CHAPTER II

LITERATURE REVIEWS

2.1 Overview of the literature

Enzyme crystallization is a cornerstone of structural biology and bioprocessing, pivotal for both structure elucidation and the purification of biocatalysts at industrial scales (Grossmann & McClements, 2023; Wegner et al., 2024). The practice has evolved from niche laboratory techniques to scalable processes, reaching as far as the production of high-purity enzymes such as papain, lysozyme, catalase (Mitsuda & Yasumatsu, 1955), glucose isomerase, asparaginases and **α**-amylase...et.al for food, pharmaceutical, and other high-value applications (Boonkerd & Wantha, 2024; Liu, Hou, & Li, 2020; Y. Liu, M. Pietzsch, & Ulrich, 2013). In recent years, solvent freeze-out (SFO) crystallization has emerged as a promising, energy-efficient method for enzyme crystallization that addresses many limitations of traditional techniques (Ming et al., 2021).

This chapter provides a comprehensive literature review focusing on papain crystallization using SFO, including a rigorous treatment of enzyme crystallization theory—encompassing supersaturation, nucleation mechanisms, and kinetics. It further discusses the construction and application of phase diagrams, methods for solubility measurement and nucleation mapping, and provides comparative analysis of cooling, antisolvent, and SFO methods. Finally, it synthesizes recent studies (2020–2024) on the crystallization of papain and other benchmark enzymes, connecting theory to practical outcomes with exhaustive support from contemporary peer-reviewed literature.

2.1.1 Crystallization technologies

Normal crystallization process is part of the nature, taking place in several natural phenomena, for instance in the formation of solid such as snowflakes, sugar and salts. Moreover, biological lives parts are also included calcium carbonate and calcium phosphate combine in bones, teeth and cell eggs of animals.

Crystallization is known as a purification technique, as a separation process, as a branch of particle technology, but in its physical definition it is a supramolecular phenomenon of molecules, ions or atoms randomly arranged to form ordered three-dimensional ions or a matrix molecule called crystal (Davey & Garside, 2001). This phenomenon can occur inside a liquid and consequently a phase change occurs in a solution, then solid crystalline material is obtained there.

The crystallization is fundamentally governed by thermodynamic principles: a solid (crystal) will form spontaneously from a solution when the Gibbs free energy change (ΔG) for the process is negative. Crystallization proceeds only when the solution is supersaturated, that is, when the actual solute concentration exceeds the equilibrium solubility at a given temperature and composition (Lewis, Seckler, Kramer, & Rosmalen, 2015; Mullin, 2001). The magnitude and method of supersaturation generation whether by cooling, addition of antisolvent, evaporation, or SFO—determines the crystallization kinetics and thus the attainable crystal size and purity.

Phase diagrams provide essential maps indicating the regions of solubility, metastable, and precipitation for a given protein under specified conditions (protein and precipitant concentration, pH, temperature, ionic strength) (Asherie, 2004; Mullin, 2001; Nyvlt, 1984).

- Solubility curve**:** Boundary where solution and crystal are in equilibrium (no net growth or dissolution).
- Nucleation curve: Separates the metastable and labile zones—nucleation becomes statistically observable.
- Labile/precipitation zone**:** Rapidly formed aggregates/out-of-order structures dominate; this is a zone to avoid if high-quality crystals are sought.

1) Measuring solubility

The typical approach to determine a phase diagram involves stepwise variation of one or more parameters, incubating until equilibrium, and then measuring protein concentration or phase transitions using:

- Spectroscopic methods (e.g., UV absorbance).

- Dynamic light scattering for aggregation.
- Thermal analysis (DSC) for phase transitions.
- Cloud-point method for LLPS mapping.

The van't Hoff equation (2.1) is routinely used for temperature & solubility relationships (Hentschel, Hansen, Egelhaaf, & Platten, 2021):

$$\ln(C_2/C_1) = (\Delta H/R)\cdot(1/T_1 - 1/T_2) \quad (2.1)$$

Where C_1, C_2 are equilibrium concentrations at temperatures T_1, T_2, ΔH is enthalpy of dissolution.

2) Nucleation mapping and metastable zone width:

Between the solubility curve (where crystals dissolve) and the nucleation limit (labile zone), lies the metastable zone: supersaturated but nucleation is kinetically hindered. Crystals grow in this zone only if seeds are present. Metastable zone width (MSZW) is strongly dependent on process parameters like cooling or antisolvent addition rates and agitation intensity (Noor, Camacho, Ma, & Mahmud, 2020). MSZW can be measured by techniques such as:

- Polythermal experiments (heating/cooling cycles).
- Induction time analysis (e.g., detection of light scattering or turbidity increase).
- On-line sensors (FBRM, Raman).

These experiments define the quasi-kinetic boundaries of the phase diagram, which depend on cooling or antisolvent addition rates and system scale. Pseudo-phase diagrams combine solubility, nucleation, and aggregate formation data, providing pragmatic guides for crystallization protocol design (Sacher, Bolf, & Sejdi, 2024).

2.1.1.1 Cooling crystallization and phase diagram

Phase diagrams map temperature (T) and solute concentration (c) to liquid–solid equilibrium in heterogeneous systems. Since pressure has little effect, the equilibrium data appear as a single line in a T vs c plot, often called a solubility diagram (Ulrich & Stelzer, 2014). This solubility curve separates undersaturated solutions (below) from supersaturated ones (above), the latter being

necessary for crystallization since c exceeds the equilibrium solubility. Supersaturation drives both nucleation and growth. At high supersaturation, primary nucleation begins along the nucleation curve (labile-zone boundary), whereas the region between the solubility and nucleation curves is the metastable zone. The metastable zone permits growth of existing crystals without new nuclei forming. The width of this metastable zone represents the maximum supersaturation achievable before nucleation occurs (Giulietti et al., 2001). In cooling crystallization, lowering T reduces the equilibrium solubility, moving the system sequentially from undersaturated into metastable and then labile regions (Figure 2.1). By controlling the cooling rate and agitation, one can target either gentle crystal growth (within the metastable zone) or rapid nucleation (crossing into the labile zone).

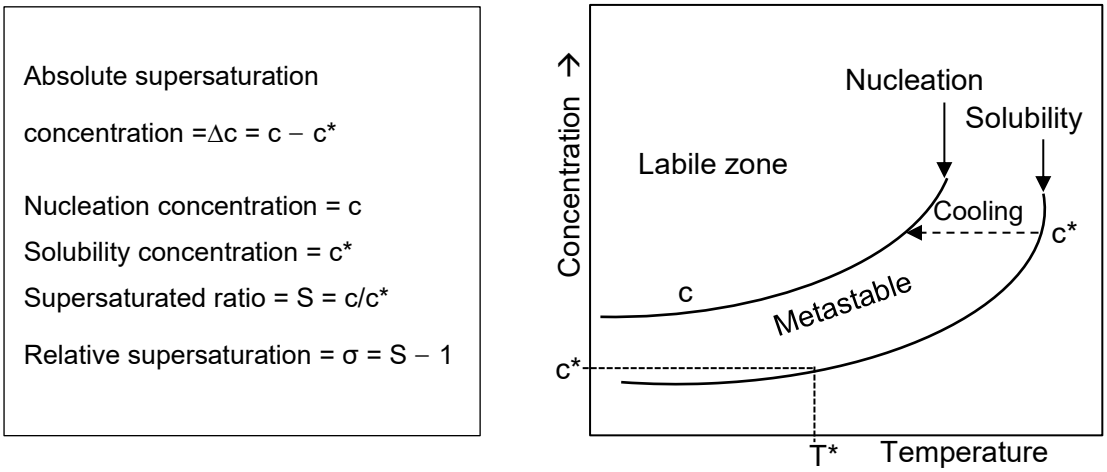

Figure 2.1: Conceptual solubility and nucleation diagram for cooling crystallization. Adapted from (Giulietti et al., 2001).

Supersaturation is achieved by lowering the temperature, exploiting the temperature dependence of protein solubility. This method is simple, common, and adaptable for scale-up.

1) Key parameters:
   - Initial and final temperature.
   - Cooling rate (slower rates favor larger crystals and less nucleation).

- Agitation (influences secondary nucleation and crystal habit).

2) Advantages:
   - No need for organic or additional chemicals.
   - Reversible: if no crystals form, system can be reheated and retried.
   - Well suited to temperature-sensitive proteins.
3) Limitations:
   - Some proteins have weak or atypical temperature dependence in solubility.
   - High minimum nucleation supersaturation.
   - Thermal denaturation risk for sensitive enzymes (mitigated by mild temperature windows).
4) Example (lysozyme):

Well-characterized lysozyme crystallization at 4 to 20 °C in sodium acetate buffer with NaCl is a standard benchmark system. Cooling rate in the range 0.03–0.2 °C/min is typically optimal for tetragonal crystals with narrow size distributions (Maosoongnern, Flood, Flood, & Ulrich, 2016; Tang, XH, Liu, JJ, & Zhang, 2018; Wang, Li, Zhang, & Wang, 2023).

2.1.1.2 Antisolvent crystallization

A miscible antisolvent (ethanol, acetone, acetonitrile., et.al) is added to the protein solution. Because the protein is much less soluble in the antisolvent, its supersaturation abruptly increases, triggering nucleation (Jia et al., 2022). The solubility at a temperature and an agitation are controlled. Crystallizing agents (anti-solvent) addition drive to the supersaturation region by different mode as in Figure 2.2. The metastable zone permits growth of existing crystals without new nuclei forming.

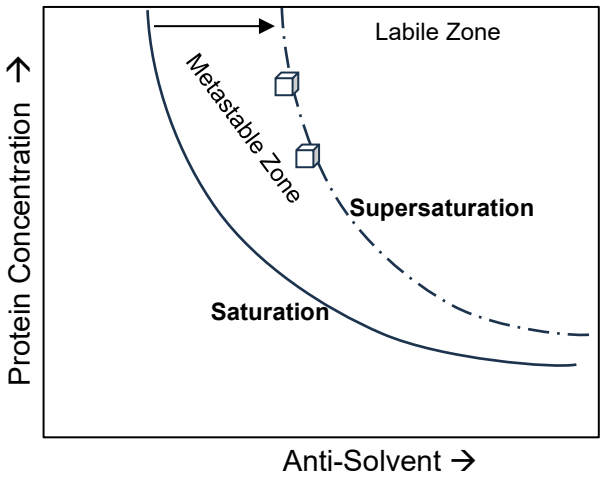

Figure 2.2: Conceptual solubility/nucleation diagram for the antisolvent technique. Adapted from (Jia et al., 2022).

1) Key process parameters:
   - Type of antisolvent and ratio to solvent.
   - Addition rate, mode (batch, dropwise, gradient).
   - Mixing and agitation.
   - Temperature.
2) Advantages:
   - Operational at ambient temperature: suited for thermally labile proteins.
   - Rapid supersaturation: enables formation of nano- or micro-scale crystals.
   - Polymorph, morphology, and particle size can be controlled by process optimization.
3) Limitations:
   - Local supersaturation inhomogeneity; possible aggregation and wide particle size distributions if mixing is suboptimal.
   - Purification and recovery of antisolvent required.
   - Potential for partial denaturation if harsh antisolvents or excessive concentration are used.

4) Example (papain):

Recent studies report ethanol as an effective antisolvent for papain (1:4 S:AS, into 30 mg/mL papain solution) yielding nanosized particles with retained activity under controlled addition and mixing (in Figure 2.3). Acetone and acetonitrile were less effective, often producing amorphous aggregates (Boonkerd, Hao, & Wantha, 2024; Boonkerd & Wantha, 2024).

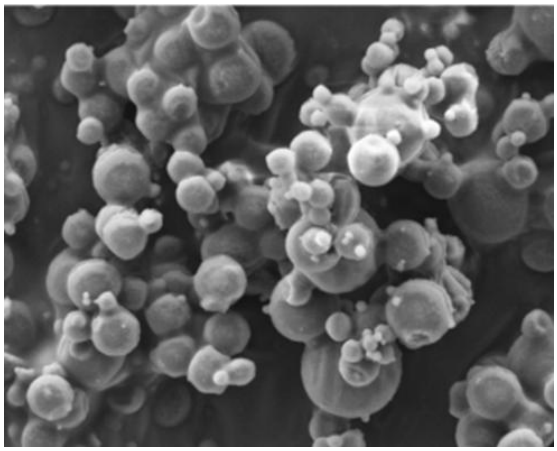

Figure 2.3: SEM photomicrographs of nanoparticle papain precipitated at different solvent-to-antisolvent ratios (1:4); reproduced from with permission (Boonkerd et al., 2024; Boonkerd & Wantha, 2024).

2.1.1.3 Solvent freeze-out crystallization (SFO) technology

Solid layer crystallization has been applied for decades to recover target compounds from mixtures by forming a crystalline layer on a cooled surface. In this process, a component of the melt undergoes a phase change and deposits as a solid layer on the heat exchanger surface. Crystal growth proceeds perpendicular to the cooled surface toward the bulk liquid (mother liquor), driven by the temperature gradient between the equilibrium temperature at the crystal–liquid interface and the bulk melt temperature. This gradient represents the difference in equilibrium conditions between the solid and liquid phases (Ryu & Ulrich, 2018; Ulrich et al., 1996).

In a typical setup (Figure 2.4), a tube cooled internally by a circulating coolant is immersed in a temperature-controlled vessel containing the feed melt. A thermostat regulates the surface temperature. Because the temperature

difference controls both growth rate and product purity, an optimal cooling program is essential. As the crystal layer thickens, it acts as a thermal insulator, reducing heat transfer. To maintain a constant driving force and steady growth rate, the cooling intensity must be gradually increased. Without this adjustment, the process slows and eventually stops when thermal equilibrium is reached. The growing layer experiences a thermal gradient due to the release of latent heat of crystallization, which must pass through the crystal layer and crystallizer wall (Borbón & Ulrich, 2012).

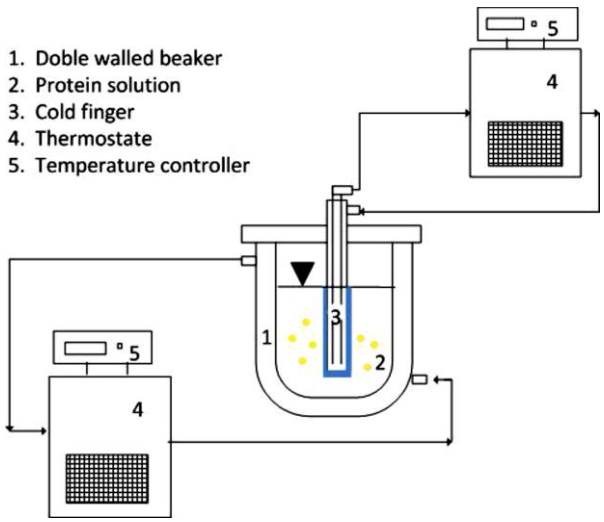

Figure 2.4: Set up of a simple equipment for solvent freeze out crystallization of proteins. Adapted from (Borbón & Ulrich, 2012).

In suspension crystallization, crystals are formed directly in the bulk liquid. These crystals are typically of high purity, but even small amounts of entrained mother liquor can significantly reduce product quality. Efficient solid–liquid separation is therefore critical. Temperature and supersaturation control are particularly important for concentrated feeds, where supersaturation is highly temperature-dependent. In suspension systems, a large surface area for crystallization (on the order of 10^{4} m²/m³) allows relatively low growth rates (10^{-7} to 10^{-9} m/s) to achieve acceptable production rates. Such low growth rates reduce impurity incorporation, enabling high separation efficiency in a single step (Ulrich et al., 1996). Suspension crystallization also offers high crystal production rates per unit equipment volume.

In SFO crystallization, two processes occur simultaneously:

- Solvent crystallization (e.g., water) as a solid layer on the cooled surface — analogous to melt crystallization.
- Solute crystallization (e.g., proteins) within the remaining liquid phase — analogous to suspension crystallization.

Ice formation is governed by the phase separation between ice and dissolved substances in water, while protein crystallization is dictated by the phase behavior of the aqueous protein solution (e.g., enzymes). Only when system conditions fall within the respective crystallization regions will nucleation and growth occur. For solid-layer melt crystallization, the solution adjacent to the cooled surface must be below the A–B line in the simplified binary phase diagram of water and solute (Figure 2.5, left). For protein solution crystallization, the bulk conditions must lie between the solubility curve and the metastable liquid–liquid boundary in the generic protein phase diagram (Figure 2.5, right) (Borbon & Ulrich, 2013).

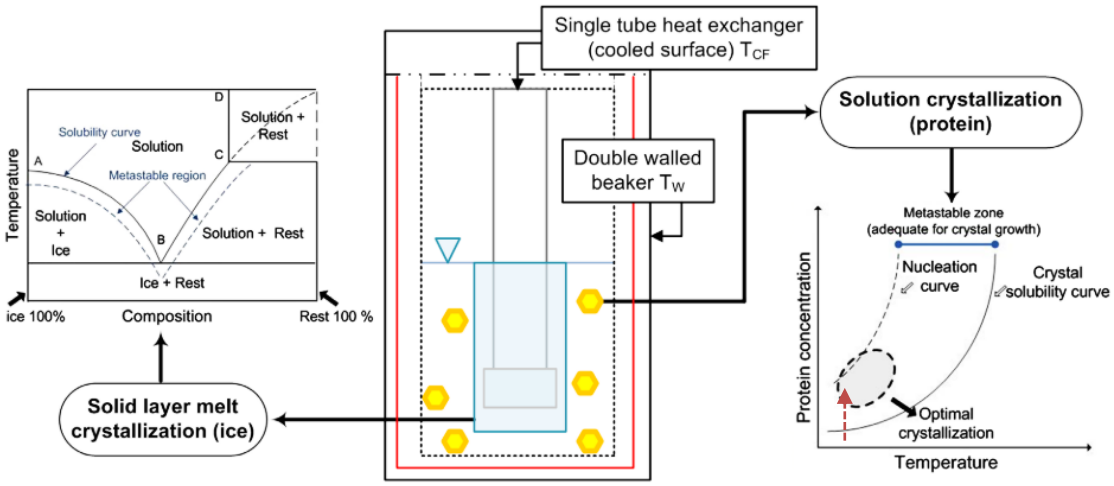

Figure 2.5: Mechanism of the solvent freeze out (SFO) crystallization process. Two crystallization processes take place. One is the crystallization of melt (water-ice) on a cooled surface. The other is the crystallization of the protein within the solution. Adapted from (Borbon & Ulrich, 2013).

SFO integrates aspects of freeze concentration and crystallization. The process involves controlled freezing of the solvent (usually water), thus concentrating the protein and promoting supersaturation. As the "ice" of solvent

separates, the unfrozen solution becomes supersaturated, allowing the protein to crystallize (Ryu & Ulrich, 2018).

1) Key process parameters:
    - Cooling rate and cold surface temperature (cold finger or freeze coil).
    - Ice growth rate/time at fixed temperature.
    - Initial protein concentration and ionic strength (salt type/concentration).
    - Agitation and mixing regime.
2) Advantages:
    - Low-temperature operation preserves protein activity and structure.
    - Significantly reduced salt/precipitant usage compared to traditional methods.
    - High yields and high purity due to the concurrent purification during ice crystallization.
    - Scalable and suitable for industrial bioprocessing.
    - Offers a mild environment; minimal chemical exposure; unlike salt-heavy traditional methods.
3) Limitations:
    - Requires precise thermal control.
    - Potential for protein inclusion (entrapment) in ice, reducing yield; requires optimized ice growth and agitation conditions for maximal protein exclusion.
    - May be less documented for certain enzyme classes and complex feeds.
4) Recent industrial relevance of SFO:

The SFO has been used for purification of industrial enzymes such as lysozyme showing high activity retention and eco-friendly profiles (Ming et al., 2021; Ryu & Ulrich, 2018). Previous works success the proteins purification

from mixtures by SFO method for three in here review as lysozyme, urease and recombinant l-asparaginase.

In approach, the solid layer formed on the cooled surface is composed primarily of the solvent, while the remaining liquid phase contains the target protein and other solutes. A key challenge is that small droplets of protein solution can become trapped within the frozen solvent layer, leading to product loss. Ryu *et al.* (2012) investigated how cooling rate and protein concentration influence this phenomenon. For an ice layer approximately 1 cm thick, reducing the cold-finger cooling rate from 0.4 K/min to 0.1 K/min lowered protein loss to ice from ≈40 % to ≈23 % (Ryu & Ulrich, 2018).

For hen egg-white lysozyme (HEWL), crystallization conditions were first identified through screening experiments and represented in pseudo-phase diagrams. These data guided the selection of initial SFO parameters. In this system, ice formation generated supersaturation in the remaining liquid, enabling lysozyme to crystallize. Using a feed containing 15 mg/mL lysozyme and 1.7 mg/mL ovalbumin, the SFO process achieved a crystallization yield of 69 %, producing crystals with an average size of 77.8 μm in ≈15 h. SDS-PAGE confirmed the identity of the crystals as lysozyme, and enzymatic assays verified that activity was retained (Borbón & Ulrich, 2012).

Urease was crystallized from jack bean meal extracts using SFO, and product purity was evaluated by specific activity measurements and SDS-PAGE. Compared with conventional salt-induced crystallization, SFO provided greater control over process conditions. Experimental variation of operating parameters highlighted the importance of ice-layer quality in determining crystallization performance. Although the process was not fully optimized, it yielded urease crystals of satisfactory purity and reasonable recovery, with higher enzyme activity preserved relative to traditional methods (Xiaoxi Yu et al., 2015).

Recombinant L-asparaginase II from *Escherichia coli* fermentation broth was also purified at laboratory scale using SFO. Initial crystallization parameters were determined via batch screening. Subsequent freeze-out crystallization with ethanol as the solvent produced rhombic (diamond-shaped)

protein crystals. Purity was assessed by SDS-PAGE and UV spectrophotometry, which confirmed effective removal of DNA contaminants. Enzymatic activity assays showed that the catalytic function of L-asparaginase II was maintained after purification (Yu et al., 2017).

2.1.2 Direct methodological comparisons

From the above sections of crystallization methodologies review that can summary in short comparison the advantages and weakness of each method under table 2.1.

Table 2.1: Summary comparison of crystallization methods.

Crystallization Method →	**Cooling**	**Antisolvent**	**SFO**
Supersaturation Source	Lowering temperature	Compositional (adding non-solvent)	Solvent solidification/removal
Temperature Profile	Decreasing	Constant/Ambient	Decreasing
Speed	Moderate	Rapid	Moderate
Activity Retention	High	High (if controlled)	High
Scalability	High	High	High
Process Complexity	Simple	Moderate	Moderate–Complex
Salt/Additive Use	Low	Moderate	Very low

*Note: “low/moderate/very low” relative to salt/additive mass per unit product.

2.2 Protein crystallization

Proteins are linear polymers of amino acids linked by peptide bonds, folding into defined secondary (α-helices, β-sheets), tertiary, and, for multimeric proteins, quaternary structures through a combination of hydrogen bonding, hydrophobic

interactions, and disulfide bridges (Bennema, 1992; M., Tymoczko, & Stryer, 2007). Their surface charge depends on ionizable side chains and solution pH, reaching zero net charge at the isoelectric point (pI), where solubility is minimal (M. et al., 2007; Mcpherson, 1990).

Water molecules hydrating protein surfaces are essential for solubility. Removing this hydration layer drives "salting-out" and can precipitate the proteins by adding salts or polymers; conversely, low concentrations of certain salts enhance solubility ("salting-in") in figure 2.6 (Mcpherson, 1990). Crystallization occurs when the system's free energy is minimized by transferring protein molecules from solution into an ordered crystal lattice; analogous to the fully hydrated state satisfying charge and bonding requirements.

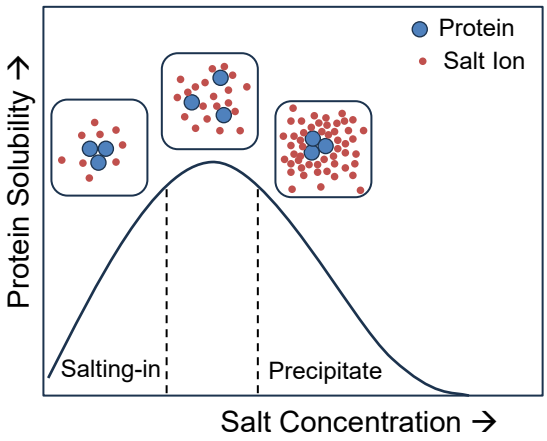

Figure 2.6: Effect of salt on macromolecule solubility (Mcpherson, 1990).

The thermodynamic driver is supersaturation ($S=C/C_{eq}$), achieved by altering temperature, protein concentration, pH (Wang et al., 2023), or adding precipitants (Weber, 1997). Crystallization requires ($S>1$) mild supersaturation promotes growth of existing crystals, while higher supersaturation triggers primary nucleation.

Phase diagrams (temperature vs. concentration) map liquid–solid equilibrium and define three regions:

- Undersaturated ($C<C_{eq}$) — no crystallization.
- Metastable (between solubility and nucleation curves) — crystal growth only.

- Labile (beyond the nucleation curve) — spontaneous nucleation (Alderton & Fevold, 1946).

Accurate phase-boundary data guide process design. The width of the metastable zone quantifies the allowable supersaturation before unwanted nucleation occurs as shown in Figure 2.7.

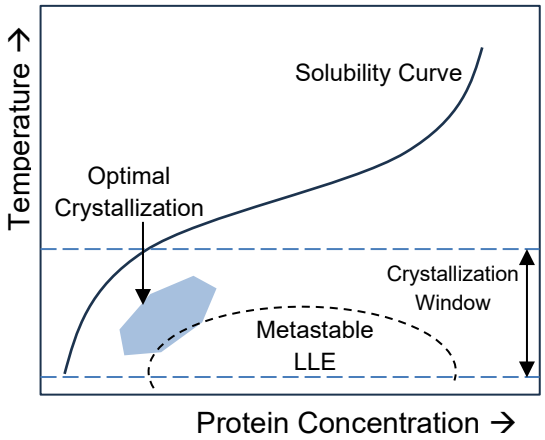

Figure 2.7: Typical phase diagram with crystallization window where the optimal crystallization region is found near the metastable liquid–liquid equilibrium (LLE) (Curtis & L.Lue, 2005).

At the molecular level, crystallization behavior correlates with protein–protein and protein–salt interactions, often described by the osmotic second virial coefficient (B_2). A narrow "crystallization window" exists between the solubility limit and the onset of liquid–liquid phase separation, within which optimal nucleation and growth occur (Curtis & L.Lue, 2005).

Because each protein's phase behavior depends on its unique surface chemistry and solution environment, empirical screening across pH, ionic strength, temperature, and precipitants and representation as pseudo–phase diagrams, it is essential (Wang et al., 2023). Only a few model proteins (e.g., lysozyme) (Alderton & Fevold, 1946) have well–characterized thermodynamic parameters, underscoring the need for systematic condition mapping in novel systems.

In this kind of experiments the conditions of the protein systems are varied in a wide range and the results are observed and analyzed. Furthermore, results can be summarized in pseudo–phase diagrams. The conditions under determined phase were

observed that is located on them and then a broader knowledge of the phase behavior is available.

2.3 Industrial crystallization of protein

Protein crystallization plays a vital role across pharmaceutical, cosmetic, and food industries. Crystalline proteins offer enhanced stability, purity, and handling properties, making them valuable in both formulation and processing. Studies show that protein crystals can be thermodynamically more stable than their dissolved counterparts, with stabilization energies (Drenth & Haas, 1992). Biopharmaceuticals, including therapeutic proteins and industrial enzymes, are high-value products. For example, lysozyme (from egg white) and papain (from papaya) are widely used for food preservation and antibacterial applications. Proteins are also incorporated into cosmetics for skin and hair protection, and in food processing to improve texture, elasticity, and nutritional value — such as through transglutaminase-mediated cross-linking (Grzonka, Kasprzykowski, & Wiczk, 2007; Kieliszek & Misiewicz, 2013).

Industrial crystallization, or mass crystallization, is employed to produce crystalline intermediates or final products with controlled purity, morphology, and physical properties (Giulietti et al., 2001; Nyvlt, 1984). Crystallization techniques vary by how supersaturation is achieved — commonly through evaporation, cooling, salting-out, or antisolvent addition (Albert M. Schwartz, 2000; Borbon & Ulrich, 2013; Drenth & Haas, 1992; Hekmat, Hebel, Joswig, Schmidt, & Weuster-Botz, 2007; Liu et al., 2020; Schmidt et al., 2005; Tam et al., 2011; Weber, 1997; Wiencek, 1999; Yi-Bin Lin et al., 2008).

Designing a crystallization process requires consideration of product specifications: purity, activity, crystal size and distribution, polymorphism, and downstream compatibility (Nyvlt, 1984; Roy & Abraham, 2003). Supersaturation is still the key variable, governing nucleation and growth kinetics. Its control determines crystal quality, yield, and reproducibility. Because crystallization occurs in a dynamic liquid–solid interface, managing non-equilibrium conditions is essential for process optimization.

2.4 Papain and crystallization of papain

2.4.1 Papain

The solvent freeze-out (SFO) technique has demonstrated effective protein purification across several model systems, including lysozyme, urease, and recombinant L-asparaginase, as outlined in the preceding section. These studies highlight SFO's potential for producing high-purity protein crystals while retaining enzymatic activity. Building on this foundation, the present work extends the application of SFO to papain, a cysteine protease of significant industrial and biomedical importance.

Papain is a proteolytic enzyme belonging to the cysteine protease family (molecular mass 21–30 kDa), characterized by a catalytic dyad of Cys25 and His159 (Figure 2.8) and stabilized by three disulfide bonds (Kamphuis et al., 1984). It is primarily sourced from the latex of *Carica papaya* L. (family Caricaceae), though it can also be obtained from the fruit's skin, leaves, or sap. Dried papaya latex contains approximately 57 % protein, with the protease fraction comprising papain (10 %), chymopapain (45 %), and other proteases (20%), each with a molecular weight near 23 kDa (Chaiwut, Nitsawang, & Shank, 2007; Macalood, Vicente, Boniao, Gorospe, & Roa, 2013). Crude latex papain exhibits protease activities of 2655 U/g at pH 5.5 and 285 U/g at pH 9.0.

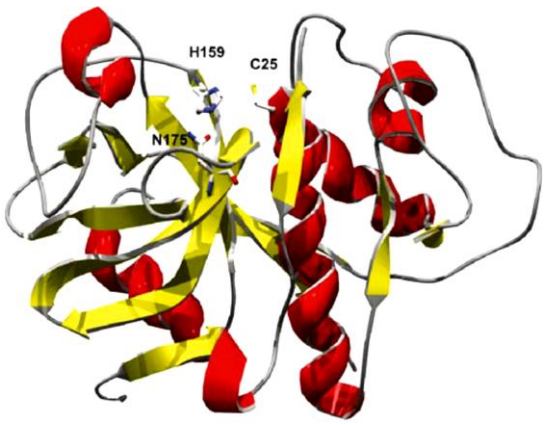

Figure 2.8: Ribbon representation of the three-dimensional structure of papain active site at His159 and Cys25 (Amri & Mamboya, 2012; Grzonka et al., 2007; Kamphuis et al., 1984).

An isoelectric point (pI) of 8.75, papain is optimally active between pH 5.0 and 7.0 at 50–60 °C, retaining most of its activity under moderate heating but showing progressive loss at higher temperatures. Commercial papain, often sold as a yellowish-white powder, is typically stored at ≤ 4 °C to preserve activity. Reported kinetic parameters include a Michaelis–Menten constant K_m and V_{max} (Mathias Elsson, Anondho Wijanarko, Heri Hermansyah, & Sahlan, 2019).

Papain's versatility underpins its widespread use in detergents, leather processing, brewing, meat tenderization, pharmaceuticals, and waste treatment. Medically, it has been applied to manage conditions such as leaky gut syndrome, hypochlorhydria, and gluten intolerance, and has shown analgesic and anti-inflammatory effects in allergic sinusitis without reported side effects (Amri & Mamboya, 2012). In drug discovery, papain has served as a model enzyme for developing selective inhibitors of cathepsin K and cathepsin L. Antimicrobial studies have demonstrated that papain from papaya seeds can inhibit *Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Shigella flexneri* (Parsaeimehr et al., 2014).

As showed in Mathias Elsson study characteristic of commercial papain in papain-casein mixture and used the Lowry protein assay method (Mathias Elsson et al., 2019). Commercial preparations, such as the "Paya" brand meat tenderizer (Figure 2.9), contain papain mixed with sugar and salt. Although the exact enzymatic activity of such products is often unknown, they have been used in experimental studies and present an interesting candidate for crystallization trials.

Figure 2.9: Commercial enzyme Papain brand "Paya" reaction with casein (Mathias Elsson et al., 2019).

Notably, a 2016 study compared lyophilized latex and purified papain from *C. papaya* for activity against *Strongyloides venezuelensis* eggs and larvae. High latex concentrations exhibited partial toxicity to parasite forms, suggesting that compounds other than papain contributed to the observed effects. Purified papain at 2.8 mg/mL produced similar egg-hatching inhibition to latex diluted 1:1000 in the egg hatching test (EHT) (Moraes, Levenhagen, Costa-Netto, Costa-Cruz, & Rodrigues, 2016).

Studies on papain (2025) show that organic cosolvents can markedly affect its kinetics and structure. In BAPNA hydrolysis assays (with 10 % DMSO for substrate solubility), methanol and ethanol raised K_m without altering V_{max}, lowering catalytic efficiency. Acetonitrile acted as a reversible mixed-competitive inhibitor (IC_{50} in the low millimolar range) and induced a blue shift in tryptophan fluorescence, implying conformational tightening. Molecular dynamics simulations confirmed that acetonitrile competes with BAPNA at the active site and that solvent polarity modulates substrate binding. These insights inform optimal solvent selection for papain-based biosensor design (Sirirak, Tamdee, Sawatthitileat, Thaithong, & Sirasunthorn, 2025).

1) Productions and exportation of Thailand's papaya:

From website List of countries by papaya production - Wikipedia according to the list of countries by papaya production (Wikipedia), Thailand produced 165,605 tons of papaya in 2022, while India produced 5,341,000 tons. The estimated global production that year was 13,822,328 metric tons, representing a 1.9 % decrease from 14,086,181 tons in 2021. India remained the dominant producer, contributing over 38 % of the world's total output. These figures highlight both the agricultural capacity and the global demand for papaya, underscoring the importance of research across multiple fields that including agricultural technology, extraction processes, and production methods for applications in food, cosmetics, and pharmaceuticals.

In Asia, many universities continue to investigate the benefits of papaya latex. In Thailand, for example, Chiang Mai University has conducted extensive studies on extraction techniques (Chaiwut et al., 2007; Manosroi, Chankhampan, Pattamapun, Manosroi, & Manosroi, 2014), culminating in dissertations focused on modifying papaya-derived enzymes for use in cosmetic and pharmaceutical products

(Nekoueinaeini, Aliahmadi, & Soleimani, 2024). Given this context, advancing research on papain purification, particularly its crystallization, would be highly valuable for Thailand, enabling the development of high-purity enzyme products for diverse industrial applications.

2) The conclusion of papain proteolytic activity determination as review in table 2.2:

Table 2.2: Papain enzymatic activity studies.

Buffers	Sources	Enzymatic activity	pH	Max, T (°C)	Method/ Substrate
	Liquid papain by Arinor Limited, Belfour House 46-54, Great Titchfield Street, London	250.0 U/g	5.0-7.0	60	Caseinolytic method (casein digestion) (Lukin, 2020)
Acetate 0.2M	Dried crude latex of C. papaya L (code P-1301)	2655.0 U/g	5.5	55	Caseinolytic method (Macalood et al., 2013)
In water	Boonkerd's work. Commercial papain	Residual activity >90%	7	25	Ruth's method, BAEE, BAPNA hydrolysis (Boonkerd & Wantha, 2024)
In water, methanol, ethanol, DMSO, acetonitrile.	Sirirak's work. Commercial papain activity effected by different solvents	Catalytic efficiency reduced by acetonitrile	-	25	Ruth's method, BAPNA hydrolysis (Sirirak et al., 2025)

Given papain's industrial relevance, biochemical stability, and prior evidence of successful crystallization in other proteins via SFO, it represents a

compelling target for further investigation. The following sections detail the experimental strategies employed to achieve high purity papain crystals using the SFO method.

2.4.2 Papain crystallization review

First, papain represents another useful protein for studying electron microscopically the fibre to crystal transformation process, as well as for the understanding of the formation of varying crystalline state. The crystals of papain exhibit longitudinal and transverse periodicities and are clearly shown to be formed like needle (Figure 2.10) by the coalescence of parallel bundles of individual fibrils. Thin cross-sections of the thicker crystals show a complex honeycomb structure with pronounced aqueous channels (Harris, 1983).

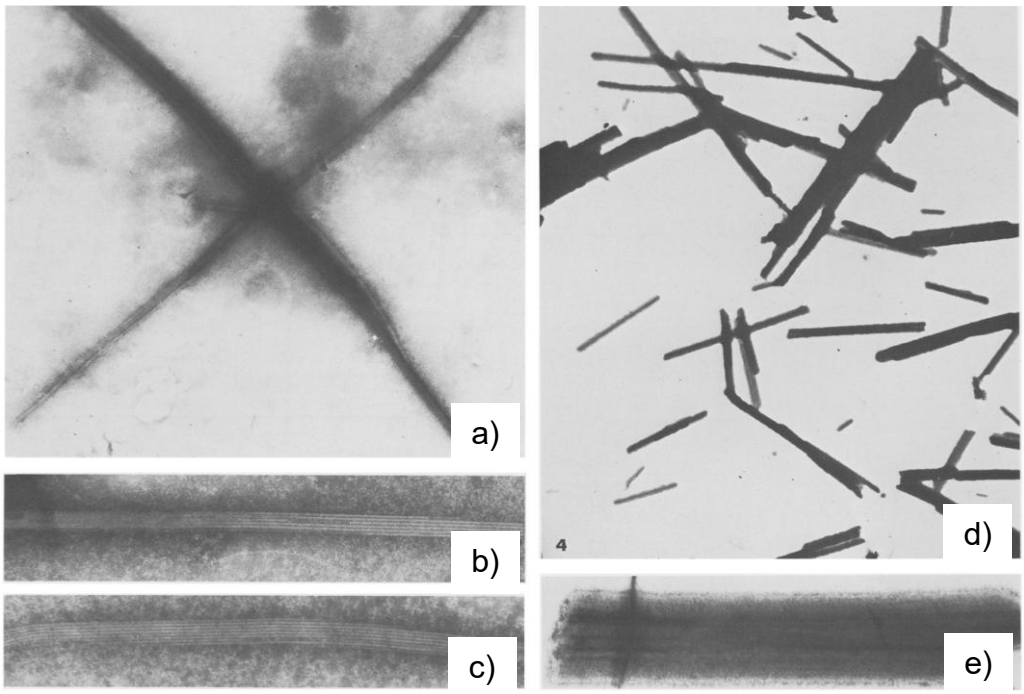

Figure 2.10: Needle-like crystals of papain present in the suspension originating from Sigma. The needles contain parallel fibrils, which exhibit a pronounced zigzag helical periodicity. Negatively stained with 2% sodium phosphor tungstate (pH 7.0). (a) x18,000, (b) x78,000, (c) x78,000, (d) x6,000, (e) x30,000, respectively. Adapted from (Harris, 1983).

Second, the crystallization of a Kunitz-type Papain Protease Inhibitor (PPI) that was isolated from the latex of green Carica papaya fruits. PPI has a molecular weight of 23 kDa and two disulfide bridges. PPI was crystallized by the hanging-drop vapour-diffusion method using Hampton Research Crystal Screen and checked with X-ray by a merohedral twin law of diffraction method. The Prismatic crystals (crystal form I) were obtained after a week of incubation in 2.0 M ammonium sulfate. Crystals diffract resolution of 2.6 Å (Mohamed Azarkan et al., 2006) as shown in Figure 2.11.

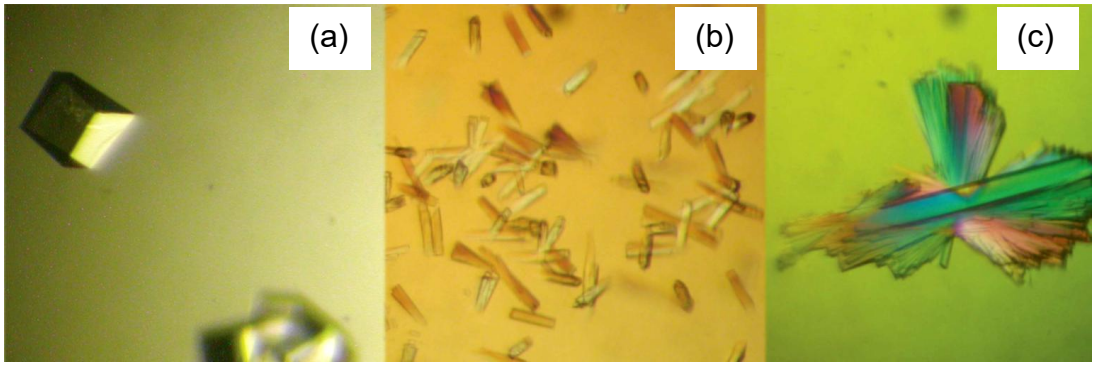

Figure 2.11: Crystals of papain protease inhibitor (PPI). (a) Crystal form I (prismatic) grown in 2.0 M sodium formate, 20 mM sodium acetate pH 4.6. (b) Original crystals of crystal form II grown at 293 K in 1.8 M ammonium sulfate, 100 mM MES pH 6.5, 10 mM cobalt chloride. (c) Typical growth crystals of crystal form II of PPI grown at 283 K after optimization. The clusters of needles are only loosely attached to each other and the large single crystal in the middle was picked out for data collection. Adapted from (Mohamed Azarkan et al., 2006).

Ethanol antisolvent protocols for papain (1:4 S:AS, controlled addition/mixing) (Boonkerd & Wantha, 2024) report nanoscale particles with retained activity, whereas methanol under similar droplet-addition regimes frequently yields amorphous aggregates and gelation; this contrast underscores solvent-specific protein–solvent interactions and mixing-driven local supersaturation as key determinants. In contrast, SFO builds supersaturation by solvent removal at near–freezing bulk temperatures, decoupling protein nucleation from harsh compositional shocks and minimizing denaturation risk.

2.4.2.1 Phase diagram of papain

Papain (papaya latex from Sigma/Aldrich) was studied solubility and nucleation in polyethylene glycol (PEG) reagent solution at three points temperature in Figure 2.12 (Yi-Bin Lin et al., 2008).

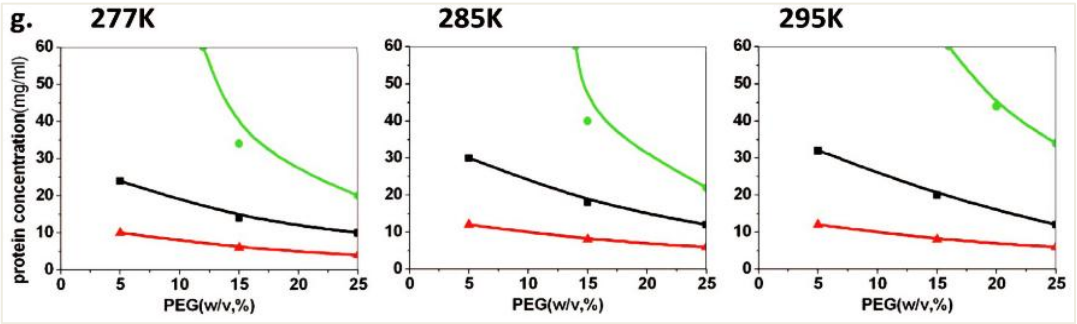

Figure 2.12: Phase-diagrams of papain: Red solubility curve was determined from the residual concentration in equilibrium with crystals 50-days after the initiation of crystallization at varying temperatures from 4 °C, 12 °C and 22 °C, left to right respectively. Nucleation and precipitation curves are plotted in black and green, respectively. Adapted from (Yi-Bin Lin et al., 2008).

2.4.2.2 Related studies of papain using XRD and SEM

In Figure 2.13 (a) X-ray diffraction (XRD) analysis was conducted using a Da Vinci PXRD instrument (Germany) with a scan range of 2θ = 5–60°; step size of 0.05°; scan rate 4°/min. Papain crystals were prepared using three different precipitation conditions: 50% unsaturation ammonium sulfate, 20% w/w PEG 6000, and a combination of both. The XRD patterns revealed distinct peaks at 23.29 degree and 28.47 degree for crystals precipitated with ammonium sulfate, which matched the raw papain profile. Crystals obtained from the combined precipitant exhibited a prominent peak at 20.69 degree, indicating enhanced crystallinity. In contrast, PEG 6000 alone produced weaker and broader peaks, suggesting lower crystallinity (Qi Hao et al., 2024).

Scanning electron microscopy (SEM) imaging (Figure 2.13, b) was performed using a Quanta 450 FEG-SEM. Air-dried crystals were sputter-coated and mounted on stubs for observation. The SEM micrographs showed block-shaped crystals with sharp, well-defined edges and corners. A uniform crystal habit was

consistently observed across batches prepared with the combined precipitant and L-cysteine dosing (Qi Hao et al., 2024). These results were used for discussion.

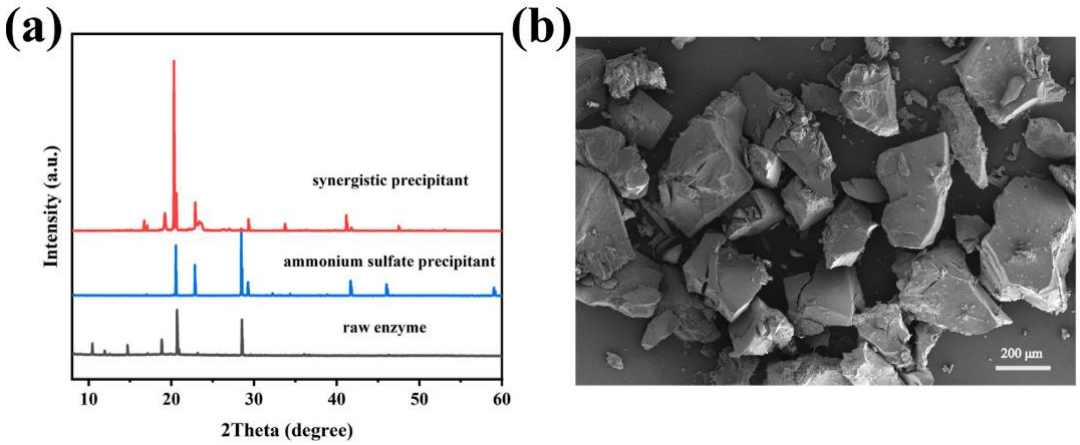

Figure 2.13: a) XRD pattern of papain crystals under different precipitation conditions. b) SEM micrographs showing crystal morphology of papain under combined precipitant. Adapted from (Qi Hao et al., 2024).

2.5 Conclusion

Advances in enzyme crystallization science have produced a toolkit of methods: from classical cooling and antisolvent techniques to solvent freeze-out (SFO) are suitable for a diversity of protein systems and industrial requirements. Each method offers unique strengths: cooling for simplicity and gentleness, antisolvent for rapidity and small size control, and SFO for environmental friendliness, reduced additive use, industrial scalability, and enzyme integrity retention.

In the context of papain, antisolvent crystallization with ethanol at a 1:4 ratio yields highly active, uniform nanocrystals-like, as validated by robust gravimetric and activity assays. Lysozyme now serve as models for SFO and precipitant-free crystallization, underscoring the generality and transferability of modern approaches. Urease, asparaginase, lysozyme, et.al though more complex, also benefit from these advances, with stabilization agents and solvent systems broadening the operational window for successful, high-yield crystallization.

Future directions include expanded mechanistic modeling (linking thermodynamic and kinetic parameters to process outcomes), digital twin and machine-learning–guided process optimization, seamless integration with downstream

purification, and further scale-up studies to ensure industrial impact and environmental sustainability.

To our knowledge, quantitative solubility and nucleation boundaries for papain in water/buffer–methanol systems have not been reported; this gap is addressed here using gravimetry and pseudo–phase mapping. Preliminary of cooling, antisolvent technique were conducted to comparison with SFO. Methanol is using for antisolvent. Enzyme activity determination was followed Ruth's method and PXRD also analyst with reference works (Boonkerd, Sirilak, ...et.al).