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Automated measurement of apparent protein solubility to rapidly assess complex parameter interactions

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ABSTRACT

Characterization of protein solubility in downstream processing steps is important to either prevent protein aggregation, e.g. during inclusion body refolding, hydrophobic interaction chromatography and formulation or to decrease solubility, e.g. for selective precipitation or crystallization. In general we distinguish between thermodynamic solubility at equilibrium and kinetically driven apparent solubility.

In our study we used a high throughput screening method established on a liquid handling robot to rapidly assess an apparent solubility of lysozyme and its dependence on parameters such as pH, ionic strength and additive concentration. Combinatorial effects were measured in a reasonable amount of time with high data density and low material consumption.

Parameter interactions were observed between solvent pH and temperature. With increasing margin of pH from the isoelectric point, the effect of temperature was more pronounced. In addition, we found an influence of ionic strength on the additive induced changes in apparent solubility for all systems. PEG 300 and Tween 20 improved lysozyme apparent solubility at high salt concentrations. For sorbitol and sucrose, two distinct regions of maximum apparent solubility were found depending on the additive concentration. While an explanation for single parameter effects was possible, e.g. for pH by correlating net charge and solubility, this became difficult with increasing number of parameters. By reducing the experimental effort, it was possible to build a solid data basis to elucidate the mechanism of lysozyme aggregation and to find industrial relevant regions of increased solubility. Our approach is thus a powerful tool not only for process optimization but also for an increased understanding of precipitation.

Keywords: High throughput experimentation; Cohn equation; Protein solubility; Precipitation; Formulation; Excipient screen

1. Introduction

Protein solubility plays a major role in bioprocess development when considering aggregation of both target as well as contaminating protein as it not only reduces process yields but might also disturb process steps by clogging membranes and chromatographic columns. Especially in inclusion body refolding processes, aggregation due to strong interactions between folding intermediates with higher surface hydrophobicity is one of the major obstacles during process development and buffer optimization. In formulation studies, solvent compounds are investigated for their effect on long-term protein solubility to guarantee for constant product quality. An overview on protein aggregation and its consequences on bioprocess development is given in two reviews by Cromwell et al. and Wang et al. (Cromwell et al., 2006; Wang, 2005).

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Most of published data deal with solubility in the context of crystallization for protein structure determination. Although a high interest exists in both, fundamental research and industrial process development, up to now it is hardly possible to describe the effects of parameters like pH and ionic strength on protein solubility by simple models. This said, a basis of simple solubility models exist, examining basic theoretical treatments, e.g. the Melander and Horvath model based on molal surface tension increment of neutral salts (Melander et al., 1977; Horvath et al., 1976), the preferential interaction model by Timasheff and Arakawa (1988); Arakawa et al. (1985), or combinations of these (Nfor et al., 2011). However none of those was set into industrial practice so far. Therefore optimization has to be done empirically with some guidelines based on experience. With an increasing number of parameters, high throughput methods have to be established to significantly reduce material costs and time consumption.

Fig. 1A shows a schematic protein phase diagram. Solubility is often determined by measuring the equilibrium concentration with protein crystals being present in a solution. This yields information on the thermodynamic solubility curve, meaning that protein does not precipitate or crystallize at concentrations below this line in an infinite amount of time (Asherie, 2004). For processes with rather fast changes in buffer composition or protein concentration, the protein solution will presumably be far away from this equilibrium and supersaturation occurs. As the nucleation kinetics of crystals in this region is dependent on the rate at which supersaturation is created, in fast process steps protein precipitation will appear more likely as precipitation processes usually have fast kinetics (Asherie, 2004; Garcia-Ruiz, 2003). Therefore knowledge of the precipitation curve will be sufficient in many cases for an appropriate development of a biotechnological process. Even for studies on long-term storage stability the precipitation curve describes the upper limit of a potentially interesting region in the phase diagram and should be assessed in order to decrease the size of the parameter space for more detailed investigations. Knowledge of the precipitation zone is also a prerequisite for a rational process development to optimize crystallization of proteins for structure determination (Anderson et al., 2006; Zhu et al., 2006; Santesson et al., 2003). In purification processes such as chromatography, protein concentration and solvent composition change quickly and as a consequence the thermodynamic equilibrium is not always reached especially not for low levels of supersaturation and the formation of crystals or aggregates might not occur directly as it is dependent on the kinetics of both processes. Nevertheless, protein solutions show a limited storage stability, which has to be taken into account for example if the protein solution has to be stored before proceeding with the next process step (Asherie, 2004).

For the optimization of protein solubility numerous parameters have to be considered that can be divided into physical parameters and parameters describing the chemical environment of the protein. Important physical parameters affecting protein solubility are mechanical stress induced for example by intense mixing or a high flux through a membrane, surface or interface adsorption and temperature. Important chemical parameters are solvent pH, ionic strength, salt type and protein surface hydrophobicity. As proteins display differences in net charge at different pH values according to their individual titration curve, the optimum pH for protein solubility is protein specific and usually far away from the isoelectric point (p*I*) of the protein as electrostatic repulsion dominates. Effects of ionic strength and salt type on solubility are complex and depend strongly on the solution pH (Asherie, 2004). An empirical hierarchy of salt types based on their effect on protein solubility was first published by Hofmeister (1888) and since then frequently verified by other researchers though pH was not adjusted in his studies. Ionic strength effects can qualitatively be divided into a salting-in region, where low salt concentrations increase the solubility of a given protein and a salting-out region usually at high salt concentrations where proteins have the tendency to precipitate. Thus the

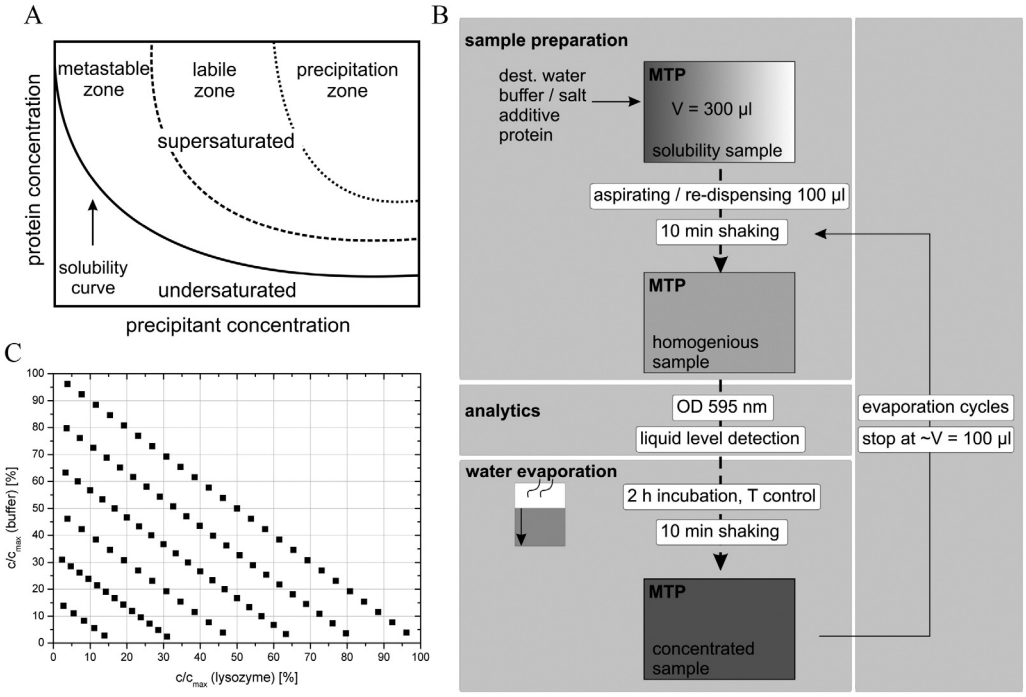

Fig. 1 – HTS process schematics. (A) Schematic protein phase diagram (adapted from Melander et al., 1977). (B) Flow scheme of the automated solubility screening process. (C) Experimental starting conditions for the determination of solubility curves in different buffer or salt systems and lysozyme.