CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemicals and reagents

Crystalline papain (white powder; CAS No. 9001–73–4) was obtained from three suppliers: a) Shaanxi Yuantai Biological Technology Co., Ltd. (YT0829), b) Tianjin HEOWNS Biochemical Technology Co., Ltd. (P–01102; provided with support from NERCICT, Tianjin University), c) Sisco Research Laboratories Pvt. Ltd (SRL; 2× USP grade, 14049), and d) Crystalline Lysozyme from Sigma-Aldrich (62971-50G-F) as following in Figure 3.1.

Sodium acetate trihydrate, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), hydrochloric acid, ammonium sulfate, and methanol were purchased from RCI LabScan Limited (Thailand). Tris(hydroxymethyl)aminomethane was purchased from Carlo Erba. Glacial acetic acid was purchased from QReC (New Zealand).

Nα–Benzoyl–DL–arginine–4–nitroanilide hydrochloride (BAPNA; B4875–1G) was obtained from Sigma–Aldrich, and L–cysteine hydrochloride monohydrate (GRM046) from Himedia. All Chemicals and reagents were of analytical grade and used without further purification.

Figure 3.1: Papain powder from a) Shaanxi Yuantai Biological Technology Co., Ltd (YT0829), b) Tianjin HEOWNS Biochemical Technology Co., Ltd (P-01102), c) Sisco Research Laboratories Pvt. Ltd (SRL) (papain 2xUSP, 14049) and d) Lysozyme powder from Sigma-Aldrich.

3.2 Methods and apparatus

3.2.1 Preliminary lysozyme study to validate SFO setup and phase mapping tools

The phase-diagram is important information for crystallization. In this case of enzyme crystallization, the solubility of lysozyme was measured by Focused Beam Reflectance Measurement (FBRM) to study the effect of temperatures and salt concentration, and then solvent freeze-out (SFO) process was applied.

FBRM is an industry-standard measurement technique used for in-process measurement of particles. A highly precise chord length distribution (CLD), which is sensitive to both particle size and count, is reported in real-time without the need for sampling or sample preparation. No shape is assumed and the measurement can be applied at full process concentrations in opaque or translucent slurries and

emulsions. It is capable of monitoring particles from ranging 0.5-1000 μm at most process concentrations and within a temperature range of −10 to 120 °C. As showed in Figure 3.2 is the Particle Track™ G600B with FBRM® technology from METTLER TOLEDO used in lysozyme solubility study.

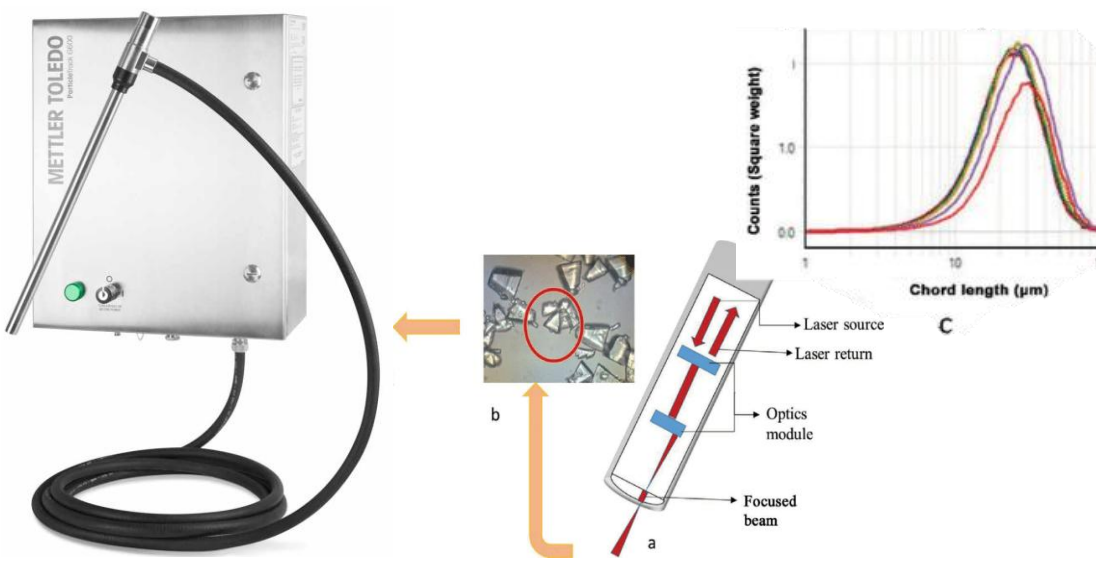

Figure 3.2: The Particle-Track™ G600B with FBRM (technology from METTLER TOLEDO) and its working principle.

The focus beam (probe a) was dived into solution b to measure the number and size of all the particles. The data were then displayed on the PC monitor as show in diagram C, which presents square wight versus particle size in micron.

The solubility measurement of lysozyme was conducted in 50 mL crystallizer which temperature was controlled by circulate water bath. The FBRM probe was positioned into solution in crystallizer and experiment began at −10 °C. The temperature was then gradually increased at a rate of 0.1 °C/min, negative temperatures were of particular interest as previous work (Yi-Bin Lin et al., 2008) have only reported lysozyme crystallization at temperatures no less than 4 degree C. Temperatures were recorded in real-time using a thermocouple. Complete dissolution of solid lysozyme was confirmed by FBRM that showed the zero particle count at the corresponding time. Those are the solubility points. Amount of solid lysozyme and salt (ammonium sulphate) were prepared in gram. The salt used were 0.4, 0.6, 0.7 M

dissolved in deionize water. These were to determine how different salt concentrations affected in the solubility (mg/mL) of lysozyme at various temperature. For each concentration, the temperature was increased until the solubility point was record. This process is illustrated in Figure 3.3. The highest temperature was 6 ℃. The result was plotted as concentration versus temperatures each salt concentration.

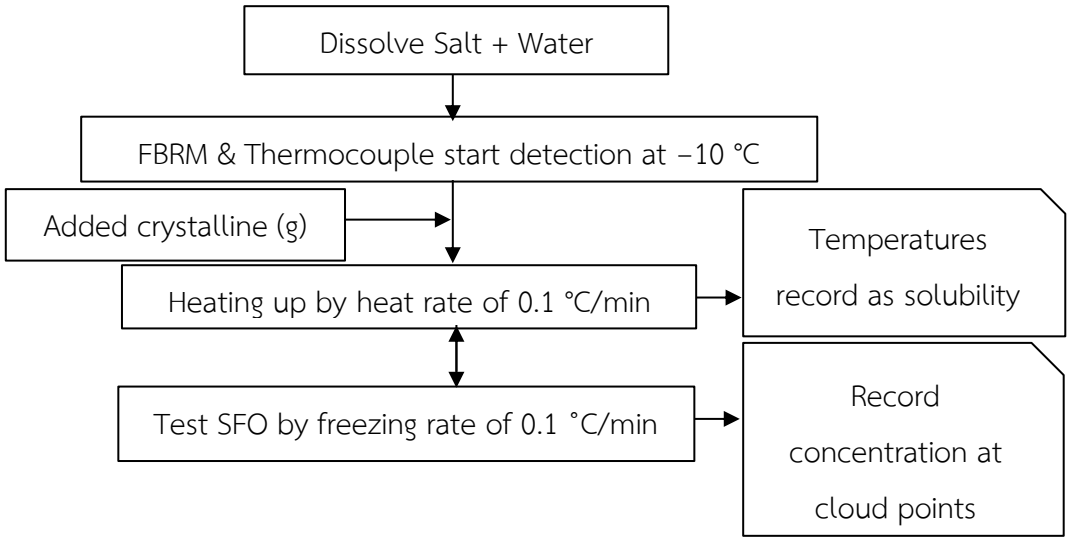

Figure 3.3: The process of solubility measurement for lysozyme and crystallization by the SFO technique.

The SFO crystallizer set up is shown in Figure 3.4. It consisted of a double wall beaker size as 100 mL and a freeze coil made from glass tube with an outside diameter of 2 cm. The first circulating water bath that controlled temperature of beaker was set up at 0 ℃. The freeze coil was controlled the temperature by second cooling system, which gradually decreased the temperature step-by-step initiating the formation of ice. The cooling rate was set to 0.1 ℃/min from 0 ℃ and decreasing to −10 ℃ (held at −10 ℃ for five minutes), to −15 ℃ (held for ten minutes) and finally down to −18 ℃ (held for approximately 14hours). By these controlled cooling process, the solution temperature of gradually decreased from 0 ℃ to minimum approximately −8 ℃ to avoid protein loss to the ice phase. At solution temperature between −1 ℃ to −4 ℃, the nucleation was observed by visually through the crystallizer and under a microscope. After 15hours, all crystals were collected by filtration as soon as possible while ensuring that the temperature did not higher than 5 ℃ in order to avoid re-

dissolution. Solid collected on the filter paper was stored in a desiccator for 4 days. The result was presented in a solution temperature and time diagram.

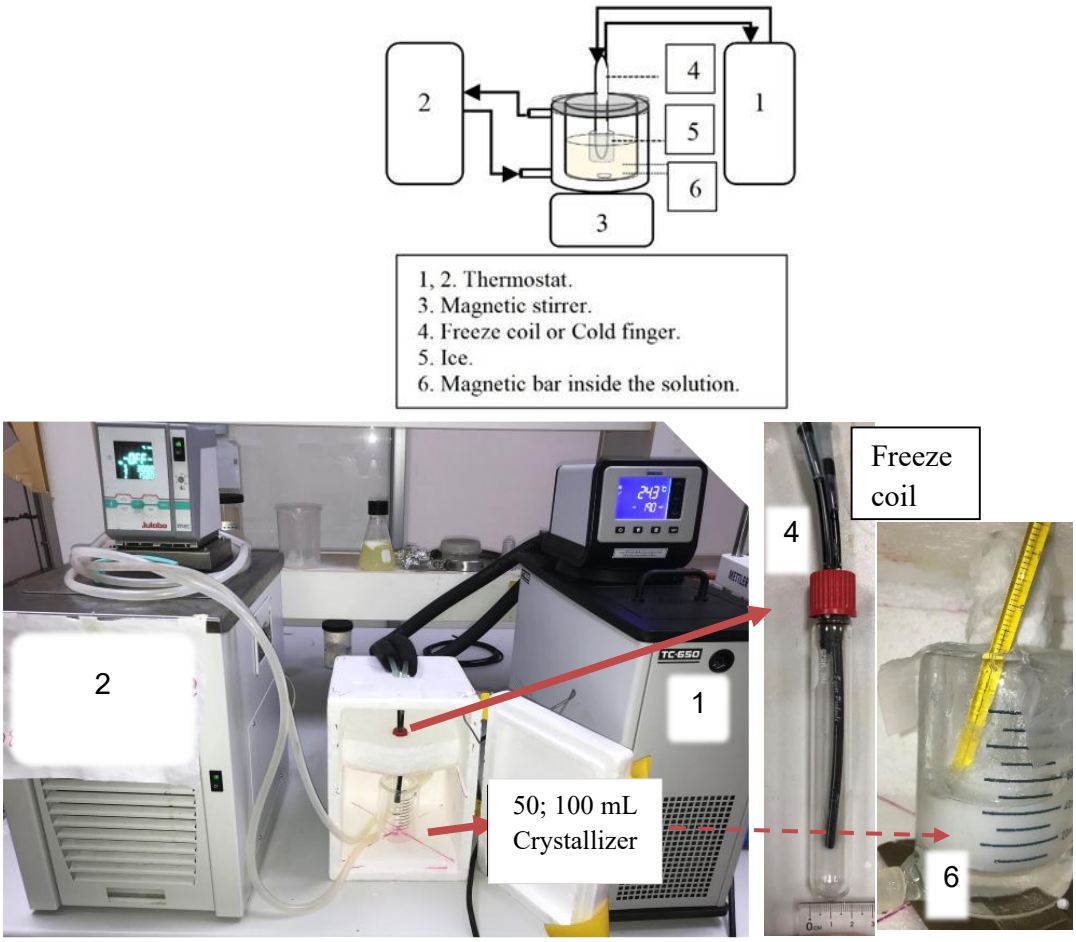

Figure 3.4: SFO crystallizer setup.

3.2.2 Solubility of papain (gravimetric and RI correlation)

The solubility (saturation concentration) of commercial crystalline papain was determined using the gravimetric method (Yu et al., 2017). An excess amount of papain powder was dissolved in three different solvents: DI water, acetate buffer (0.05M, pH5), and acetate buffer mixed methanol (0–60 % w/w). The mixtures were stirred at set controlled temperatures ranging from −8 to 30 °C for at least 48 hours to ensure saturation as shown in Figure 3.6. The saturated solutions were then filtrated using 0.22 micron syringe filters and analyzed using a digital refractometer (Refractive Index, RI) (Mat Yunus W. Mahmood & Azizan, 1988). To validate relationship between the RI and concentration, parallel filtrated samples were evaporated to

complete dryness at 60 °C, 48 hours, and the residual mass was determined gravimetrically. A linear correlation between RI and concentration was then plotted as shown in Figure 3.5. Notably, all solvents without papain exhibited a baseline RI of 1.3330.

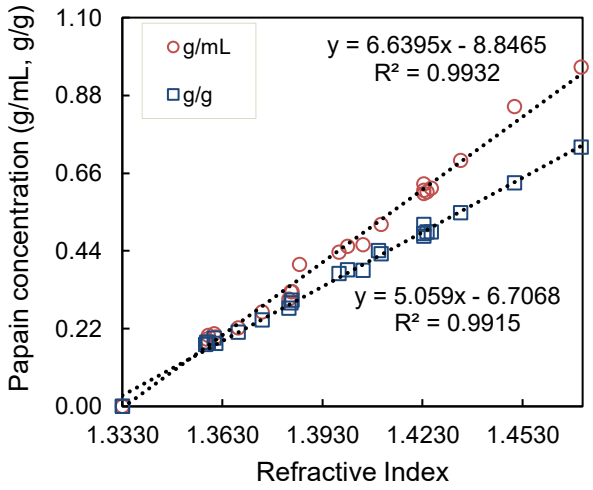

Figure 3.5: Refractive index (RI) vs. papain concentration (g/mL, g/g) in DI water, acetate buffer (pH 5.0), and buffer-methanol mixtures.

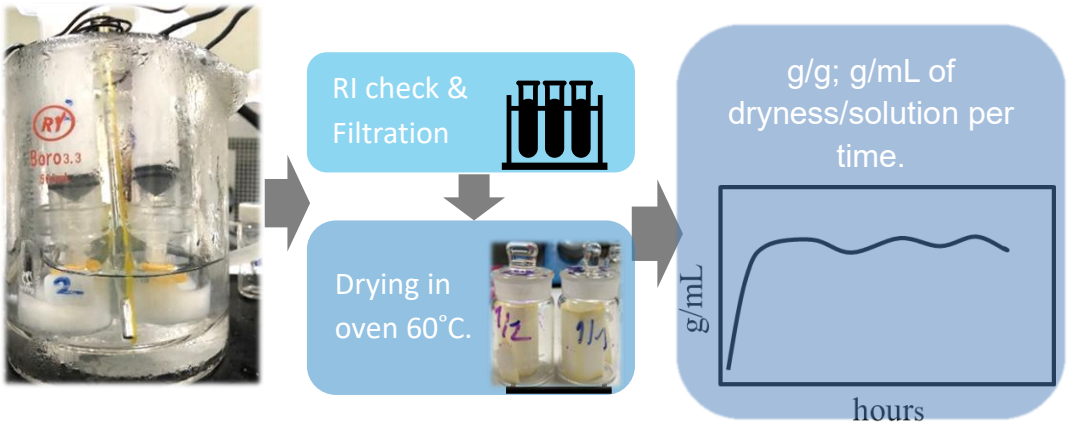

Figure 3.6: The process of solubility measurement for papain by gravimetric method. At −8, 0, 10, 20, 30 °C, in water, acetate buffer, and buffer mixed methanol (0–60 % w/w). Using a 0.22 µm syringe filter prior to drying.

3.2.3 Cooling crystallization of papain

To test the cooling crystallization, saturated papain solutions were prepared in two solvent systems: DI water (natural pH 6.7–7), 0.05 M acetate buffer

(pH 5.0, used to stabilize catalytic activity (Kamphuis et al., 1984)). Due to the elevated viscosity of concentrated papain solutions at 20–30 °C, a controlled slow-cooling protocol was employed to ensure reproducible nucleation. Filtered solutions (1 mL aliquots in sealed vials) were cooled from 30 °C to 5 °C at a rate of 0.005 °C/min using a programmable recirculating thermostat. A micro magnetic stir bar provided intermittent agitation 100 rpm and zero speed. The cloud point was slowly occurred. Therefore, the solution was monitored using a handheld camera and samples were taken for microscope observation. The temperature at which cloudiness first appeared was measured as the nucleation point. The cooling crystallizer set up is shown in Figure 3.7.

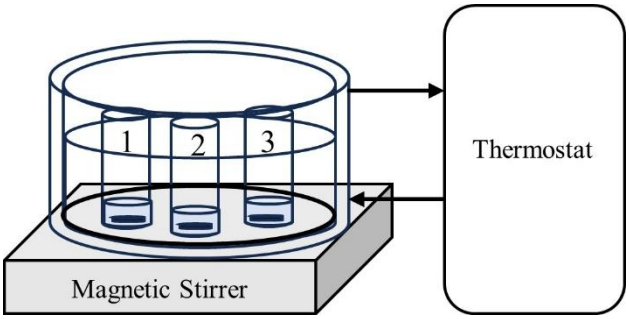

Figure 3.7: Cooling crystallizer set up.

3.2.4 Antisolvent crystallization of papain

Near-saturated papain solutions were prepared in 0.05 M acetate buffer (pH 5.0) and buffer-methanol mixtures (pre-determined via solubility assays). The solutions were equilibrated under agitation 120 rpm for 2 hours to ensure homogeneity, followed by syringe filtration (0.22 μm) to remove undissolved particulates. A jacketed crystallizer (50 mL) was charged with 10 mL of the filtered solution, maintained at 20 and 0 °C (±0.5 °C) via a circulating chiller during methanol addition. Agitation was provided by a PTFE-coated stir bar (Ø0.5 × L2.49 cm) at 120 rpm. The Eazy-Viewer camera (model 400, Mettler-Toledo) was installed for capturing solid cluster in solution and the Dinoe-lite digital camera was for real-time observation to the crystallizer until supersaturation (cloud point) was reached.

Methanol was introduced stepwise at manually rate of 0.2 mL/20 min after a 20-min equilibration period at saturation. Methanol addition continued until

cloud point (nucleation onset) and the gelation threshold (amorphous precipitation) was observed. Methanol consumption was minimized by terminating addition at gelation onset, which methanol was quantified as the weight fraction (w/w) of total solution.

3.2.5 Solvent freeze-out crystallization of papain

The solvent freeze-out (SFO) crystallization technique was applied for papain solution at pH 5 to induce supersaturation near the freezing point of water (Borbon & Ulrich, 2013). The experimental setup (Figure 3.4) consisted of 50 mL jacketed crystallizer integrated with a glass cold finger (freezing coil) and dual thermostatic controls. The first recirculating chiller cooled the freeze coil at a rate of 0.02 °C/min (−0.2 °C per 10 min manually) via stepwise temperature reduction protocol to minimize papain entrapment in ice until the final temperature (Ming et al., 2021; Ryu & Ulrich, 2018). The second thermostat stabilized the bulk solution at near-equilibrium conditions.

An initial 7 mL of near-saturated papain solution (pre-filtered, through a 0.22 µm syringe filter) was loaded into the crystallizer. Final cold finger temperatures were constrained to −12.6 °C (bulk solution temperature ranging from −1.5 to 0 °C) and −14 °C (bulk solution at 1 °C) to prevent excessive ice formation and minimize papain loss. These conditions were determined based on our preliminary study of the SFO process for papain. Solvent freezing concentrated the solution to a residual volume of 1-3 mL (60-80% of initial volume), achieving supersaturation levels corresponding to the phase diagram. Cooling below −14 °C resulted in uncontrolled ice propagation greater than 30% solute loss, consistent with previous observations that showed ice formation at lower temperatures (Ming et al., 2021). So, the protocol used in this study followed our earlier previous preliminary study as illustrated in Figure 3.8.

After nucleation occurred, thermal regulation of the freeze coil was terminated, and the solution was maintained at the nucleation temperature until growth ceased. Papain crystals were harvested afterward. The growth continued for approximately 72 hours to maximize crystal recovery. Papain crystals were isolated and dehydration (in silica gel desiccator until completely dry). The weight of papain

lost in the ice and the recovery of papain crystals were quantified using equations 3.1 and 3.2, respectively.

$$\text{Mass lost of papain\%} = \frac{B}{i} \times 100\% \quad (3.1)$$

$$\text{Recovery\% of crystalline papain} = \frac{C}{i} \times 100\% \quad (3.2)$$

where B is the mass (g) of papain per volume of ice (Ice melts and measure volume); i is the initial mass (g) of papain in saturated solution (7mL); C is dry mass (g) of papain crystal after the SFO process. The crystal was captured by microscope.

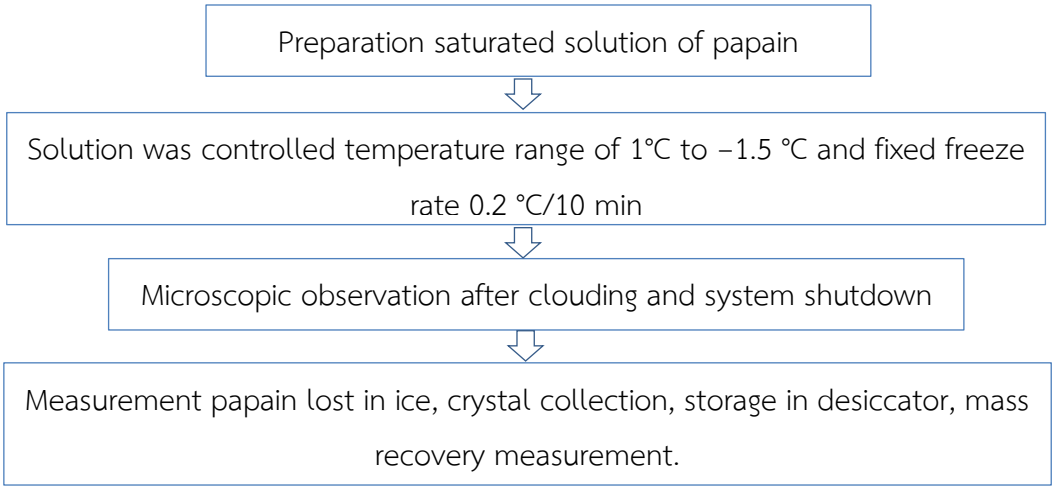

Figure 3.8: Illustration the SFO crystallization procedures for papain.

3.3 Papain concentration determination and characterization

3.3.1 Papain activity assay (BAPNA, UV–vis at 410 nm)

In this part, enzyme activity and protein concentration were determined from the UV absorption value of the protein solution, measured using a spectrophotometer. The most common use of a spectrophotometer is to measure the absorption of light at a specific wavelength. Typically, a spectrophotometer consists of two parts: namely a spectrometer for generating a light beam and a photometer for measuring the light intensity.

Usually, a cuvette containing the liquid sample is placed between the spectrometer and the photometer. The amount of light passing through the cuvette is measured by the photometer. The light absorbance can be read in a display device. The drawing of mechanism of the spectrophotometer is shown in Figure 3.9.

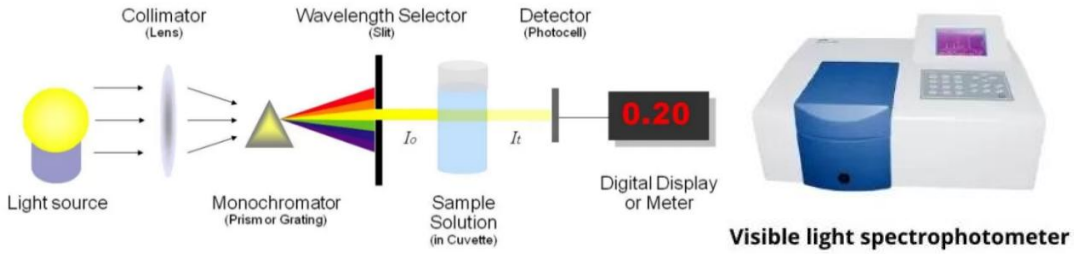

Figure 3.9: UV–vis spectrophotometer principle and equipment. Adapted from (https://microbiologynote.com/spectrophotometer-principle/).

The measurement of light absorbance is based on the Beer-Lambert law. The absorbance (also can be replaced by extinction) of a liquid sample can be expressed in linear equation, as shown in 3.3 (Law). This means that the absorbance becomes linear function with the concentration of the sample.

$$A = -\log_{10}\left(\frac{I}{I_0}\right) = \varepsilon cL \qquad (3.3)$$

Where I_0 and I are the intensities of light before and after passing through the cuvette, respectively. **ε** is the molar extinction coefficient. c is the concentration of sample solution (mol/L). L is the thickness of the cuvette which the light beams passing through (cm).

1) Steps of performing activity estimation consist of:

The enzymatic activity of commercial papain (solid storage and liquid storage at 3°C denature by time) and crystallized samples (from cooling, antisolvent, SFO processes) were quantified using the Anorn-Ruth method with BAPNA (N**α**-benzoyl-DL-arginine 4-nitroanilide hydrochloride) as substrate (Boonkerd & Wantha, 2024; Iván E. Moreno-Cortez et al., 2015; Ruth, 1970). The substrate solution was prepared by first dissolving 43.5 mg of BAPNA in 1 mL of DMSO, then diluting to 100 mL with Tris-buffer (0.05 M Tris, pH 7.5 adjusted with HCl, containing 0.005 M cysteine and 0.002 M EDTA). All solid papain samples were dissolved in DI water at a concentration of 30 mg/mL, with the enzyme concentration verified by measuring absorbance (Abs) at 280 nm (Quartz cuvette) using UV-vis spectroscopy (DR6000, Hach, USA) with DI water as blank as followed standard curve in Figure 3.10.

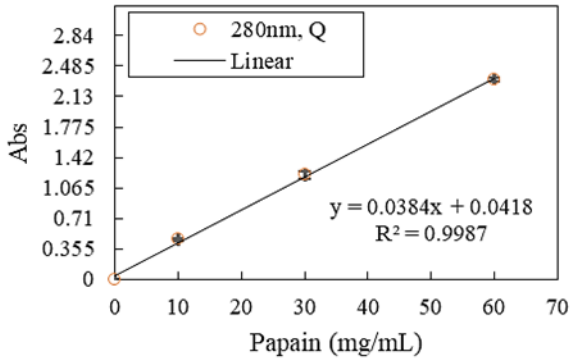

Figure 3.10: The calibration line of papain concentration in water for enzyme activity measurement.

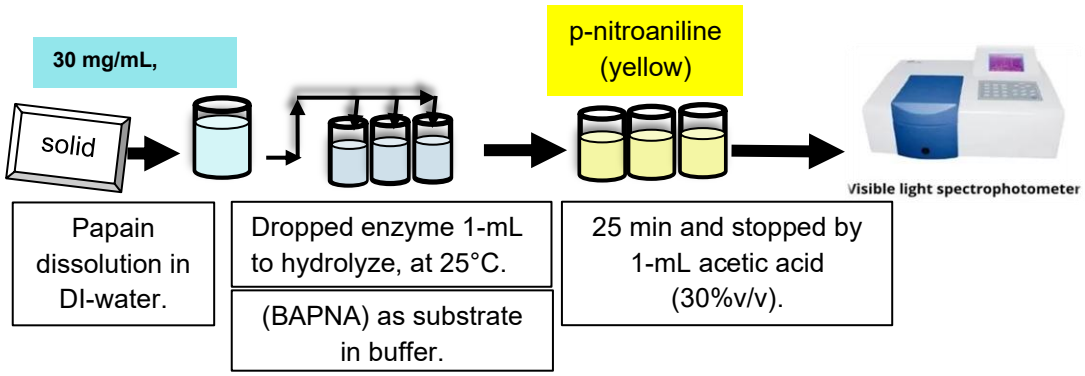

Figure 3.11: The papain activity measurement procedure with BAPNA hydrolyze.

For the activity assay, 1 mL of the papain solution was mixed with 5 mL of BAPNA substrate solution in test tubes and incubated at 25°C for 25 minutes (Figure 3.11). The reaction was terminated by adding 1 mL of 30% (v/v) acetic acid, after which the absorbance at 410 nm was measured against a blank (5 mL substrate mixed 1 mL acetic acid as the blank). The measured absorbance values, corresponding to liberated p-nitroaniline, were used to calculate BAPNA units and specific activity according to equations 3.4 and 3.5 respectively. One BAPNA units was defined as the amount of enzyme required to hydrolyze 1 micromole of substrate per minute under the specified conditions.

$$\text{BAPNA Units} = \frac{\text{Abs}_{410\text{nm}}}{25\text{min}} \times \frac{3\times1000}{8800} \quad (3.4)$$

$$\text{Specific activity} = \frac{\text{Unit}}{\text{mg}} = \frac{\text{BAPNA Units}}{30\text{mg}} \quad (3.5)$$

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

3.3.2 Crystal characterizations

The morphology of papain crystal sample was observed by microscope. The crush fine powder by an agate mortar and pestle. The fine powder was carefully transferred on a sample holder. The crystal was examined by powder X-ray diffraction (D2 Phaser, Bruker) using Cu Kα radiation (λ = 1.5418 Å) with a scan range of 10–50° 2ϑ at 0.0025° step size. The resulting diffraction patterns were compared with reference patterns (Chandran & Nachimuthu, 2018).

Scanning Electron Microscope (JSM-6010LV) was also used to monitor powder structure of commercialize papain, SFO recovery crystal of papain and from others methods.