CHAPTER I

INTRODUCTION

1.1 Background and Significance

The demand for therapeutic proteins and industrial enzymes in chemical, food, and pharmaceutical industries is increasing (Motoki & Seguro, 1998). Therefore, efficient methods for protein extraction and purification from their original source are necessary on an industrial scale (Tam, Chan, & Ng, 2011). Chromatography and precipitation often require many steps to achieve high purity and stability and can be cost-intensive at industrial scale (Chisti & Moo-Young, 1990; Wiencek, 1999). Protein crystallization can be a relatively economical purification step for industrial applications. Crystalline proteins offer advantages compared to the dissolved or amorphous solid in terms of drug release, handling, stability, purity, and shelf life (Drenth & Haas, 1992; Schmidt, Havekost, Klaus Kaiser, Kauling, & Henzler, 2005). Moreover, the crystallization can serve as a cost-effective and highly selective purification step in downstream processing and possibly as an alternative method to replace one or more chromatographic steps. However, the classical crystallization theory does not fully capture the behavior of all protein systems due to protein has highly complex structure that can make purification challenging. Proteins may fail to crystallize under small temperature variations, incompatible reagents, or inadequate hygiene conditions, all of which can negatively affect purity.

An industrial technique for separation and purification known as solid-layer melt crystallization, has been introduced (Ulrich, Bierwirth, & Henning, 1996). This method has been adapted to introduce solvent freeze-out (SFO) crystallization as a novel protein crystallization approach in industrial applications (Borbon & Ulrich, 2013; Ming et al., 2021). More work on SFO crystallization by Diaz Borbon and Ulrich improved this method and applied it to the separation of proteins such as lysozyme crystallized from lysozyme-ovalbumin mixture (Borbón & Ulrich, 2012; Yu, Wang, & Ulrich, 2014),

complex urease crystallized from jack bean meal (Xiaoxi Yu, Jingkang Wang, & Ulrich, 2015). Recombinant l-asparaginase II was also purified by SFO crystallization technique and the original enzymatic activity was preserved by this process (Müller, Liu, Migge, Pietzsch, & Ulrich, 2011; Yu, Wu, Huang, Ulrich, & Wang, 2017). Therefore, SFO crystallization can be considered as an alternative innovation method for protein crystallization, affordability, low environment impact (low salt requirement), low temperature operation, and the potential to preserve protein quality in this study (Ming et al., 2021).

Furthermore, the SFO method was applied for papain crystallization which included an ice-layer (solid) combined with solution crystallization to produce protein crystals. At laboratory scale, an SFO crystallizer was constructed comprising a double-walled vessel and a freezing coil (−14 ± 2 °C). During the crystallization process, the solvent in the papain solution was removed by freezing on a freeze coil as the ice layer. The solution was concentrated and became supersaturated, which promoted the formation and growth of papain crystals within the solution. By removing solvent from the system, the desired supersaturation of papain was achieved with minimal precipitant use. Therefore, this study concerns the recovery, loss of papain to the ice, and loss of enzymatic activity, which before and after using the SFO crystallizer. Accordingly, enzyme activity was quantified spectrophotometrically; solubility and nucleation were mapped; and crystal structure was examined by microscope and X-ray diffraction, in order to compare with previous conventional crystallization methods. In this study, papain was selected as a representation commercialized protein due to its valuable enzymatic properties. Papain is a cysteine protease from papaya latex, shows antimicrobial potential and has been widely used in pharmaceutical products, cosmetics, foods industry even as well-known in meat tenderization (Amri & Mamboya, 2012; Parsaeimehr, Chen, & Sargsyan, 2014). In Thailand, there are a lot of papaya cultivation. To add value to papaya-derived products and promote their use in high-value chemical industries. This study was initiated, especially considering the relatively high market price of purified papain. We hope our study contributed to the development at a cost-effective following the upstream production process.

The crystallization process begins with primary nucleation, which is influenced by various factors, including supersaturation level, agitation parameters, processing time, and the nature of antisolvent agents (Giulietti, Seckler, Derenzo, Re, & Cekinski, 2001; Mullin, 2001; Nyvlt, 1984). Although papain had been crystallized using methanol at pH 5.0 (Kamphuis, Kalk, Swarte, & Drenth, 1984), yielding needle-like crystals (Harris, 1983) and its industrial-scale crystallization behavior remains underexplored. Recent studies on batch antisolvent crystallization have identified ethanol, acetone, and acetonitrile as antisolvents, with ethanol (1:4 solvent-to-antisolvent ratio) achieving high activity retention (Boonkerd & Wantha, 2024). However, comprehensive studies the phase-diagram mapping, nucleation control, and process modeling for papain are still lacking.

This study aims to determine solubility and nucleation zones and to strengthen understanding of SFO crystallization as a purification unit for commercial papain. Scale-up supported by process modeling is proposed for future work.

1.2 Research Objectives

1.2.1. To quantify papain solubility versus temperature and methanol in water and acetate buffer.

1.2.2. Construct pseudo phase diagrams (solubility and nucleation boundaries) for cooling, antisolvent, and SFO; establish an SFO operating window minimizing ice entrapment while preserving activity.

1.3 Scope and limitations of the research

The investigation of the crystallization potential of papain using SFO was studied by setting up a series of experimental procedure to clarify its feasibility. The experimental study steps on this crystalline papain are obtained in Figure 1.1, which shows the overall work flow performed in this study.

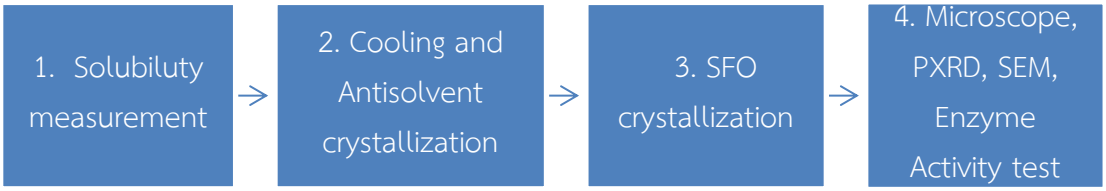

Figure 1.1: Overall workflow of papain crystallization.

First, the saturation concentration (solubility) of crystalline papain in water, acetate buffer (pH 5) and buffer with methanol (0 - 60% w/w) was measured by gravimetric method and monitored with a refractometer. Experiments were investigating at various temperature ranging from –8 to 30°C.

Second, preliminary cooling crystallization (at rate of 0.005 °C/min) and antisolvent crystallization using methanol (dropping rate of 0.2 mL/20min) were conducted along saturation lines.

Third, the SFO crystallization process was studied by conducting experiments under a controlled freezing rate of 0.02 °C/min, stepwise reduction.

Fourth, the resulting crystal was analyzed by microscope, X-ray powder diffraction (PXRD) and Scanning electron microscopy (SEM). The activity of enzyme was evaluated by the reaction efficiency of papain with the BAPNA substrate where the amount of product formation from substrate decompositions quantified over time.

1.4 Expected outcomes

This study aims to deliver: (i) pseudo phase diagrams for papain, (ii) an SFO operating window (coil –14 to –12.6 °C; bulk –1.5 to 1 °C) minimizing ice entrapment, (iii) PXRD/SEM-verified crystalline products, and (iv) activity benchmarks versus commercial references. Furthermore, it is expected to support Thailand's bioindustries in producing high-value enzyme crystals via crystallization, suitable for sale and specified application using crystallization process.