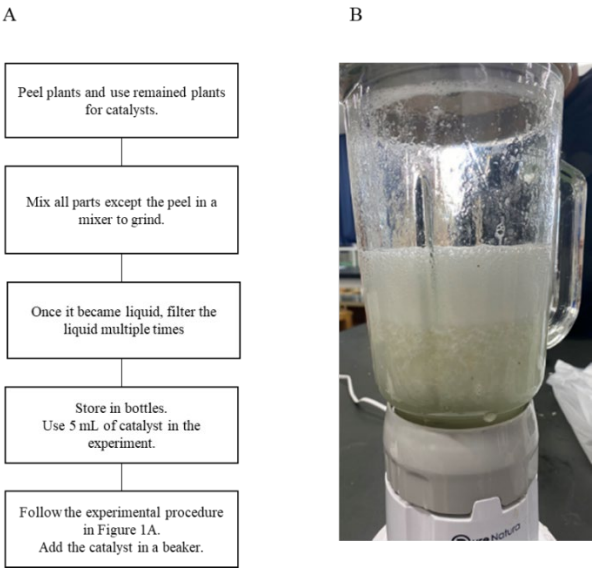

Figure 2. 2A Flowchart of procedure to create catalyst from plants. 2B Photo of grind aloe vera that is used for catalyst.

Using a Catalyst from Plants

In previous experiments, I attempted to synthesize raphide by changing external factors affecting the crystal synthesis process. However, the crystal structure itself did not change significantly. Therefore, I hypothesized that the synthesis of raphide would be possible by using as a catalyst a plant that has raphide in its cells.

The plants that are used as catalysts in this experiment were aloe vera, aloe arborescens, and kiwi fruit. The procedure for preparation of the catalysts is shown in Figure 2A and 2B, and the synthesis method followed the procedure shown in Figure 1A, with 5 mL of catalyst added to a beaker along with distilled water. The controlled experimental conditions are shown in Table 3. The conditions of the experiment were as follows: a concentration of 7.5 x 10^{-3} mol/L of the sodium oxalate solution and calcium chloride solution, a drop rate of 83 mL/h, a rotation rate of 1200 RPM, and the heated distilled water and catalyst were adjusted to keep the temperature at 80°C throughout the experiment. A process of filtration was added to remove raphide contained within the plant during the catalyst preparation process. I believed that adding this procedure would avoid the presence of raphide before the experiment.

Table 3. List of catalyst and reaction process that is used

Experiment	Catalyst	Reaction process
3-1	Aloe Vera	$Na_2C_2O_4 + CaCl_2$
3-2	Aloe Vera	$C_2H_2O_4 + Ca(OH)_2$
3-3	Aloe arborescens	$Na_2C_2O_4 + CaCl_2$
3-4	Aloe arborescens	$C_2H_2O_4 + Ca(OH)_2$
3-5	Kiwifruits	$Na_2C_2O_4 + CaCl_2$
3-6	Kiwifruits	$C_2H_2O_4 + Ca(OH)_2$

Using the amino acids to synthesis protein fiver

According to the study done by Xiuli et al. (2014), they reported that raphide in bananas have a crystal structure with the protein as the axis. I hypothesized that it would be possible to synthesize it by using amino acids which constitutes proteins. The amino acids used and their concentrations are shown in Table 4. For the selection of amino acids, I chose three amino acids contained in bananas based on the food database by the Ministry of Education, Culture, Sports, Science and Technology. For the synthesis, 5 mL of amino acid solution was added to a beaker along with distilled water, and the procedure shown in Figure 1A was followed. The concentration of the sodium oxalate solution and calcium chloride solution was fixed at 7.5 x 10^{-3} mol/L, a drop rate of 83 mL/h, and a rotation speed of 1200 RPM. However, the temperature of distilled water was set at 25°C because crystals did not precipitate at all in preliminary experiments. According to the study done by Xiuli et al. (2014), they reported that raphide in bananas have a crystal structure with the protein as the axis. I hypothesized that it would be possible to synthesize it by using amino acids which constitutes proteins, and attempted to synthesize the crystals. The amino acids used and their concentrations are shown in Table 4. For the selection of amino acids, I chose three amino acids contained in bananas based on the food database by the Ministry of Education, Culture, Sports, Science and Technology (2020). For the synthesis, 5 mL of amino acid solution was added to a beaker along with distilled water, and the procedure shown in Figure 1A was followed. The concentration of the sodium oxalate solution and calcium chloride solution was fixed at 7.5 x 10^{-3} mol/L, a drop rate of 83 mL/h, and a rotation speed of 1200 RPM. However, the temperature of distilled water was set at 25°C because crystals did not precipitate at all in preliminary experiments.

In addition, there are possibilities of precipitation of amino acid crystals. To detect amino acids, I used ninhydrin reaction on the solution before the experiment, the filtrate and the crystals. In particular, I thought that by performing the ninhydrin reaction on the solution before the experiment, I could avoid the possibility that the concentration was so low that the reaction would not appear.

Table 4. List of amino acids and reaction process that is used

Experiment	Amino acids	Concentration (mol/L)
4-1	Methionine	7.5×10^{-3} mol/L
4-2	Methionine	7.5×10^{-4} mol/L
4-3	Methionine	7.5×10^{-5} mol/L
4-4	Methionine	7.5×10^{-6} mol/L
4-5	Leucine	7.5×10^{-3} mol/L
4-6	Leucine	7.5×10^{-4} mol/L
4-7	Leucine	7.5×10^{-5} mol/L
4-8	Leucine	7.5×10^{-6} mol/L
4-9	Glycine	7.5×10^{-3} mol/L
4-10	Glycine	7.5×10^{-4} mol/L
4-11	Glycine	7.5×10^{-5} mol/L
4-12	Glycine	7.5×10^{-6} mol/L

Results

Crystals produced in the experiments were observed under an optical microscope GLB-600MBhL (600x magnification) and scanning electron microscope S-3400N. Experiments are numbered based on those described in Tables 1~4.

Result of controlled experiment

The numbers measured were measured with a memory overlay on the microscope. Comparing the average size of the crystals from Experiments 1-1~3, the crystals from Experiment 1-1 and Experiment 1-3 were 5.0 x 10^{-3} mm and 1.0 x 10^{-2} mm, respectively (Figures 3A and 3B). Comparing the crystals from Experiments 1-4~6, it was easier to observe the crystal shape when the concentration of the solution was smaller, but there was no significant difference in crystal structure. By comparing experiments 1-7~9, it was found that crystals gradually became larger and thinner as the RPM was increased. In particular, some crystals at 800RPM or higher were found to be fibrous (Figures 3C and 3D). In terms of crystal size, Experiments 1-9 had 8.0 x 10^{-2} mm, the largest among the crystals produced in experiment. Furthermore, powder X-ray crystallography was performed on the crystals generated, and the measurement results are shown in Figure 3E. In Figure 3E, the y-axis of the graph is increased for comparison. A comparison of the peaks of the specimen and the crystals produced is shown in Table 5. From the data shown, angles that occur the peaks were almost identical between the specimen and the calcium oxalate crystals produced.

Result of using a different reaction route to synthesis

Compared to the crystals generated in previous result using calcium chloride solution and sodium oxalate solution, the overall crystal size was smaller. Furthermore, the number of crystals that were extracted at once was smaller, even though the concentration of the both solutions were same.

Result of using a catalyst from plants

In experiment 3-1(Table 3) which aloe vera was used as a catalyst, raphides were found among the crystals produced, although their size did not meet the definition (Figure 4A). The crystals were also colored red (Figure 4B). Furthermore, by using a scanning electron microscope, there were crystals that fits the definition such as Figure 4C shown in this paper. However, when the reaction route was changed to one using oxalic acid and calcium hydroxide solutions under the same conditions, the number of crystals that could be extracted was small, and raphide or red crystals were not seen in the experiment (Figure 4D). Furthermore, when Aloe arborescens and kiwifruit were used as catalysts, raphides were not observed, but the crystal color became yellow (Figure 4E).

Result of using the amino acids

Microscopic observation revealed small raphides in Experiments 4-2, 4-3, 4-6, and 4-7(Table 4), where methionine and leucine were added. As in Experiment 3-1, very few crystals were needle-shaped (Figures 5A and 5B). Needle-shaped crystals were seen in the red circles in Figures 5A and 5B. However, when it observed by using scanning electron microscope, most of the crystal's face was clear, and some of it became needle shape (Figure 5C). Also, raphide was not observed in the other experiments. The ninhydrin reaction was performed on the filtrates and crystals. The reaction was observed in the filtrates from experiments 4-1, 4-5, and 4-9, in which 5 mL of 7.5 x 10^{-3} mol/L amino acid was added, but not in the filtrates from amino acid solutions at lower concentrations (Figure 5D). Considering that the solutions were too thin to react, a ninhydrin reaction was performed on the aqueous solutions before the

experiment, and found that all solutions reacted. Also, no reaction was observed in any of the crystals that was produced (Figure 5E).

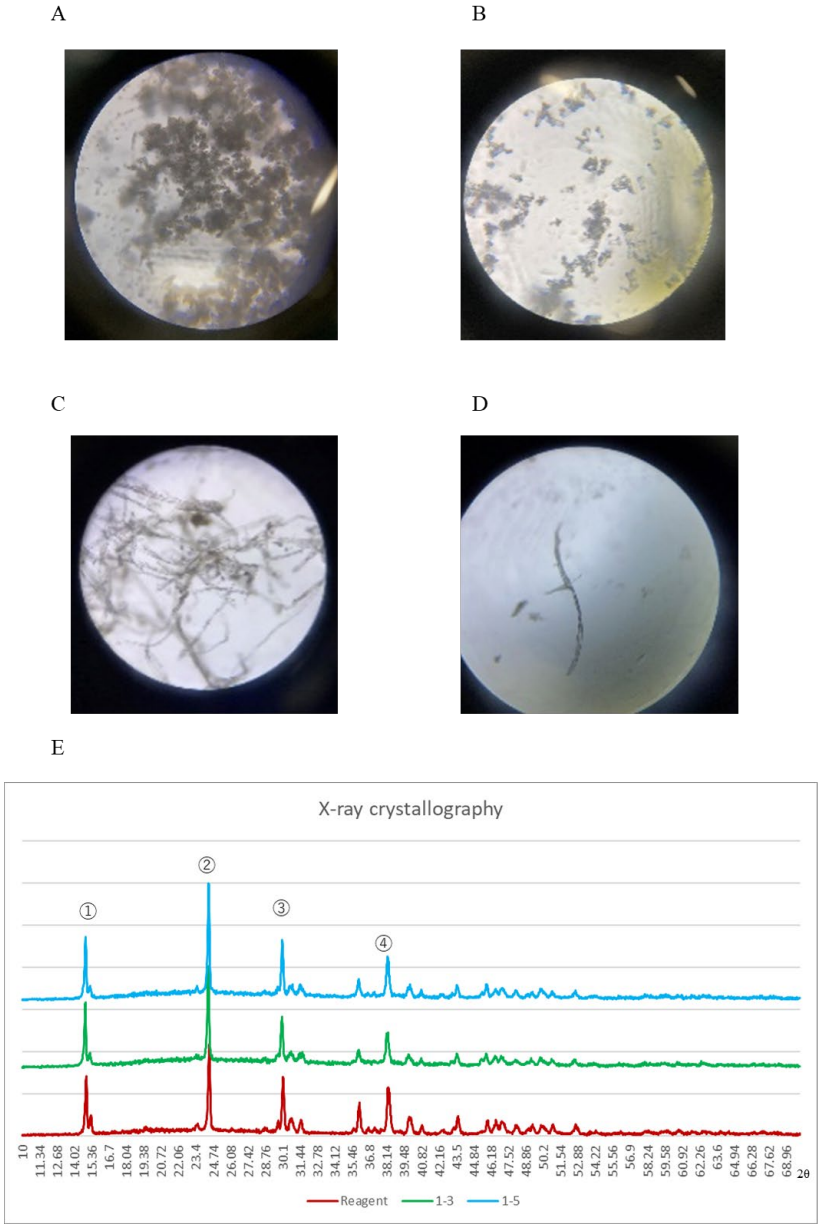

Figure 3. 3A Crystals synthesized in experiment 1-1. 3B Crystals synthesized in experiment 1-3. 3C Crystals synthesized in experiment 1-7. 3D Crystals synthesized in experiment 1-9.

Table 5. List of peaks and angles of compared graphs and its difference between the reagent.

Peak	Reagent	1-3	Difference	1-5	Difference
①	14.96°	14.88°	0.08	14.90°	0.06
②	24.44°	24.36°	0.08	24.38°	0.06
③	30.14°	30.06°	0.08	30.08°	0.06
④	38.26°	38.22°	0.04	38.20°	0.06

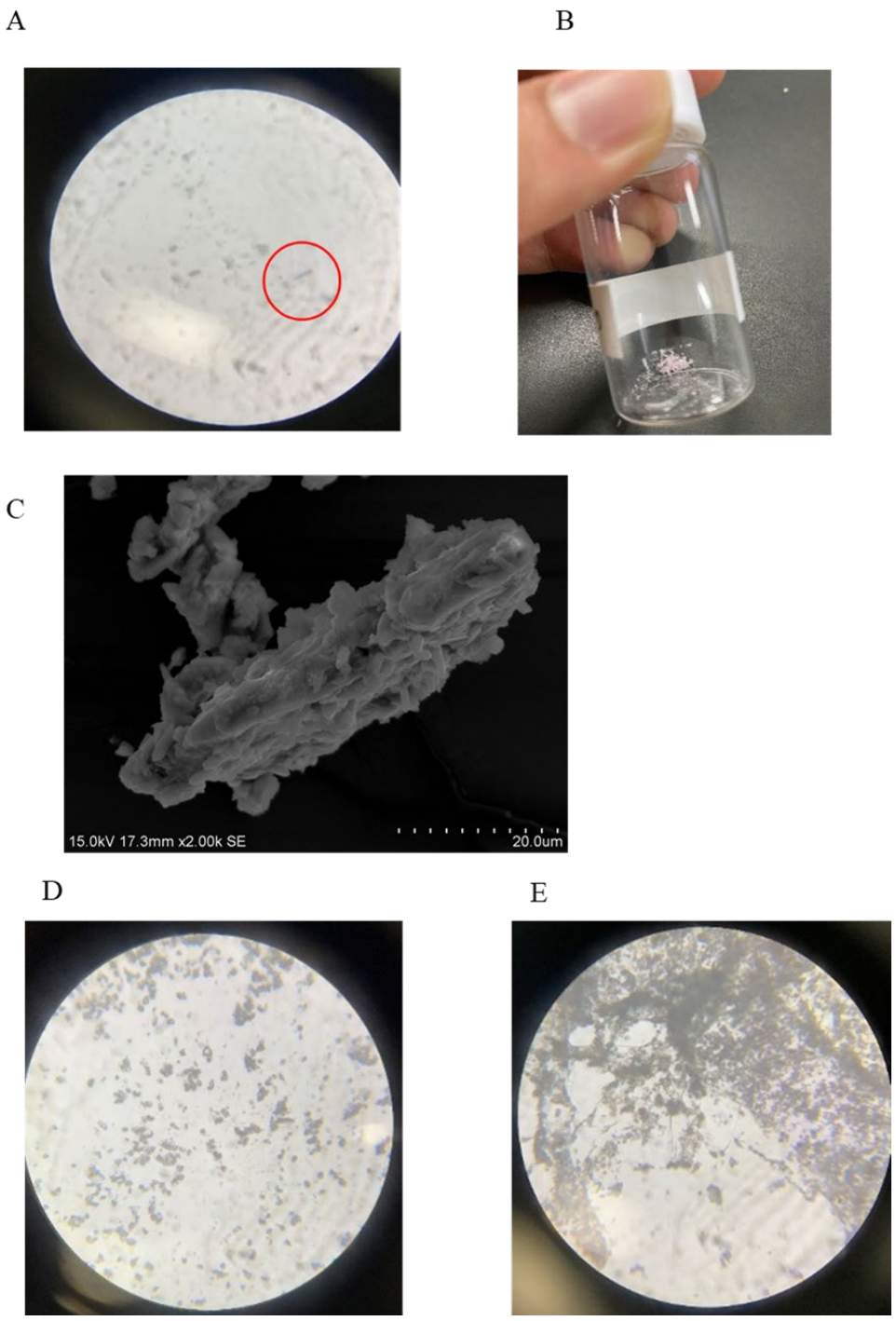

Figure 4. 4A Crystals synthesized in experiment 3-1. 4B Crystals synthesized in experiment 3-1 that shows its color red. 4C Crystals synthesized in experiment 3-1 that is observed by scanned electron microscope. 4D Crystals synthesized in experiment 3-2. 4E Crystals synthesized in experiment 3-3.

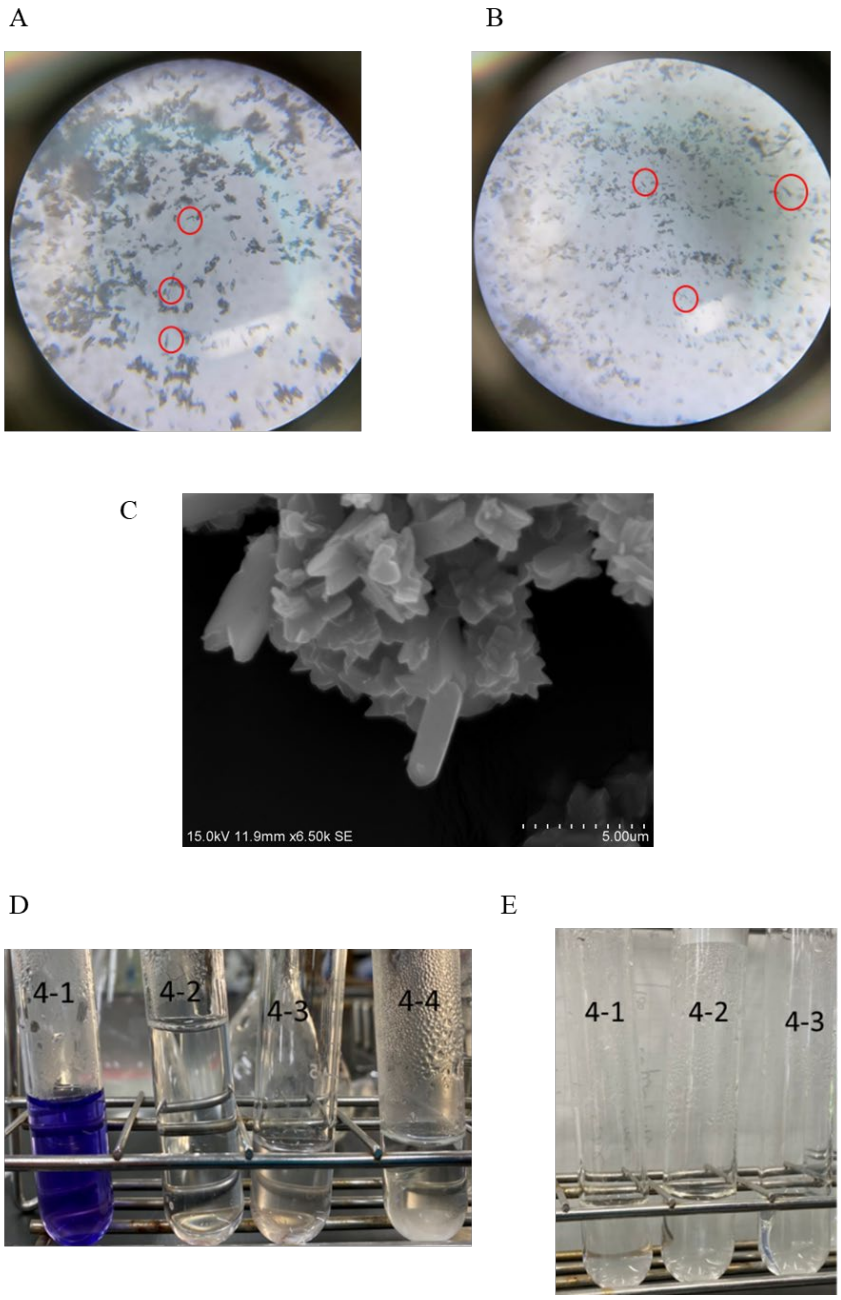

Figure 5. 5A Crystals synthesized in experiment 4-2. 5B Crystals synthesized in experiment 4-3. 5C Crystals synthesized in experiment 4-2 that is observed by scanned electron microscope. 5D Ninhydrin reaction of filtrate which is labeled by experiment number. 5E Ninhydrin reaction of crystals.

Discussion

Discussion of controlled experiment

Comparing Experiments 1-1~3, it was found that the size of the crystals increased as the drop rate was slowed down. This was as reported in the paper by Grases et al. (1990) . This indicates that drop rate is a necessary factor for synthesizing raphide.

On the other hand, the concentration of the raw material solution did not affect the crystal structure, which was different from my hypothesis. Since almost all of the reagent is ionized when dissolved in water, I considered that the ionization equilibrium was not affected by the concentration. Therefore, it can be said that there was no difference in the environment in which calcium oxalate crystals bind from the state of oxalate and calcium ions.

Also, by comparing the crystals from Experiments 1-7~9, I found that the number of rotations was a very important factor. It is thought that the force acting vertically was increased by applying rotation during crystal growth. Therefore, it can be inferred that the crystals became vertically elongated by increasing the rotation rate. However, it is also clear that it is difficult to synthesize raphide only by external factors such as drop rate and rotation speed. Even though crystals in Figure 3D are thinner than other results, they are not shaped like raphide. Furthermore, since not all crystals have the same shape, I can say that it is difficult to synthesize raphide without combining these factors with new ones.

As can be seen from the X-ray crystallography measurement results in Figure 3E and Table 5, the peaks of the specimen and the synthesized crystal appear at nearly the same angle. The difference between the peaks and the angles at which they appeared is very small (0.06°).

According to the Nagoya Institute of Technology (2004) this difference could be due to the human error. In particular, it is known that unevenness in the operation may cause the peak to move toward a smaller angle. As can be seen in Table 5, errors appear especially in the lower angles of less than 0.1°. Also, considering the fact that the crystals 1-5 are consistent in regularity with a difference of 0.08°, it can be said that the measurement results are a systematic error and that the crystals produced in this study are calcium oxalate crystals.

Discussion of using a different reaction route

As can be seen from the results, it may be difficult to consider using oxalic acid and calcium hydroxide as a new route for synthesis. In the ultimate goal of mass production, a method that can extract target product more would be preferred. However, when the concentrations of the two reagents used were the same as previous experiment the amount of crystal extracted was much smaller. One possible reason is that the distilled water that was used for crystallization was neutral. Oxalic acid is known as a mild acid, and it is difficult to ionize hydrogen ions in a neutral environment. In other words, oxalic acid ions, which are necessary for the formation of crystals, are also difficult to ionize. Therefore, I thought that by making the solvent in the beaker where the drops are made basic, the ionization equilibrium would be disrupted and oxalic acid would ionize more oxalate ions, which would combine with calcium ions to precipitate more crystals. So, I thought that crystals would be less likely to form if the synthesis method (Figure 1A), in which the solvent is neutral, is used with the reaction pathway in Figure 1D.

Discussion of using a catalyst from plants

As can be seen from the results, some of the crystals synthesized in the experiment using aloe vera as a catalyst had a raphide structure. There were two major changes with the use of aloe vera as a catalyst: one was that the crystals became red which was not observed in the experiments with not using aloe vera. In the experiment without the catalyst, calcium oxalate was observed to be white crystals. However, the reddish coloration in the crystals suggests that the

growth process of the crystals was affected by using aloe vera. Furthermore, the use of aloe vera as a catalyst allowed us to confirm raphide for the first time by scanning electron microscope. There are possibilities that this crystal came from original plants, but filtration was done when the aloe vera was made as catalyst (Figure 2A). Therefore, I can say that using aloe vera is an important factor in order to produce raphide artificially. However, experiments using Aloe arborescens and kiwifruit as catalysts showed different results from those of aloe vera. In the case of Aloe arborescens, the color of the crystals changed, suggesting that the crystal growth process had an effect, but the target structure could not be found. Although Aloe arborescens and aloe vera are similar in biological classification to the genus aloe, a major difference emerged when comparing them in terms of catalytic properties in this experiment. Since raphides were observed only in aloe vera, I believe that a comparison of the components of aloe vera, Aloe arborescens, and kiwifruit will reveal elements that aid in the synthesis of raphide. Furthermore, there were not any raphide observed in the experiment when a catalyst was added to the reaction pathway shown in Figure 1D. The amount of precipitate recovered was very small, even though the conditions were the same as in Experiment 3-1, where raphide was observed. Based on the previous discussion, it can be said that synthesis in aqueous oxalic acid and calcium hydroxide solutions may be difficult only under basic conditions.

Discussion of using the amino acids

Raphides were observed in experiments 4-2, 4-3, 4-6, and 4-7. Especially on experiment 4-2, by the observation of scanning electron microscope there are some raphide crystal seen from the sample. A possible reason for the formation of raphide is that the amino acid or protein axis reported by Xiuli et al. (2014) aided crystal growth.

As can be seen in Figures 5D and 5E, when the ninhydrin reaction was performed on the crystals, no reaction occurred except for the filtrate in the experiment with the higher concentration of the aqueous amino acid solution. This suggests that the amino acids reacted together in the formation of the crystals and were no longer detected. One possible reaction is that the amino acids formed a protein and also formed cocrystal. In experiments 4-2, 4-3, 4-6, and 4-7, where raphides were observed, the protein acted as the axis of the crystal and helped it grow, similar to the raphide metabolized in bananas reported by Xiuli et al. (2014).

Furthermore, through this experiment, it can be inferred that the ratio of the ingredient and amino acid that reacted would weigh ingredients more than amino acids. In experiments 4-1, 4-5, and 4-9, where equal amounts of the ingredients were added, amino acids were confirmed in the filtrate, unlike in the other experiments. This was thought to be because the amino acids could not react with the raw material of calcium oxalate crystals. In particular, if the case reported by Xiuli et al. (2014) and raphide identified in this study are similar, it is unlikely that the oxalate and calcium ions are equal in ratio since they gather around the amino acid as an axis. So, I can say that amino acids were detected in the filtrate in a sample with a dense aqueous amino acid solution.

Conclusion

In this study, I was able to synthesize crystals with sharp needles, similar to raphide defined by Konno (2016). There are three things I have discovered through my research.

One is that drop rate and rotation rate are two important factors in changing the crystal structure. Comparing experiments 1-1 and 1-3, the crystals were about twice as large as those obtained by slowing down the drop rate (Figures 3A and 3B). In addition, as seen in 1-8 and 1-9, the crystals became elongated by increasing the rotation speed, and the crystal size was 10 times larger than that of 1-3 (Figures 3C and 3D). Furthermore, X-ray crystallography confirmed that the crystals produced in this study were calcium oxalate crystals. Therefore, the experimental method used in this study is suitable for the synthesis of calcium oxalate crystals.

I also revealed that various reaction pathways can be used to synthesize raphide. Although there are differences in the post-reaction conditions of the two different reaction pathways, it was found that the synthesis method that makes the most of their characteristics, such as reaction under basic conditions, can be considered.

Furthermore, adding substances other than the ingredients was found to be important. Among the catalysts used in this study, raphides were observed in experiment 3-1 using aloe vera and in experiments 4-2, 4-3, 4-6, and 4-7 using amino acids (Figures 4C, 5A, 5B, and 5C). For these crystals, I can say from the experimental process and the results of the ninhydrin reaction that these crystals were generated in this study. Furthermore, there were several experiments in which amino acids were not detected in both the filtrate and the crystals with respect to the amino acid reagent. In particular, the fact that no amino acids were detected in the experiments in which raphides were observed led us to believe that the same reaction occurred as with the banana raphide identified by Xiuli et al. (2014).

Through these findings, there are two future activities we would like to do: first, I would like to experiment on how the structure of the crystal's changes depending on the combination of amino acids. Through previous research and this study, we have learned that the composition of the axis amino acid, protein, is a very important factor. In this study, I used three amino acids as the target experiment to focus on the change of one amino acid. Since we were able to clarify the relationship between amino acids and crystal growth through our research, I would like to use multiple types of amino acids in a single experiment, focusing on the perspective of protein in the future. In addition, I would like to verify the "needle effect" reported by Konno (2016) using crystals generated in this study. If the needle effect is put to practical use, as described in the introduction, it could be used in various fields such as biotechnological agrochemicals, anticancer drugs, and DDS. For this purpose, I would like to investigate the properties of raphide produced by artificial synthesis.

Limitations

In our study, I was not able to investigate all crystals using SEM, because of limited resources. However, we used reactions such as ninhydrin reaction to observe whether the crystal is amino acid or not. In addition, I was not able to use many amino acids to observe whether the reaction of protein fiber synthesis works for this experiment. This is because I believe it would be hard to discuss whether which amino acid worked to synthesis in such condition.

Acknowledgements

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