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Investigation on the role of ion exchange resin in the crystallization process for the purification of vancomycin

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Abstract−We investigated the cause of a difference in the crystallization aspect depending on a surface area-increasing material (ion exchange resin) in the vancomycin crystallization process with increased surface area per volume of reaction solution (S/V). The result in terms of whether or not crystallization occurred was reversed when the ionic form of the ion exchange resin was altered. In addition, it was shown that the pH range of a solution for vancomycin crystallization was 4-7. Eventually, it was confirmed that vancomycin crystallization was affected by a change in pH of a crystallization solution depending on the ionic form of the ion exchange resin. Furthermore, in the absence of ion exchange resin, the time required for crystallization increased as the pH rose from 4-7. In addition, the size of the vancomycin crystal increased as the pH decreased.

Keywords: Vancomycin, Crystallization, Surface Area-increasing Material, Ion Exchange Resin, Ionic Form

INTRODUCTION

Vancomycin, the first glycopeptide antibiotic isolated from *Amycolatopsis orientalis* (*Streptomyces orientalis*, *Nocardia orientalis*) in Borneo by Eli Lilly in 1956, inhibits the synthesis of cell walls of Gram-positive bacteria, thus killing them [1,2]. Vancomycin is effective in the treatment of methicillin resistant *Staphylococcus aureus* (MRSA) and also is widely used to treat endocarditis in those allergic to penicillin or cephalosporin. In addition, it is generally used in heart surgery using prostheses, orthopedic surgery and brain surgery such as insertion of a ventriculoperitoneal shunt as a preventive medicine against MRSA infection [3].

Several steps of isolation and purification are needed to purify vancomycin obtained from microbial fermentation. The content of vancomycin as registered in the United States Pharmacopeia (USP) and the European Pharmacopoeia (EP) is strictly provided for, as is the content of total impurities and the content of each impurity. In the U.S., the content of vancomycin must be more than 88% according to HPLC analysis as proposed the by the USP, and no material except vancomycin must be more than 4% [4]. In Europe, the content of vancomycin must be more than 93% according to the EP and no material except vancomycin must be more than 4%, as in the USP. Several steps of an isolation and purification process are needed to meet the requirements, and a crystallization process is commonly conducted for the final purification step in the production of high purity drugs such as antibiotics. The crystallization technique is one method of producing and extracting solid matter from a liquid or gas mixture [5,6], and it is a core technology in the control of physical properties and forms of a crystalline material as well as isolation and purification of a specific material from a mixture. The minimum crystal particles initially formed from the solution are called nuclei, and nucleation is classified into homogeneous nucleation, where nuclei are generated in the liquid state due to supersaturation, and heterogeneous nucleation, where nuclei are generated with the assistance of external surfaces (external impure particles, reactor wall, mixer, etc.). Homogeneous nucleation can voluntarily induce nucleation by high supersaturation so that the nucleation rate is fast. On the contrary, the heterogeneous nucleation rate is rather slow due to relatively low supersaturation. The supersaturation of a solute with high solubility such as a bioproduct is generally limited and a crystal particle is formed by heterogeneous nucleation in a number of cases [7]. In the case of vancomycin, nucleation takes a very long time and the nucleation rate is shortened due to the effect of a surface area-increasing material of the reactor so that it can be called heterogeneous nucleation. Crystallization not only improves the quality of the final product but also produces a high value-added product. It is a simple, energy efficient and environmentally friendly process that is widely applicable and has a low fixed investment cost [8]. A method to produce high-purity and high-yield vancomycin by optimization of key process variables (solvent, temperature, time, conductivity, pH, agitation speed, initial vancomycin concentration, etc.) in the vancomycin crystallization process was reported in 2010 [9]. However, crystal formation required a long period of time (~24 hr), resulting in low productivity in the mass-production process. An improved crystallization process with increased surface area per volume of reaction solution (S/V) by using glass beads or ion exchange resins was developed in 2011 and 2012 so that the vancomycin crystallization time could be shortened by ~12 hr [10,11]. However, the reason why the vancomycin crystallization efficiency varied depending on the type of surface area-increasing material (ion exchange resin) remained unclear. Thus, we intended to establish ultimately an effective vancomycin crystallization strategy by investigating the cause of this difference in crystallization efficiency in

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this study.

MATERIALS AND METHODS

1. Preparation of Vancomycin Sample

Vancomycin used in this experiment was obtained through the fermentation of the microorganism *N. orientalis* isolated from soil. Bacterial cells were removed from the fermentation solution containing vancomycin, which was then purified [12]. The solution was consecutively passed through cation exchange, anion exchange and porous cation exchange resins and eluted with ammonia to obtain vancomycin in the form of hydrochlorate. Impurities such as pigment and protein were removed using alumina and a weak acidic cation exchange resin. The resulting product (vancomycin purity: 88%) was used for the crystallization process.

2. Vancomycin Analysis

An HPLC system (SCL-10AVP, Shimadzu, Japan) and Candenza CW-C18 column (4.6×100 mm, 3 μm, Imtakt, Japan) were used for analysis of vancomycin at 260 nm using a UV detector. Mobile phase A was prepared by mixing 1,000 mL of distilled water and 1 mL of formic acid. Mobile phase B was prepared by mixing 1,000 mL of acetonitrile and 1 mL of formic acid. The analysis was performed in a gradient and isocratic mode for 20 min. Elution was performed in a gradient using a mixture of mobile phase A and mobile phase B varying from 95 : 5 to 30 : 70 for the first 10 min. After that, the mixture of mobile phase A and mobile phase B (95 : 5) was isocratic from 10 min to 20 min. The flow rate was 0.8 mL/min and the injection volume was 20 μL [11]. The concentration was calculated using the peak area acquired with the standard materials. Each sample was analyzed in triplicate.

3. Crystallization Method

A crystal is produced and developed on several surfaces (crystallizer wall, agitator surface, etc.) inside a crystallizer in the crystallization process because these surfaces eventually play a role in providing the surface area required for crystallization. A diagram of the vancomycin crystallization process with increased surface area per volume of reaction solution is shown in Fig. 1. First, a sample was dissolved in distilled water of pH 2.5 (with 1 N hydrochloric acid) and conductivity of 20 ms/cm (with sodium chloride). Since sodium chloride has no influence on H^+, the pH was adjusted first. Acetone (3.5 mL) was slowly added drop-wise to the vancomycin solution (1.0 mL) while stirring. Ion exchange resin was then added to increase the surface area per volume of reaction solution (S/V), and the solution was maintained at 10 °C to induce a vancomycin crystal. A cation exchange resin (Amberlite 200, Amberlite IR 120 (Na), Amberlite IR 120 (H), or Amberlite IRC 50; Rohm and Haas, USA) or an anion exchange resin (Amberlite IRA 400 (Cl), Amberlite IRA 400 (OH), or Amberlite IRA (910); Rohm and Haas) was used. To change the ionic form of the ion exchange resin, an ion exchange resin whose ionic form was Na^+ and OH^- was stored in 1 N HCl solution for 24 hr and an ion exchange resin whose ionic form was H^+ and Cl^- was stored in 1 N NaOH for 24 hr. Then, it

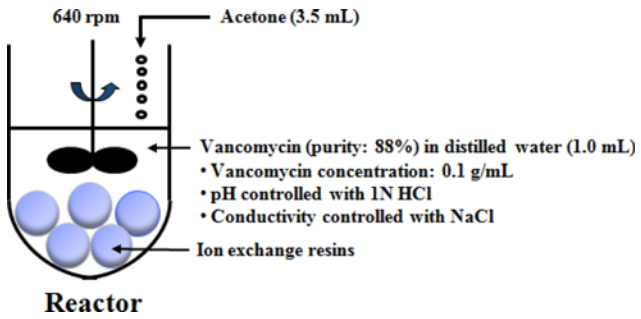

Fig. 1. Schematic diagram of increased surface area crystallization using ion exchange resin for purification of vancomycin.

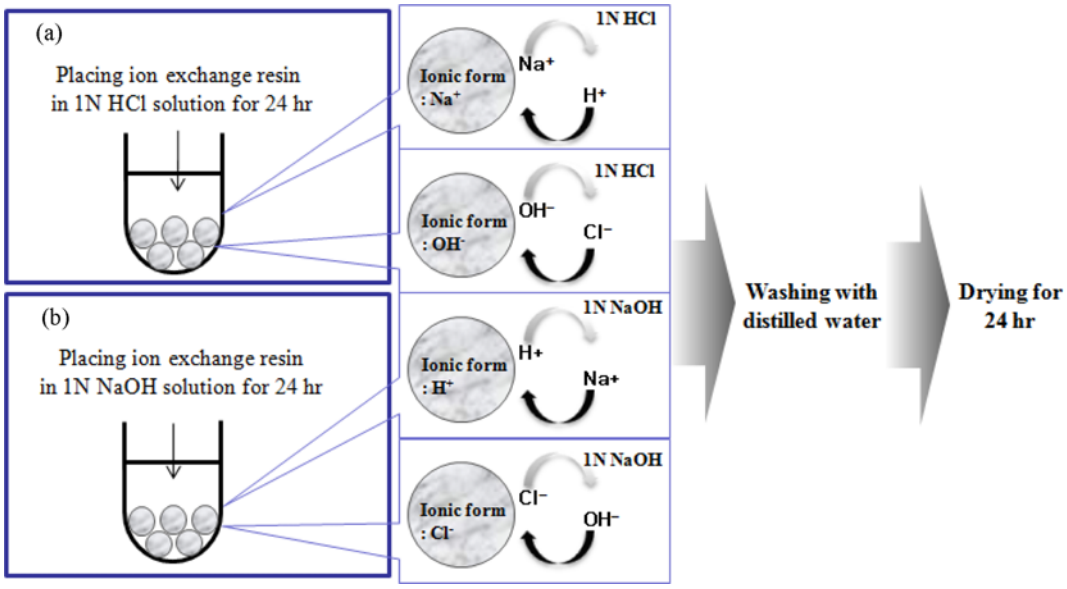

Fig. 2. Schematic diagram of the ion exchange of ion exchange resin. (a) Ion exchange resin with Na^+ or OH^- ionic form [Amberlite IR 120 (Na), Amberlite IRA 400 (OH), Amberlite 200]; and (b) ion exchange resin with H^+ or Cl^- ionic form [Amberlite IR 120 (H), Amberlite IRA 400 (Cl), Amberlite IRC 50, Amberlite IRA 910].

Table 1. Effect of ionic form on vancomycin crystallization at 12 hr

Ion exchange resin	Ionic form before ion exchange	Vancomycin crystallization	Ionic form after ion exchange	Vancomycin crystallization
Amberlite IR 120 (Na)	Na^+	Yes	H^+	No
Amberlite IR 120 (H)	H^+	No	Na^+	Yes
Amberlite IRA 400 (OH)	OH^-	No	Cl^-	Yes
Amberlite IRA 400 (Cl)	Cl^-	Yes	OH^-	No
Amberlite IRC 50	H^+	Yes	Na^+	No
Amberlite IRA 910 (Cl)	Cl^-	Yes	OH^-	No
Amberlite 200	Na^+	Yes	H^+	No

was washed with distilled water and dried for 24 hr (Fig. 2). In this process, the ionic form was changed from H^+ to Na^+, from Na^+ to H^+, from OH^- to Cl^- and from Cl^- to OH^-. All types of ion exchange resins used in this study were dried for 1 day at 60 °C prior to use in experiments. Vancomycin crystallization was conducted using ion exchange resin prepared for investigating the effect of the ionic form. Furthermore, to investigate which aspect of vancomycin crystallization depends on the pH of the crystallization solution, crystallization was performed with changing pH (4.3-7.2) of a solution to which no surface area-increasing material was added. The pH was adjusted by adding HCl or NaOH and the solutions were stored at 10 °C for 24, 36 and 48 hr to induce a vancomycin crystal in each pH environment. The vancomycin crystal produced was filtered using filter paper (150 mm, Whatman) and also washed with acetone to remove impurities from the crystal surface at the same time. In addition, HPLC and XRD analysis were conducted to determine the purity, yield and crystallizability of vancomycin after 24 hr of vacuum drying.

4. Analysis of Vancomycin Morphology and Size

The morphology and size of vancomycin particles formed during crystallization were determined using a video microscope (SV-35 Video Microscope System; Sometech, Korea) [9,10]. Precipitates were observed under high magnification (500×). Crystal morphology and size were determined from video images using the IT-Plus System (Sometech).

5. XRD Analysis

The morphology of vancomycin was analyzed by means of an X-ray diffractometer (MiniFlex 600, Rigaku) operated by the WINHRD 3000 program. XRD measurements were performed in the 5 to 25° 2θ range using CuKα radiation (40 kV, 15 mA) as the X-ray source. The amount of each sample was about 50 mg.

RESULTS AND DISCUSSION

1. Effect of Ionic Form of the Ion Exchange Resin

In the vancomycin crystallization process with increased S/V, the vancomycin crystallization efficiency varied depending on the type of ion exchange resin use to increase the surface area [10,11]. In the control group, to which a surface area-increasing material was not added, the vancomycin crystal was formed at 24 hr of crystallization, whereas the vancomycin crystal was formed at 12 hr of crystallization when a cation exchange resin (Amberlite 200, Amberlite IR 120 (Na), Amberlite IRC 50) or anion exchange resin (Amberlite IRA 400 (Cl), Amberlite IRA 910) was used to increase the surface area so that crystallization efficiency could be considerably improved. However, the vancomycin crystal was not formed 24 hr later when Amberlite IR 120 (H), a cation exchange resin, or Amberlite IRA 400 (OH), an anion exchange resin, was used to increase the surface area [11]. To determine the reason why the crystallization efficiency varied depending on the material used to increase the surface area, we investigated the effect of the ionic form of the ion exchange resins used to increase surface area. The ionic forms of the cation exchange resins Amberlite 200, Amberlite IR 120 (Na), Amberlite IRC 50, and Amberlite IR 120 (H) and the anion exchange resins Amberlite IRA 400 (Cl), Amberlite IRA (910), and Amberlite IRA 400 (OH) are Na^+, Na^+, H^+, H^+, Cl^-, Cl^-, OH^-, respectively. To determine the effect of the ionic form of the ion exchange resin on vancomycin crystallization, the ionic form of the ion exchange resin was changed from H^+ to Na^+, from Na^+ to H^+, from OH^- to Cl^- and from Cl^- to OH^-, by ion exchange methods (Fig. 2) for crystallization and they were stored for 12 hr to induce vancomycin crystal formation. Table 1 shows whether or not the crystal was formed before and after ion exchange of the resin used for the crystallization process. For all types of ion exchange resin used for crystallization, the results before and after ion exchange were reversed; if the ionic form of the ion exchange resin was changed, whether or not the vancomycin crystal was formed was completely changed. Based on this result, we decided that there is a correlation between the ionic form of the ion exchange resin and crystal formation and the time required for crystallization. To confirm these results, we studied the effect of pH on the crystallization process. The pH has been known as a crucial process variable in the vancomycin crystallization process [9]. In the crystallization process (Fig. 1), the pH of a solution increased to 4.4-4.7 when the aqueous solution containing vancomycin (pH: 2.5) was added to acetone. When ion exchange resin was added to the crystallization solution to increase the surface area, the pH of the solution was changed depending on the ionic form of the ion exchange resin because its ionic form includes ions affecting pH. The ionic forms Na^+ and Cl^- do not affect pH, but H^+ and OH^- do. When the ionic form was H^+, the pH of the solution decreased because the H^+ concentration increased due to the exchange of Na^+ and H^+ in the solution. On the other hand, when the ionic form was OH^-, the pH increased because HCl in the solution was neutralized due to an increase in OH^- concentration in the solution by the exchange of Cl^- for OH^-. An ion exchange resin whose ionic form was Na^+ and

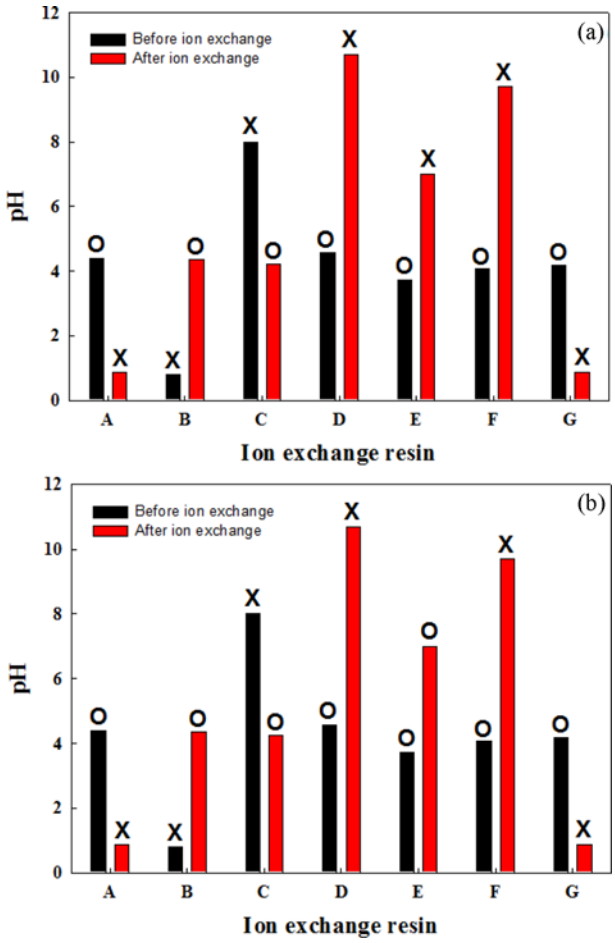

Fig. 3. Effect of ion exchange and pH on the vancomycin crystallization at 12 hr (a) and 24 hr (b). A: Amberlite IR 120 (Na), B: Amberlite IR 120 (H), C: Amberlite IRA 400 (OH), D: Amberlite IRA 400 (Cl), E: Amberlite IRC 50, F: Amberlite IRA 910, G: Amberlite 200). O: Crystallization, X: No crystallization.

Cl^- did not affect the pH of the solution. It may be because ion exchange did not occur owing to sufficient Na^+ and Cl^- caused by the addition of NaCl to control the conductivity of the crystallization solution. When an ion exchange resin whose ionic form was Na^+ and Cl^- was added, the pH was changed in the solution without added NaCl, but the conductivity and pH of the solution did not change when NaCl was added. In the crystallization process with increased surface area, the pH of the crystallization solution and whether or not the crystal was formed were determined and are shown in Fig. 3. Vancomycin crystal was formed when the pH of the solution before and after ion exchange of the ion exchange resin was 4-5 at 12 hr of crystallization (Fig. 3(a)). In the case of crystallization after ion exchange of Amberlite IRC 50, (pH of solution: ~7.0), a crystal was not formed at 12 hr of crystallization, but it was formed at 24 hr of crystallization (Fig. 3(b)). In other words, the vancomycin crystal was formed in the solution at a pH of 4-7 before and after ion exchange of the ion exchange resin at 24 hr of crystallization. Eventually, it was confirmed that changes in the pH of the crystallization solution depending on the ionic form of the

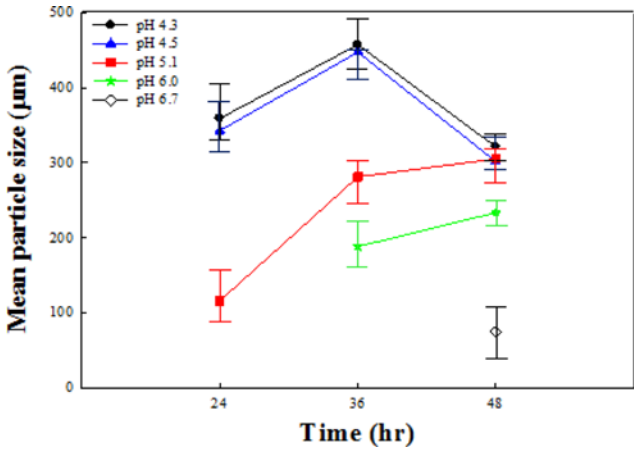

Fig. 4. The relationship between particle size and pH during vancomycin crystallization.

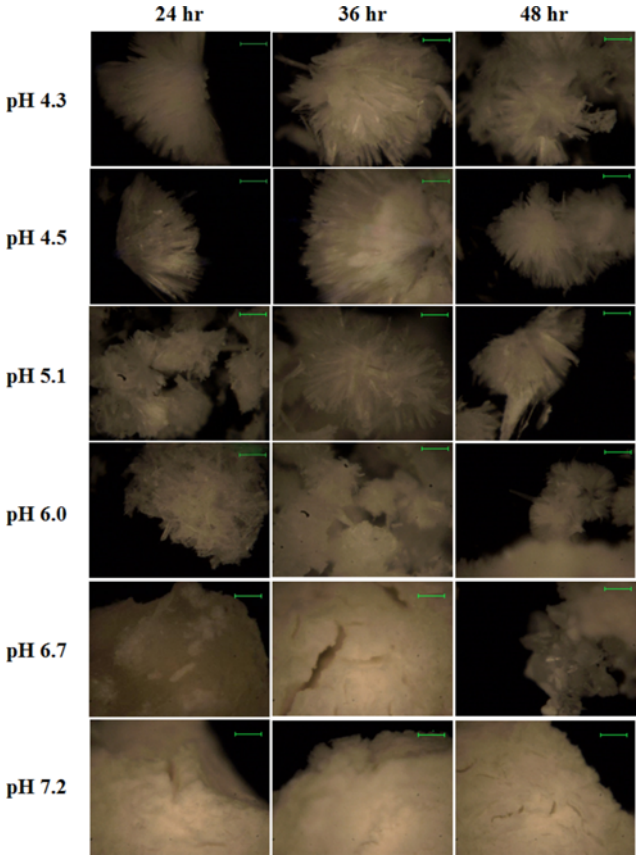

Fig. 5. Electron micrograph of vancomycin formed by crystallization at various pHs. Scale bar indicates 100 μm.

ion exchange resin influence vancomycin crystallization. It was also confirmed that there was a correlation between the pH of the solution and the time required for crystallization.

2. Effect of pH of the Crystallization Solution

To investigate in detail the effect of pH on the vancomycin crystallization process, crystallization was conducted with changes in the pH of the crystallization solution to 4.3-7.2 without adding ion ex-

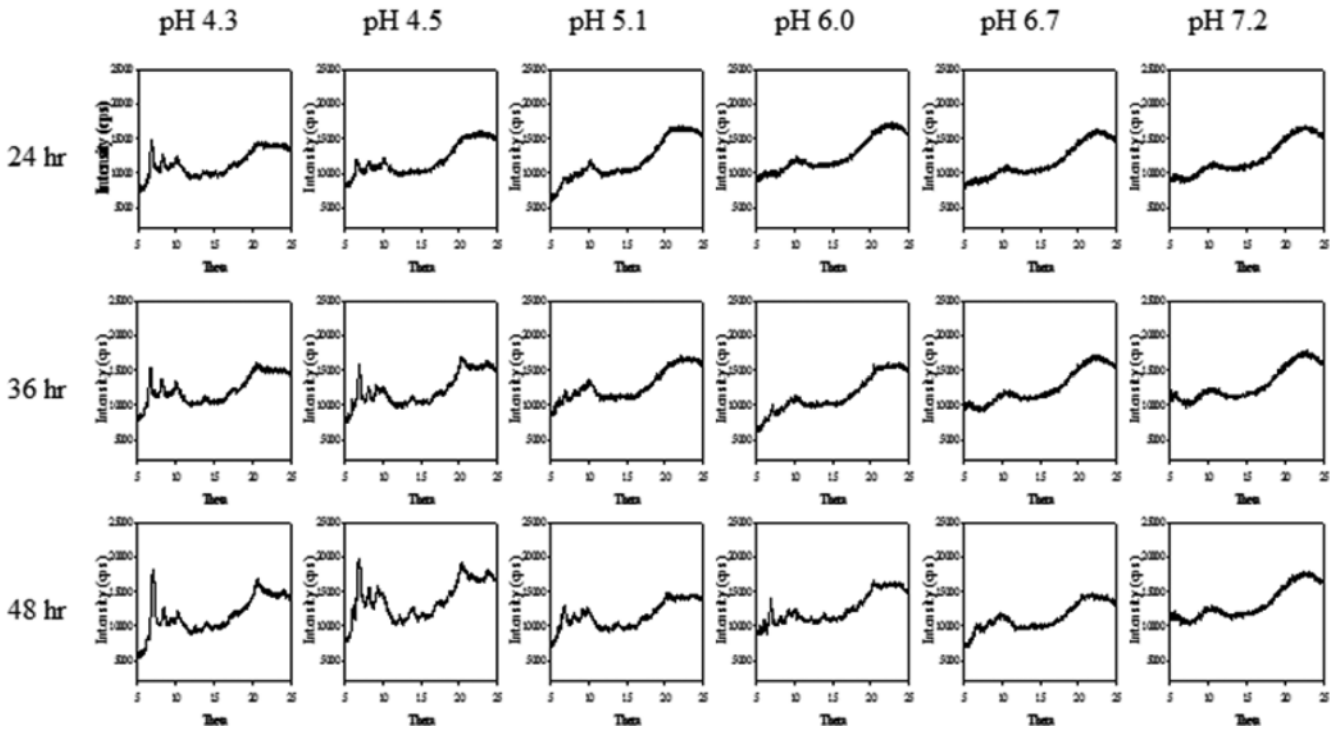

Fig. 6. XRD patterns of vancomycin formed by crystallization at various pHs.

change resin. As shown in Figs. 4-6, the time required for crystallization increased as the pH of the solution increased. At 24 hr of crystallization, a crystal was formed at a pH of 4.3-5.1 and up to pH 6.0 at 38 hr and pH 6.7 at 48 hr. However, the vancomycin crystal was not formed at a pH over 7 even as time passed. We observed the vancomycin crystal via electron microscope analysis (Fig. 5) under the condition in which the crystal was successfully formed and also confirmed crystallization by observing significant peaks via XRD analysis (Fig. 6). Based on the results of electron microscopy and XRD analysis, the crystallization time corresponded and the crystallizability of vancomycin increased as the crystallization time passed. Thus, we could confirm that the pH range of the solution to form vancomycin crystal is 4-7. In addition, the particle size of the vancomycin crystal tended to decrease as the pH of the crystallization solution increased (Fig. 4). Such a phenomenon is related to the crystallization time because the crystal growth time increases due to rapid crystal formation at lower pH. However, in the case of pH 4.3 and 4.5, the particle size decreased after 36 hr of crystallization, which might have been caused by crystal breakage due to frequent impacts of crystal particles or by secondary nucleation, making crystal particles smaller. Crystal was not formed at a pH of 4 or lower, which was caused by a difference in vancomycin solubility depending on pH. Vancomycin solubility was 2.97-17.7 mg/mL at a pH of 4-7 whereas it rapidly increased (more than 60 times) at a pH 4 or lower so that crystal formation was difficult [13]. According to the result of a previous study [14], vancomycin is stable at a pH of 2-7, and in this study we also observed that the color of vancomycin turned purple at a pH 7 or higher in the vancomycin crystallization, indicating that vancomycin had been denaturalized. There was no significant difference in the purity and yield of vancomycin according to the pH of the crystallization solution and the crystallization time (data not shown). The purity of the vancomycin sample for crystallization was 88% and it was 92-96% after 24 hr of crystallization. In addition, the yield was 95% and it rarely changed depending on the crystallization time.

CONCLUSIONS

We investigated the cause of the difference in the crystallization aspect depending on the surface area-increasing material (ion exchange resin) used in the vancomycin crystallization process with increased S/V. To confirm the effect of the ionic form of the ion exchange resin on vancomycin crystallization, the ionic forms of the cation exchange resins Amberlite 200, Amberlite IR 120 (Na), Amberlite IRC 50 and Amberlite IR 120 (H) and the anion exchange resins Amberlite IRA 400 (Cl), Amberlite IRA (910) and Amberlite IRA 400 (OH) used in the crystallization were changed from H^+ to Na^+, from Na^+ to H^+, from OH^- to Cl^- and from Cl^- to OH^-, respectively, using ion exchange methods. Whether or not crystal was formed before and after ion exchange was reversed for all of the ion exchange resins used in the study, the result completely changed if the ionic form of the ion exchange resin was altered. It was shown that the vancomycin crystal was formed at a pH of 4-7 before and after ion exchange of the ion exchange resin at 24 hr of crystallization. Eventually, it was confirmed that vancomycin crystallization was affected by changes in pH of the crystallization solution depending on the ionic form of the ion exchange resin. Furthermore, in the absence of ion exchange resin, the time required for crystallization increased as the pH rose from 4-7. This result was also confirmed by electron microscopy and XRD analysis, and it

was confirmed that the crystallizability of vancomycin increased as crystallization time passed. In addition, the particle size of the vancomycin crystal tended to decrease as the pH of the crystallization solution increased. There was no significant difference in the purity (92-96%) and the yield (>90%) depending on the pH of the crystallization solution and the crystallization time.

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