APPENDIX A

SUPPLEMENTARY INFORMATION

A.1 The induction time measurement by PVM100 (easy-viewer by Mettler Toledo) for papain cooling crystallization.

Papain saturation at 30 degree C in water was cooling by a rate of 0.005 degree C/min to 9 degree C (30 hours usage) with 50-100 rpm agitation (magnetic stir bar) and easy-viewer probe was added for observation the cloud point. In Figure A.1(a) the easy-viewer monitor showed uncount particle and clearly solution and after 15 hours, the cloud point started counting in Figure A.1(b). So, the total induction time was approximately 35 hours to nucleation, about 7 hours to growth and for none agitation was usaged about 50 hours to nucleation.

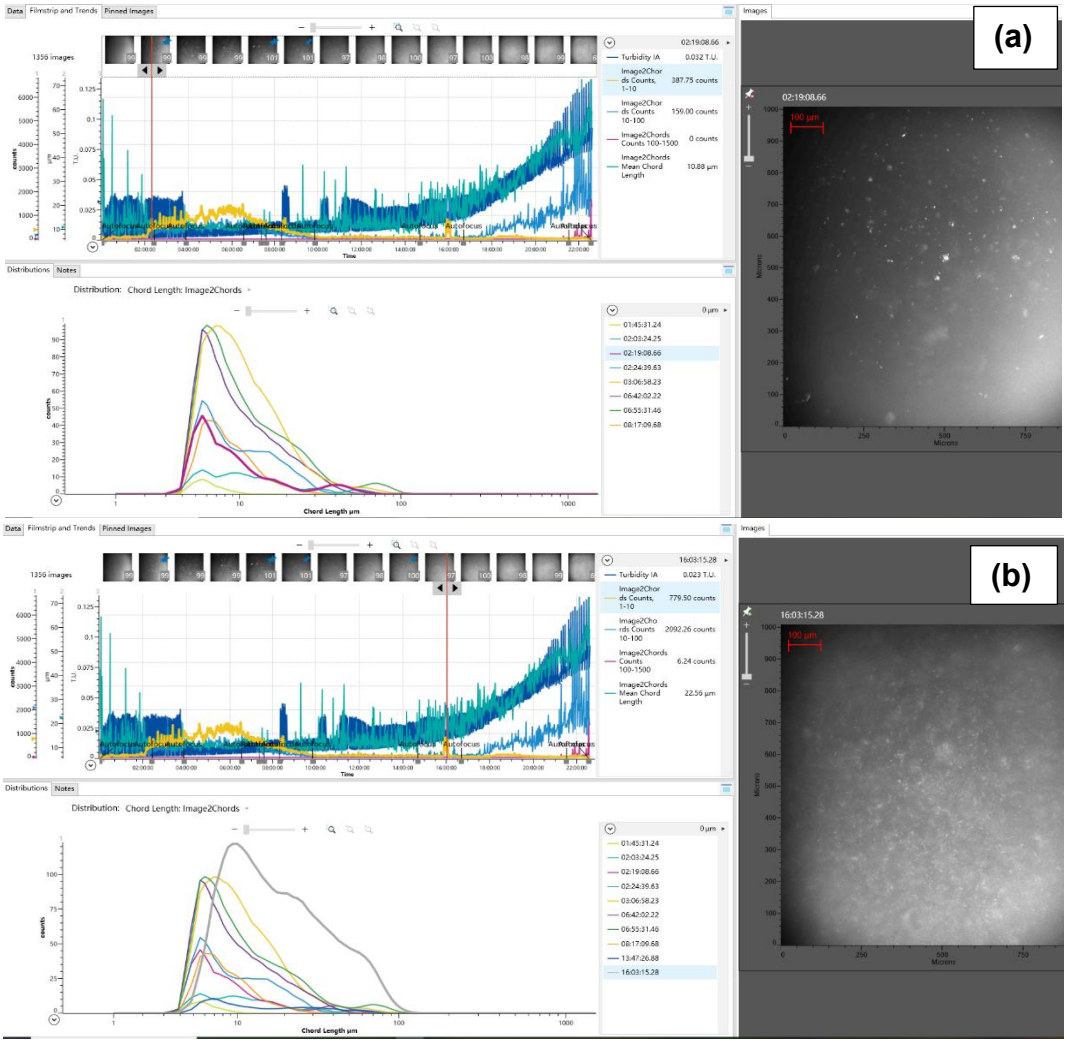

Figure A.1 Easy-viewer (100) probe observation the cloud point.

APPENDIX B

PUBLICATION AND PRESENTATIONS

B.1 List of publications

Chonut Xaiyathoumma, Penchit Chitnumsub and Lek Wantha (2025). Papain Crystallization Using Solvent Freeze-Out Crystallizer. **The 2025 Pure and Applied Chemistry International Conference (PACCON 2025),** E-proceeding April 30th, 2025.


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NP-P-33

Papain Crystallization Using Solvent Freeze-Out Crystallizer

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Abstract:

To enhance the efficiency of industrial enzyme crystallization, important parameters such as the solubility curve must be understood. The solubility of papain was measured using the gravity method in an acetate buffer solution (pH 5) and in a buffer solution with methanol concentrations ranging from 0 to 0.6 weight fraction, while controlling the temperature at 20°C, 10°C, 0°C, and -8°C. Nucleation was studied by starting using saturated solutions. The solvent freeze-out (SFO) crystallization technique, conducted near the freezing point of water. The results showed that methanol reduced the solubility of crystalline papain, resulting in smaller nucleation at 0°C. Amorphous solid papain was obtained from the antisolvent crystallization. However, methanol had no effect on the enzyme activity of papain. Papain crystals obtained by the SFO method had a needle-like shape. The activity of the recovered papain crystals remained stable, with a 57% recovery, and 20% recovery for the saturation concentration of 0.4 g/mL and 0.33 g/mL, respectively.

1. Introduction

Crystallization enables the preservation of macromolecule properties and serves as an alternative method in the enzyme purification in food and pharmaceutical industries[1,2]. The crystallization of papain is quite challenging due to the various driving forces involved. Papain has an against microorganism ability and it is also intimate in the skincare's production area[3,4]. Therefore, it would be valuable if crystalline papain is incorporated into the supply chain. There are two mechanisms of crystallization including crystal nucleation and crystal growth. Nucleation is the initial stage of crystal formation. Supersaturation, agitation, time, and type or amount of anti-dissolution agent also affect nucleation[5,6]. Papain is also crystallized difficulty, but it has been successfully crystallized using methanol as a precipitant at pH5[7] with needle-like crystals[8]. Therefore, the methodology for crystallizing papain on an industrial scale should continue to be studied and refined. The effect of the amount of methanol on solubility should be considered before crystallization study. The solubility measurement using the gravimetric method is consistent, and nucleation studies by anti-solvent, monitoring by Focus Beam Reflectance Measurement (FBRM) or easy-viewer in solution, are also more suitable for industrial scale-up purposes[9]. However, from the preliminary crystallization of papain using methanol and measuring nucleation particles with FBRM, it was found that the solution sometimes becomes cloudy, but no particles were detectable. This is related to Andrea Sauter's criticism of the two-step nucleation process for some protein crystallizations[10]. In the first step, a solute or macroscopic dense liquid phase forms a metastable intermediate phase (nano-sized), followed by the second step, where nucleation occurs within that phase. This is consistent with a 2021 review that revises the solvent freeze-out (SFO) technology as an efficient method for protein crystallization[11]. The SFO process performs crystallization near the freezing point of water. The water content in protein/enzyme solution will be decreased by formation of ice on the freezing coil, which increases the solution concentration until the nucleation point is reached. However, this method has many parameters that need to be considered. In this study, a laboratory scale SFO crystallizer was set up. The cooling rate of freezing coil was fixed at 0.02°C/min as a step-down of temperature to avoid papain lost in ice[11]. The SFO process was prepared at close to 0°C saturation. The saturation of the solution was measured by the gravimetric method and with various methanol fraction. Supersaturation measurements using anti-solvent were also conducted and observed with an Easy-Viewer camera under the liquid. Methanol was added stepwise at a rate of 0.2 mL/20 min. It provides slow nucleation, to define first cloudy zone well and reduce wasting papain solution[12]. Therefore, this work focuses on nucleation points observation in the SFO process, and comparison products using methanol versus using the SFO method.

2. Materials and Methods

2.1 Materials

Crystalline papain (white powder) was purchased from Shaanxi Yuantai Biological Technology Co., Ltd (YT0829) and crystalline papain 2xUSP (14049) was purchased from Sisco Research Laboratories Pvt. Ltd (SRL). Sodium acetate trihydrate, ethylenediaminetetraacetic acid (EDTA), dimethylsulfide (DMSO), hydrochloric acid, and methanol were purchased from RCI LabscanLimited. Tris (hydroxymethyl-aminomethane) was from Carlo Erba. Glacial acetic acid was purchased from QReC New Zealand. Nα-Benzoyl-DL-arginine-4-nitroanilide-hydrochloride (BAPNA, B4875-1G) was purchased from Sigma-Aldrich. L-cysteine hydrochloride monohydrate (GRM046) was purchased from Himedia. All chemicals and reagents used were of analytical grade.

2.2 Solubility measurement

The solubility (saturation) was measured by the gravity method[12]. An excess amount of papain powder was dissolved in the acetate buffer solution (pH5) and in the buffer solution plus methanol at weight fractions of 0 to 0.6 while controlling the temperature at 20, 10, 0, and -8°C to observe the saturated points. The clear solution was sampled using syringe filters and monitored with a refractometer (Refractive Index, RI) every hour. The filtrated solution was then evaporated to dryness to determine the solution concentration.

2.3 Solvent Freeze-Out Crystallization

The solvent freeze-out (SFO) crystallization technique near the freezing point of water was conducted for papain solution at pH 5.[13]. The SFO crystallizer is a 50-mL jacket crystallizer assembly with a glass cold finger (freezing coil) that is temperature-controlled by a second thermostat. The setup is shown in Figure 1. The first thermostat controlled the jacket's temperature for saturation solution. The second thermostat manually lowered the temperature of the freezing coil at a rate not exceeding 0.20°C every 10 minutes (0.02°C/min), maintaining a stepwise reduction to the final temperature to prevent papain loss in the ice.[11, 14].

The final temperature of the freezing coil depended on the different saturation temperatures and continued freezing until the concentration of the papain solution reached supersaturation. Approximately 1-3 mL of the papain solution remained from the initial 7 mL, or about 14-42% of the final volume of solution. After crystallization occurred, the solution was maintained at the same nucleation temperature until growth ceased, and the papain crystals were harvested. The growth continued for approximately 72 hours. The weight of papain lost in the ice of the freezing coil and the recovery of papain crystals were calculated by Equations 1 and 2, respectively. The nucleation points, or supersaturation points, were marked on the phase diagram, as shown in Figure 3.

$$\text{Mass lost papain\%} = \frac{B}{i} \times 100\% \quad (1)$$

$$\text{Recovery\% of crystalline papain} = \frac{C}{i} \times 100\% \quad (2)$$

where B is the mass (g) of papain in ice per volume; i is the initial mass (g) of papain in saturated solution before the SFO process launching; C is dry mass (g) of papain crystal after the process. The crystal was captured by microscope.

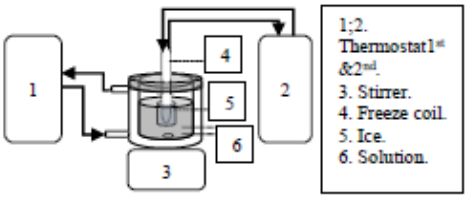

Figure 1. The SFO crystallizer set-up.

2.4 Assays of Enzymatic Activity and PXRD

The enzymatic activity of papain from commercials supplier, an anti-solvent, and the SFO process were measured by Anorn Ruth's method[15-17]. Crystalized papain was confirmed by powder X-ray diffraction (PXRD, D2-Phaser, BRUKER).

The substrate solution was prepared by dissolving 43.5 mg of BAPNA in 1 mL of DMSO and the volume was adjusted to 100 mL with Tris-buffer (0.05 M Tris, pH 7.5 by HCl, containing 0.005 M cysteine and 0.002 M EDTA). All the solid papain was dissolved in DI-water at a concentration of 30 mg/mL and measured by UV-vis spectroscopy (DR6000, Hach, Ames, IA, USA) at 280 nm (Quartz U-shaped cuvette, Di-water was blank), to confirm the concentration. One milliliter of the papain solution was reacted with 5 mL of the BAPNA substrate in test tubes at 25°C for 25 min. The reaction was stopped by adding 1 mL of 30%v/v acetic acid before measuring the absorbance by UV-vis at 410 nm (5 mL substrate + 1 mL acetic acid was used as the blank), corresponding to the liberated p-nitroaniline. The absorbance values of the product were used in calculation by Equation 3 for substrate hydrolysis (BAPNA Units) and specific activity as Units per mg of papain. In the same way, the BAPNA activity is

defined as the enzyme hydrolyzing 1 micromole of substrate per minute.

$$\text{BAPNA Units}=\frac{Abs_{410nm}}{25min}\times\frac{3\times1000}{8800} \quad (3)$$

$$\text{Specific activity}=\frac{\text{Units}}{mg}=\frac{\text{BAPNA Units}}{30mg} \quad (4)$$

where Abs_{410nm} is the absorbance of product p-nitroaniline (yellow); t is 25 min of enzymatic reaction; and 8800 M^{-1} cm^{-1} is the p-nitroaniline molar extinction coefficient at 410 nm. The enzyme activity was measured immediately after complete dissolution.

3. Results & Discussion

3.1 Solubility

The solubility of papain in mixture of acetate buffer (pH5) and methanol is illustrated in Figure 2. The result showed that the papain solubility decreased with increasing methanol weight fraction and decreasing temperature.

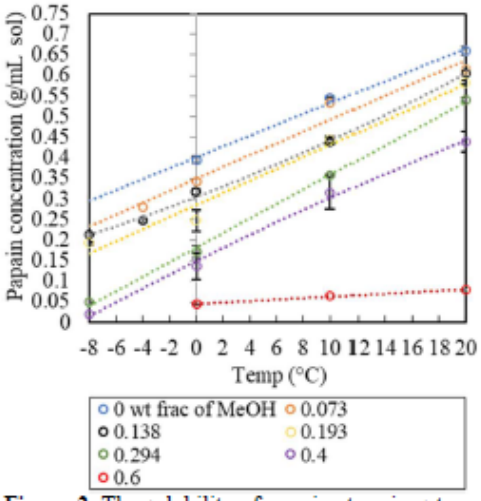

Figure 2. The solubility of papain at various temperatures and methanol fractions.

3.2 The SFO Crystallization

Solvent freeze-out crystallization was carried out at three concentration levels: supersaturation (0.472 g/mL), saturation (0.4 g/mL), and undersaturation (0.33 g/mL), respectively to observe the nucleation point with different temperatures (-1.5, -0.5, and 1°C). The phase diagram used for the SFO process of papain is shown in Figure 3. The nucleation point was quite high.

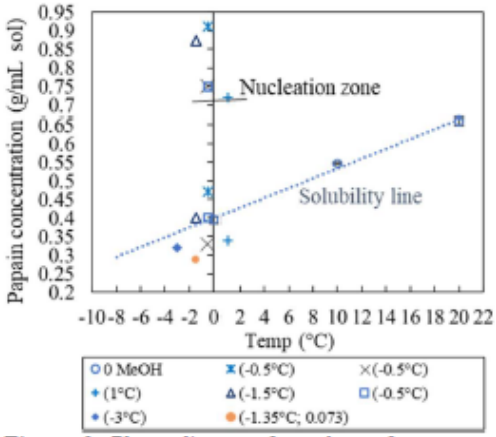

Figure 3. Phase diagram for solvent freeze-out crystallization of papain solutions.

Figure 4 describes the control of the freezing coil temperature and the solution temperature of the saturated papain solution (0.47 g/mL, 6.6 mL) from the point the coil was immersed in the solution (-3°C) until the freezing and nucleation occurred. Ice started to form on the freezing coil at -5°C, and ice growth continued at -6.8 °C until the final coil temperature reached -12.6 °C. The temperature was held constant at -12.6°C until the papain solution volume was reduced to 2-3 mL, at which point stirring was initiated at 120 rpm for 10 min. Then, the solution was kept until nucleation, which occurred at hour 188 (24 hours after stirring). At this point, the freezing coil was turned off. The solution concentration was measured immediately at the nucleation point by reflectometer and it was kept at constant temperature until growth stopped. Crystals harvesting and characterization followed this. The nucleation process, crystal growth images, and photomicrographs of papain crystals obtained from SFO crystallization are shown in Figure 5. At the time of nucleation, the final volume was 2.6 mL, and the nucleation concentration was 0.91 g/mL (Figure 3).

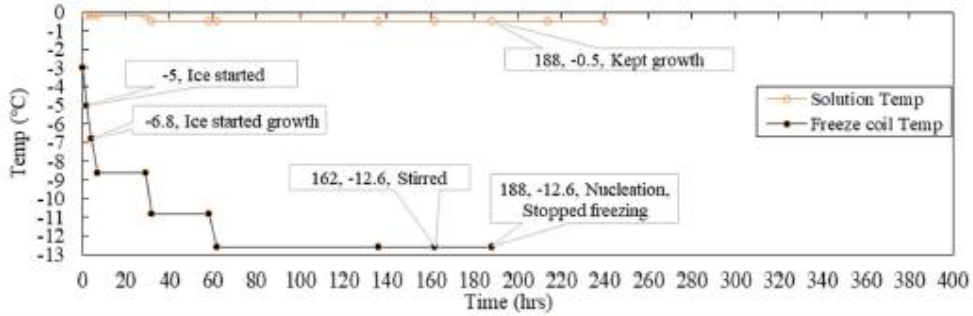

Figure 4. The temperature reduction profile of the freezing coil for initial 0.47 g/mL papain solution at -0.5°C.

Figure 5. The SFO crystallizer and photomicrographs of papain crystals obtained from SFO crystallization of initial 0.47 g/mL papain solution at -0.5 °C.

Figure 6 describes the control of the freezing coil temperature and the solution temperature of the saturated papain solution 0.4 g/mL (7 mL) from the point the coil was immersed in the solution (-3 °C) until the freezing and nucleation occurred. Ice started growing at -6.6 °C and continued until the final coil temperature reached -12.6 °C. The temperature was held constant at -12.6 °C, and stirring was initiated at 120 rpm for 10 min at hour 148. Then, the solution was observed for 72 hours and re-stirred at hour 233 due to no nucleation occurring. Nucleation started at hour 311 (3 days + 10 hours after stirring). It appeared a bit cloudy and was left for another 20 hours to confirm the process. The freezing coil was turned off at hour 331. At this point, the solution concentration was 0.7449 g/mL (2 mL), and it was maintained. The crystals were harvested after 72 hours. The nucleation process, crystal growth images, and photomicrographs of papain crystals obtained from SFO crystallization are shown in Figure 7.

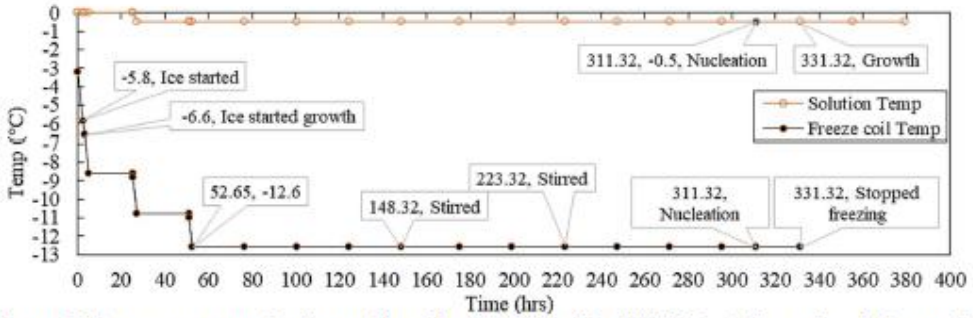

Figure 6. The temperature reduction profile of the freezing coil for initial 0.4 g/mL papain solution at -0.5°C.

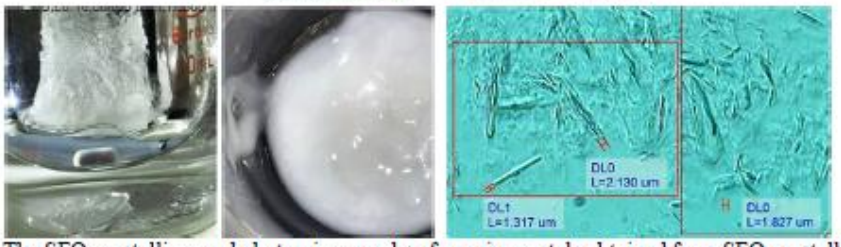

Figure 7. The SFO crystallizer and photomicrographs of papain crystals obtained from SFO crystallization of initial 0.4 g/mL papain solution at -0.5°C.

Figure 8 describes the control of the freezing coil temperature and the solution temperature of the saturated papain solution 0.33 g/mL (7 mL) from the point the coil was immersed in the solution (-3 °C) until the freezing and nucleation occurred. Ice started growing at -3.6 °C, and the final coil temperature reached -12.6 °C, at hour 60. The temperature was held constant at -12.6°C, and stirring started at hour 140 and re-stirred at 182. The solution was then observed for 72 hours and re-stirred at hour 254 due to no nucleation occurring, although it became slightly sticky. The freezing coil was turned off at hour 254, and the solution was stored. A slight nucleation was observed at hour 278. The solution concentration was 0.7482 g/mL (1.9 mL), and it was kept for further growth. The crystals were harvested after 72 hours. So, for this initial concentration the freezing coil should be turned off at around -12.2°C to avoid high viscosity. The nucleation process, crystal growth images, and photomicrographs of papain crystals obtained from SFO crystallization are shown in Figure 9.

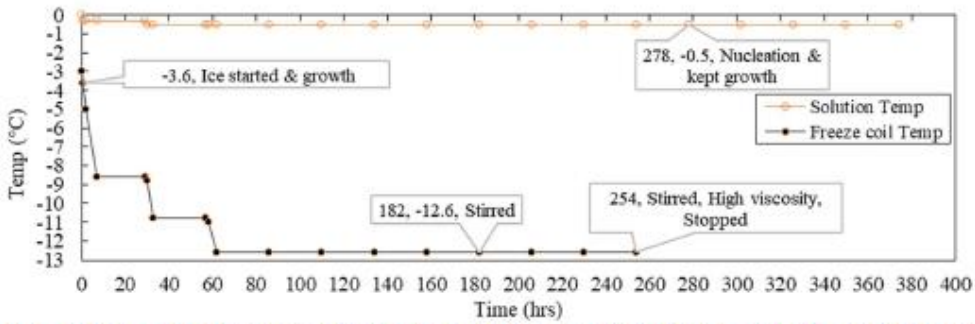

Figure 8. The temperature reduction profile of the freezing coil for initial 0.33 g/mL papain solution at -0.5°C.

Figure 9. The SFO crystallizer and photomicrographs of papain crystals obtained from SFO crystallization of initial 0.33 g/mL papain solution at -0.5°C.

The SFO process for 0.47, 0.4, and 0.33 g/mL initial papain concentration at -0.5°C influenced the percent recovery, as shown in Table 1. The percent recoveries were 64%, 57%, and 20% for 0.47 g/mL, 0.4 g/mL, 0.33 g/mL, respectively. However, all three concentrations unlost more than 26%, indicating that a freezing rate of 0.02°C/min[11] is effective. The initial

concentrations of 0.4 and 0.33 g/mL took time to nucleate more than 200 hours. However, at an initial 0.33 g/mL papain solution reached nucleation after the freezing coil was stopped, likely due to the high viscosity that developed on the surface of the papain solution. This is because the lower concentration allowed the ice to grow faster than at the higher initial papain concentration. Even though, all the recovered crystals exhibited a needle-like crystal shape and growth, which indicates the crystal shape of papain.[8]

Table 1. Comparison of the percent lost into the freezing coil and recovery of papain crystallization using SFO at solution temperature of -0.5 °C.

Initial Concentration, g/mL	Final Volume, mL	%Recovery	%Lost in Ice
0.47	2.6	64	21
0.4	2	57	26
0.33	1.9	20	21

3.3 The Enzymatic Activity and PXRD

The papain powder was dissolved in DI water, and its concentration was prepared and calculated from the prepared calibration curve shown in Figure 10. The calibration curve of papain was measured at 280 nm before reacting with the substrate. The parched crystalline papain, papain solid obtained from antisolvent crystallization using methanol, and papain crystals obtained from the SFO crystallization were each tested at 30 mg/mL for reaction with the substrate and the resulting product was measured at 410 nm, as shown in Figure 11. The activity was then calculated, and the specific activity is shown in Figure 12. The results showed no reduction in activity. This work was further examined using PXRD, as shown in Figure 13. The product obtained from the SFO process had high crystallinity, while the product obtained from anti-solvent crystallization was amorphous.

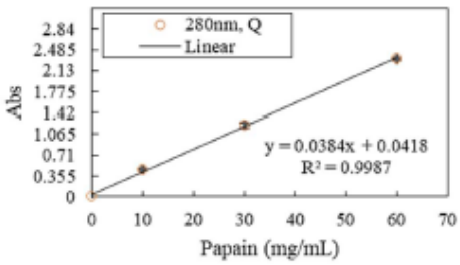

Figure 10. The calibration for determining the concentration obtained from UV-vis spectroscopy at 280 nm.

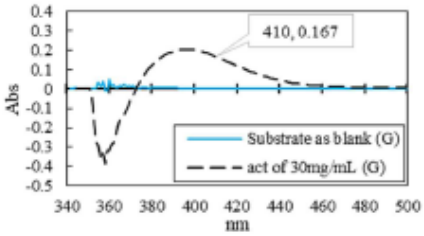

Figure 11. The product was analyzed using UV-vis spectroscopy at 410 nm.

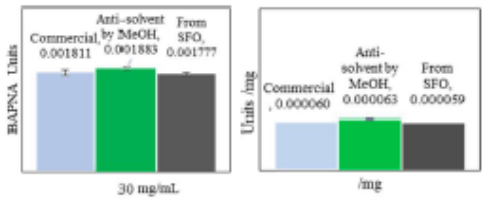

Figure 12. Enzyme activity of purchased papain (left), antisolvent crystallization (middle), and SFO (right).

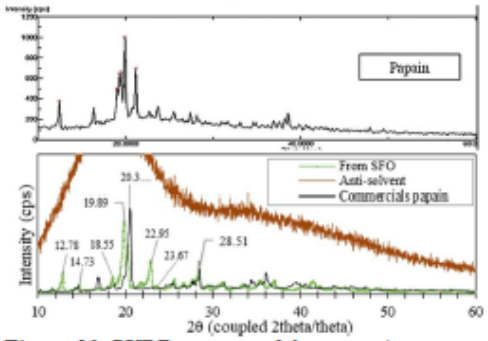

Figure 13. PXRD patterns of these papain crystals obtained from this work (Bottom) and previous work (Top)[18].

4. Conclusion

The SFO crystallization itself involves complex parameters that need to be controlled. However, this study successfully crystallized papain from solution at a solution temperature of -0.5°C, with concentrations ranging from 0.33 to 0.47 g/mL, and a final freezing coil temperature of -12.6°C. The crystals obtained from the SFO crystallization were needle-like. In contrast, antisolvent crystallization using methanol produced amorphous-like. Furthermore, the effect of methanol on the solubility of papain is consistent, and the specific activity remains stable.

Acknowledgments

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B.2 List of presentations

Chonut Xaiyathoumma, Penchit Chitnumsub and Lek Wantha (2022). Preliminary Study of Lysozyme Crystallization in Ammonium Sulfate Solution using Solvent Freeze-Out Crystallizer. **The 31st Thai Institute of Chemical Engineering and Applied Chemistry Conference (TIChE2022),** which held online on March 15th-16th, 2022, organized by The Thai Institute of Chemical Engineering and Applied Chemistry (TIChE) and the Department of Chemical Engineering, Naresuan University, Thailand. (Poster presentation)

Chonut Xaiyathoumma, Penchit Chitnumsub and Lek Wantha (2023). The Study of **α**-amylase Crystallization from Commercializes **α**-amylase Broth with various Salt Precipitants. **The 32nd Thai Institute of Chemical Engineering and Applied Chemistry Conference (TIChE2023)**, Nakhon Pathom, Thailand, March 16th-17th, 2023, organized by The Thai Institute of Chemical Engineering and Applied Chemistry (TIChE) and the Department of Chemical Engineering, Silpakorn University. (Oral presentation)

Chonut Xaiyathoumma, Penchit Chitnumsub and Lek Wantha (2023). The Nucleation Zone Screening of Crystalline Papain from Solution by Dropwise Methanol Cooling Crystallization. **The 12th International Symposium on Nano & Supramolecular Chemistry (ISNSC-12) & The 3rd Thailand Biorefinery Symposium (TBioS-3)**, Chiang Mai, Thailand, July 23rd-26th, 2023, organized by Thammasat University's Faculty of Science and Technology. (Oral presentation)

Chonut Xaiyathoumma, Penchit Chitnumsub and Lek Wantha (2023). Nucleation of Papain using Methanol as Precipitant. **The 6th Asian Crystallization Technology Symposium (ACTS2023)**, **(Virtual Conference),** Taipei, Taiwan, September 25th-26th, 2023, organized by National Central University, National Taiwan University, National Taipei University of Technology, and Chang Gung University. (Oral presentation)

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