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An open source cryostage and software analysis method for detection of antifreeze activity

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ABSTRACT

The aim of this study is to provide the reader with a simple setup that can detect antifreeze proteins (AFP) by inhibition of ice recrystallisation in very small sample sizes. This includes an open source cryostage, a method for preparing and loading samples as well as a software analysis method. The entire setup was tested using hyperactive AFP from the cerambycid beetle, *Rhagium mordax*. Samples containing AFP were compared to buffer samples, and the results are visualised as crystal radius evolution over time and in absolute change over 30 min. Statistical analysis showed that samples containing AFP could reliably be told apart from controls after only two minutes of recrystallisation. The goal of providing a fast, cheap and easy method for detecting antifreeze proteins in solution was met, and further development of the system can be followed at https://github.com/pechano/cryostage.

1. Introduction

Organisms vary in their response to cold and freezing conditions. Some are tolerant to internal freezing, while others must avoid it to survive. Insects have a variety of behavioural responses to initially avoid the cold [24,20], but evolution has in many cases favoured the adaptation of a physiological cold response [28,19]. Plants indigenous to the temperate regions of the world often experience freezing conditions for extended periods of time, but their inability to move leaves them few options for freeze avoidance through behaviour [7,26]. For many organisms, freeze tolerance is therefore an important survival strategy. Freeze tolerant organisms typically do not freeze completely at random. Rather, the freezing process is directed by biological ice nucleating agents [20,21,29,30], found in blood and tissue. For most freeze tolerant organisms, intracellular freezing is still unwanted and damaging [26], and thus extracellular ice nucleation prevents fatal damage. Freeze avoiders often employ a certain group of biological antifreeze agents called antifreeze proteins (AFP),[1] as well as low molecular weight colligative antifreeze compounds such as polyols [34], free amino acids [20] and inorganic salts [27].

Due to biological constraints, the colligative antifreeze response only has a functional range down to a maximum of −10 °C in most species. Further protection from freezing must be provided by AFP [27,32]. Tiny ice crystals that may otherwise inoculate the supercooled body fluid are effectively neutralised through a process known as adsorption inhibition [22]. The mode of action is believed to be a function of the Kelvin effect [16], which states that vapour pressure of a surface is correlated to it's curvature. See Eq. (1) below. As the particle radius r decreases, surface vapour pressure increases due to curvature. The effect of curvature on surface vapour pressure also relates to the ice nuclei that constantly form at random in an undercooled solution. If the radius, r, is below a certain value, phase equilibrium will shift towards the liquid and the nucleus dissolves. This effect is exploited by AFP on the surface of ice crystals.

$$ln\frac{p}{p_0} = \frac{2\gamma V_m}{rRT} \quad (1)$$

where.

p: vapour pressure of surface
p_0: vapour pressure of solution

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[1] As more intricate knowledge about the mechanism of these proteins have become available in recent years, they are often referred to as **I**ce **B**inding **P**roteins or **I**ce **S**tructuring **P**roteins.

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γ: surface tension
V_m: molar volume
r: crystal radius
T: system temperature in Kelvin
R: universal gas constant

AFP adsorb irreversibly onto the ice crystal surface and prevent ice from forming underneath it [16]. Between adsorbed AFP, the ice crystal will continue to grow as a convex bulge with the minimum radius r. A denser population of adsorbed AFP will lead to lower r, which increases the vapour pressure of the convex surfaces between adsorbed AFP (Eq. (1)). This shifts the interfacial phase equillibrium towards the liquid state, effectively preventing further ice crystal growth at a given temperature. If the temperature is lowered further, the equillibrium shifts towards the crystal phase, slowly expanding the crystal with decreasing temperature. At a certain temperature, the effect breaks down and an explosive crystal growth is observed [25]. In the metastable system consisting of liquid water and tiny ice crystals, the temperature difference between the equillibrium melting point T_m and the non-equillibrium freezing point, where the antifreeze effect breaks down, is called thermal hysteresis, *TH*.

In freeze avoiding organisms, the primary function of AFP is allowing body fluids, despite the presence of tiny ice crystals, to become supercooled through thermal hysteresis. AFPs are often found in freeze tolerant organisms as well [21]. This may seem paradoxical, but is often attributed to their secondary function, which is inhibition of recrystallisation, *RI*. As an aqueous solution freezes, it often happens from several nucleation points, especially if the rate of cooling is high. A slow freezing may result in fewer nucleation points, or even just one single point of nucleation. This is typically seen in ice cubes or the surface of a frozen pond during a cold spell, both examples of finely structured ice crystals. If a large number of nucleation points initialise ice crystal growth, the final body of ice consists of a varied distribution of ice crystal orientations and sizes [14]. Variations in radius equals variation in surface vapour pressure. This leads to the phenomenon known as Ostwald ripening, which is the mechanism that drives recrystallisation of ice [3]. When water freezes, solutes are exclude from the growing crystal, leading to a sharp concentration gradient near the crystal surface. In a flash frozen sample with many tiny ice crystals, the macroscopic distribution of solutes is not significantly different from a liquid system. As time passes and Ostwald ripening is allowed to recrystallise the ice, larger and larger areas of the ice cross section will be either pure ice or pure brine, see Fig. 1. It is this clear separation between ice and its surroundings that is the basis for the detection method described in this paper. For the purpose of the method, bulk-ice and edge-ice, will henceforth refer to the large surface of the growing ice-crystal and the interfacial region in contact with water, respectively.

The recrystallisation process is believed to be a main cause of tissue damage in frozen organisms [11,5]. By inhibiting recrystallisation in frozen body fluids, AFP's can essentially extend the period of time before permanent damage occurs from freezing of tissue.

From an industrial point of view, recrystallisation has a significant impact on quality of frozen goods. Reducing the amount of recrystallisation that takes place in frozen storage of foodstuffs could therefore be of interest. Zang et al. [33] showed that addition of carrot AFP to frozen dough significantly improved frozen shelf life. The unfulfilled potential of AFP as a food additive is largely due to production issues, and as such, a new approach is perhaps in order. Naturally occurring AFPs from fish, and especially insects, have high antifreeze activity ratings in terms of thermal hysteresis. Several patents exist on both fish and insect AFP because of this. Plant antifreezes are different in the way that most do not provide a noticeable thermal hysteresis, but a rather efficient recrystallisation inhibition [8,9,17]. A potential source for future applications could be naturally occurring plant AFP.

Since the discovery of antifreeze proteins and the first quantification of their effects, thermal hysteresis has been used as an endpoint measurement for detection and activity. Thermal hysteresis activity, THA, shows a correlation to AFP concentration. There is no general formula to describe the concentration/effect relationship of all AFP's, but a functional calculation can be established on a case-to-case basis [16].

The inhibition of recrystallisation requires AFP concentrations orders of magnitude lower than detectable THA. So as a detection endpoint, IRI seems superior to THA. The effect is, however, somewhat binary over a large span of concentrations. Either there is complete IRI or there is none. This proves a challenge in terms of quantifying the effect, especially when compared to inorganic recrystallisation inhibitors, which provide a scalable inhibition over a large span of concentrations [18]. Screening for thermal hysteresis requires a time consuming and expensive setup that often includes a nanoliter osmometer. Screening for recrystallisation inhibition is different due to the nature of the mechanism. One only needs to observe ice crystals at a constant temperature for a limited amount of time to identify the presence of recrystallisation inhibition proteins.

In the past, IRI has been observed and quantified by microscopy [12,14,21]. Small samples of solution possibly containing AFP have been flash frozen and evaluated over time, by hand. There have been several method developments that automate some of the steps involved in detecting antifreeze activity, and reduce the amount of experimental sample needed. This paper seeks to combine the two ideals in order to provide the reader with a simple, low-cost setup for performing the experiments needed to

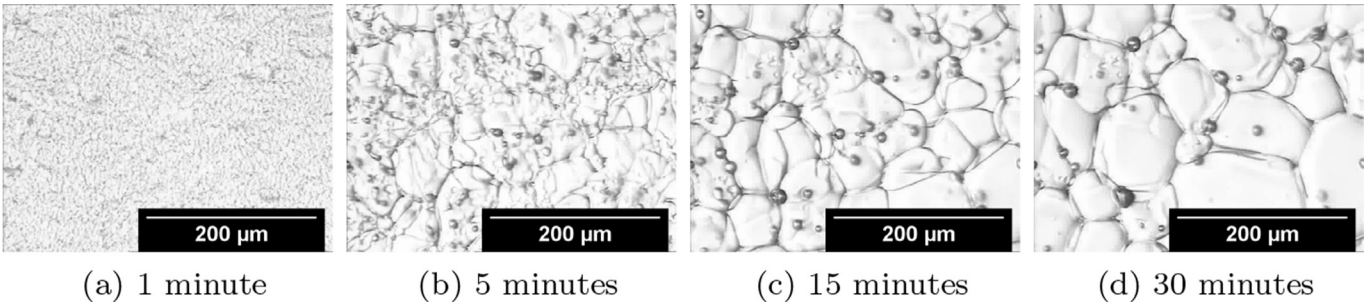

Fig. 1. Ostwald ripening or ice recrystallisation. All images are the same FOV at different points in time. The amount of edge-ice decreases with time, while the amount of bulk-ice increases. This mechanism is exploited as a detection method.