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Thermal Hysteresis and Bursting Rate in Sucrose Solutions with Antifreeze Proteins

Antifreeze proteins (AFPs) modify the ice shape and inhibit further growth leading to thermal hysteresis (TH). Numerous studies have been performed with the addtition of AFPs to preserve frozen products but the influence of sucrose on the effects of AFPs has not been investigated as of yet. Therefore, the TH activities of type I antifreeze protein (AFP I) and antifreeze glycoprotein (AFGP) were measured as a function of concentration, type of AFPs, and also sucrose concentration. The results showed that the TH values rose with increase in concentration of AFPs and sucrose concentration. The crystals experienced shape modification and grew in the *c*-axis direction in the presence of both AFPs. The bursting rate of crystals changed depending on both the concentrations of AFPs and sucrose.

Keywords: Antifreeze proteins, Ice-binding proteins, Ice crystals, Sucrose solution, Thermal hysteresis

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1 Introduction

Ice recrystallization (IR), which occurs especially during frozen storage, is an undesired process due to the potential negative effect on the quality of frozen products. As a result of recrystallization, the size, number, and shape of ice crystals are changing while the total volume of ice remains constant. The changes are significant factors affecting especially appearance, texture, and flavor release of products. Therefore, the inhibition of ice recrystallization is of great importance, not only for food industry, but also for several other areas where sensitive products are stored frozen, such as pharmaceutical products or organs and blood in medicine [1–3].

As proposed by Griffith and Ewart, ice-binding proteins (IBPs) have the unique ability to inhibit ice growth and in consequence inhibit recrystallization [4]. Regand and Goff suggested that ice structuring proteins (ISPs), which is another name for IBPs, from cold-acclimated winter wheat grass extract can be used to inhibit ice recrystallization [5]. Gaukel et al. revealed that the presence of IBPs in sucrose solutions resulted in a marked decrease in recrystallization [6]. This effect is based on their ice-binding affinity. An accepted theory for this is known as the adsorption-inhibition model which claims that antifreeze proteins (AFPs), a subset of IBPs, adsorb to the ice surface irreversibly and stimulate the surface curvature of the ice between bound AFPs [7]. Consequently, the freezing point is depressed due to an increase in surface curvature due to the Gibbs-Thomson effect.

Although irreversible binding of AFPs is still criticized, many researchers examined the adsorption of AFPs to the ice surface through fluorescence microscopy and found that AFPs adsorb to the surface of ice crystals irreversibly [8, 9]. Thus, in the presence of an AFP in a solution, ice crystal growth is inhibited during temperature decrease until a certain temperature is reached. Below this temperature, ice crystal grows quickly and "burst growth" occurs.

In literature, research focused on the growth rate of ice crystals, especially to examine the AFP-ice crystal interaction planes [10]. In addition, it was found that the melting temperature is not significantly affected [9]. The gap between the melting temperature and the freezing temperature is known as thermal hysteresis (TH). TH activity can be investigated under laboratory conditions in IBP buffer solutions. However, in product applications (e.g., food), there are many other solutes present which may affect TH activity. Therefore, in this study, AFPs were used to investigate the impact of sucrose solution concentrations on TH activity and bursting rate.

2 Materials and Methods

TH activities of type I antifreeze protein (AFP I) and antifreeze glycoprotein (AFGP) were measured in a 40 % w/v sucrose solution with antifreeze proteins' concentrations varying between 0 and 20 mg mL^{-1} (0.5, 10.0, 15.0, and 20.0). Moreover,

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several concentrations of sucrose ranging from 0 to 50 % were used to examine its effect on TH activity at a constant AFP concentration (10 mg mL^{-1}). AFP I and AFGP were provided by Nichirei Co. Ltd. AFP I is a low-molecular-weight protein (< 30 kDa) obtained from the barfin plaice (bp), also called the native bpAFP [11] with 3.5 kDa molecular weight [12]. AFGP is a purified sample of AFGP from *Eleginus gracilis* [13] with molecular weight assumed to be 15 kDa [12]. The solutions were prepared with demineralized water on a magnetic stirrer until AFPs dissolved completely. Then they were kept in Eppendorf tubes frozen at –18 °C until they were needed for further experiments. Prior to the experiment, the solution was melted at room temperature. The experimental setup in Fig. 1 was used to perform the TH measurements.

The setup consists of a polarization microscope (Nikon Eclipse LV100ND) equipped with a digital camera (Nikon DS-Ri1) and a cold stage (Linkam LTS420). The cold stage is equipped with a microscope sample slide holder (G16M) with a stainless-steel ring on a pure silver heating/cooling block. The temperature gradient between the sample and the silver block was minimized by using a thin glass cover slip (16 mm diameter and 0.13–0.17 mm thickness) inside as basis for the sample solution. Two shim spacers (outside diameter 14 mm × interior diameter 12 mm × 70 µm) were placed on the glass cover slip to provide a gap for the sample solution and enough space for an ice crystal to grow.

A sample of 0.5 µL was placed on 1 µL of an immersion oil drop in the middle of the cover slip to ensure uniform heat dispersion around the sample. Besides the uniform sample temperature, the small amount of sample provides the advantage of ensuring having only one single crystal during the crystal preparation. A small portion of immersion oil, which has the same refractive index as the glass cover slip, was used below the cover slip to provide a thermal seal between the glass and the stage. The upper surface of the sample was closed with another glass cover slip.

After completing the sample preparation, the temperature of the stage was decreased to –30 °C via liquid nitrogen in a Dewar to freeze the sample. Afterwards, the sample was heated to a temperature close to the melting point to get a single ice crystal. The crystal was then kept under these conditions for 10 min and afterwards cooled at a rate of 0.1 °C min^{-1} to detect the temperature at which the crystal swiftly grew.

The rapid, excessive, and spontaneous elongation from one end of the crystal across the *c*-axis is named the bursting of crystals. The temperature at this point was the nonequilibrium freezing temperature T_f (°C). Afterwards, the crystal was melted at the same rate to detect the melting temperature T_m (°C). The difference between these temperatures was calculated to determine the TH activity. This procedure was slightly adopted from Bar et al. [14] to accommodate the circumstances of the different equipment used in this study. Each experiment was repeated at least three times. The images were captured at a rate of 25 frames per second.

The system was tested by performing measurements in 0.1 M ammonium bicarbonate (pH 7.9) with AFP I at a concentration of 10 mg mL^{-1}. Approximately the same value with that of literature was detected. AFP I showed a TH of 0.82 °C. This value was determined to be approximately 0.8 °C at the same concentration with the same type of AFP I (native bpAFP) in 0.1 M ammonium bicarbonate (pH 7.9) [11].

The bursting rate of each crystal was measured using calibrated images by the ImageJ software [15]. Images of the ice crystals for bursting rate measurements were exported from videos via the VLC software in short time intervals (0.13–0.23 s). The scale of the images was used for calibration. The known distance of the side length of a neubauer chamber (µm) was measured in pixels and the scale was determined via set scale function by using these values as a ratio of (pixels/µm). The length measurements were performed based on this calibration. The change in length from one tip of the crystal was measured with time as illustrated in Fig. 2.

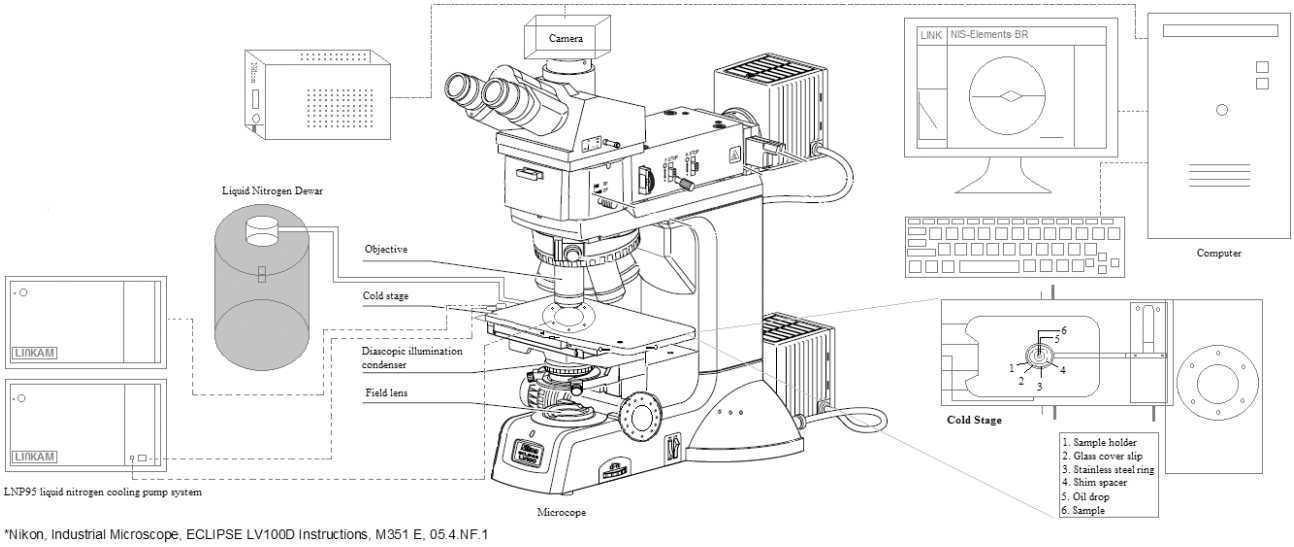

Figure 1. Experimental setup for the thermal hysteresis measurements.

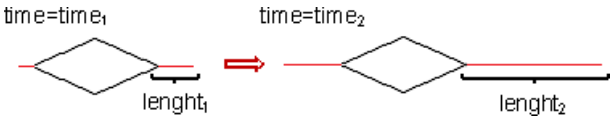

Figure 2. Schematic diagram of the determination of the bursting rate of an ice crystal.

The growth rate of the ice crystal was calculated by using the change in length as represented in Eq. (1).

$$\text{c-axis growth rate of crystal}\left(\mu\text{m s}^{-1}\right) = \left(\frac{\text{lenght}_1 - \text{lenght}_2}{\text{time}_1 - \text{time}_2}\right) \quad (1)$$

The maximum growth rate value known as the bursting rate of crystal was detected. These measurements were performed three times for each experiment.

3 Results and Discussion

3.1 TH Activities

TH activities of AFPs were determined to be a function of AFPs and sucrose concentrations. Fig. 3 shows the link between TH and the concentration of AFPs in a 40 % sucrose solution. The average TH activities of both AFP I and AFGP rise with higher concentration of proteins. For instance, at a concentration of 0.1 mg mL^{-1}, AFP I showed a TH of 0.60 °C with 0.02 °C standard deviation and AFGP exhibited a TH of 0.55 °C with 0.01 °C standard deviation. The lowest TH values were 0.43 °C and 0.37 °C in the presence of 0.05 mg mL^{-1} AFP I and AFGP, respectively, while the highest antifreeze activity values were 2.06 °C and 2.05 °C in the presence of 20 mg mL^{-1} AFP I and AFGP, respectively. Above 5 mg mL^{-1}, the TH values were decreasingly growing with increase in AFP concentration. Thus, there is a hyperbolic dependence on protein concentrations. This dependence of the TH values on AFP concentrations shows a similar trend like the results of other studies reported previously in literature [11, 16, 17].

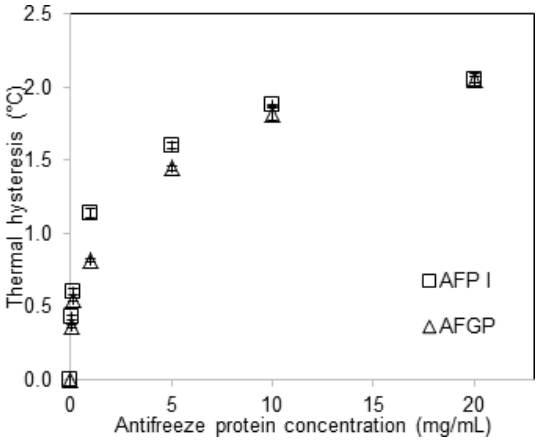

Figure 3. Comparison of TH activities of AFP I and AFGP in a solution of 40 % sucrose.

As indicated in literature, if there is an irreversible adsorption of AFPs to the ice surface, there should be a weak correlation between TH and AF(G)P concentration. However, a significant concentration dependence of TH can be observed in Fig. 3. The concentration dependence of TH, despite of an irreversible adsorption of AFPs to the ice surface, has been explained by one model suggested by Kristiansen and Zachariassen [18]. This model indicated that the reason of the concentration dependence is the AFP molecules' reversible equilibrium distribution between the ice-water interfacial region and the solution at the melting point. Contrary to this, at the temperatures which are lower than the melting point, AFP molecules adsorb irreversibly to the ice surfaces. But, there is already some suspicion regarding the irreversible adsorption of AFPs as stated by Knight and DeVries [19].

Many investigations deal with the mechanism of ice-AF(G)P interaction. Some researchers focused on the accumulation of AFPs at the ice/water interfacial region. They suggested that AFPs allow for liquid water arrangement into an ice specific surface at its hydrophobic face [20–23]. Wierzbick et al. demonstrated that there is a mechanism resembling the hydrophobic solvation effect [24] including the hydrophobic face of AFP which is related to the orientation of AFP molecules at the water/ice interface [25]. Nutt and Smith simulated the interaction and suggested that AFPs arrange a quasi ice-like layer of water on the ice-binding surface of the protein. This layer could be attached to the growing ice crystal by allowing the protein to bind to the solid-liquid interface [26].

The TH activity of AFP I is only slightly higher than that of AFGP. The similar tendency seen in this study was revealed in the review paper of Kim et al. These authors published numerous studies on this subject and compared the results of TH activity of various AFPs from different organisms in buffer solutions. According to this comparison, TH values of different types of AFP I, type I SS3 and type I HPLC6 are indicated to be approximately 0.2 °C at ~ 255 µM and 305 µM concentrations, respectively. The TH activity of AFGP 6 is slightly lower than 0.2 °C at an ~ 300 µM concentration [27]. One of the structural differences between AFP I and AFGP lies in the disaccharide groups of AFGP.

However, Tachibana et al. revealed that TH was not related to the terminal galactose of the native disaccharide [28]. In this investigation, AFGP had a TH activity of 2.05 °C at 20 mg mL^{-1} concentration (see Fig. 3). In the literature, the TH activity of AFGP with similar molecular weight (~ 14 kDa) used as in this study was measured with the nanoliter osmometer in a buffer solution and lower values than the value obtained in this study were detected. They determined the TH value at a concentration of 20 mg mL^{-1} of AFGP as 0.7 °C [29]. The TH values differences between this study and literature values may principally have various reasons: annealing time, cooling rate, ice crystal size, measurement device etc.

Takamichi et al. investigated the impact of ice crystal size on TH at two different cooling rates and found that while TH highly depends on the ice crystal size at a slow cooling rate (0.01 °C min^{-1}), the impact of the size on TH ceases at a fast cooling rate (0.20 °C min^{-1}) [30]. Therefore, the effect of ice crystal size on TH activity becomes more consequential at a slow cooling rate. Another explanation for the differences

could be that TH activities of various AFPs were measured by means of different techniques and devices under microscope in the literature [9, 31–34].

Duman reported that TH values of the beetle-derived AFP were measured to be at a level that is approximately four times higher when using the nanoliter osmometer as opposed to the capillary technique [32]. Consequently, it is clearly understood that the measurement technique has a marked effect on the results obtained. Besides all these effects, the most obvious difference is the high amount of sucrose in our investigation which probably causes the higher TH compared to the results in buffer solutions. The same effect is observed for both AFP I and AFGP. TH values obtained in the presence of AFP I reach 2.06 °C at 20 mg mL^{-1}. This value was determined to be ~ 1.0 °C at the same concentration of the same type of AFP I (native bpAFP) in 0.1 M ammonium bicarbonate (pH 7.9) [11]. This difference demonstrated that the presence of sucrose had a significant impact on TH activity of AFPs because the approximately same value was obtained with that of the literature value during the testing of the system with this AFP in this buffer solution.

This conclusion is supported by a study in which it was demonstrated that the TH activity of *Lolium* AFP was higher in sucrose solution than that obtained in water [35]. Therefore, it is assumed that the differences between TH values may also occur due to differences in the solution content and concentration. However, in the literature, there has yet to be a study which investigates the effect of sucrose concentration variation on TH activities of AFPs. For this reason, the TH activities of AFP I and AFGP in various sucrose concentrations were assessed in this study.

The change in melting and freezing points was demonstrated in detail for the purpose of examining the effect of sucrose concentration on TH activities of AFP I and AFGP. The freezing and melting points determined as a function of sucrose concentration in the presence of 10 mg mL^{-1} are displayed in Fig. 4a for AFP I and in Fig. 4b for AFGP. The difference between the lines drawn for the melting point results and for the freezing point results is described as TH or antifreeze activity of the AFPs used. The dotted lines, which are parallel to the lines drawn for the melting points, indicate the expected freezing points' lines in case of a shift in TH due to the freezing point depression of the AFPs. However, the deviation from these parallel lines grows as the sucrose concentration increases. The freezing points are shifted to lower values in a linear manner with increasing sucrose concentration. Therefore, it can be concluded that there is an additional TH increase with higher sucrose concentration without any connection to the freezing point depression.

The conformity of the measured melting points to expected values in accordance with the freezing point depression were checked by using the T_{melt}[1)] = –1.86m equation which is valid for dilute aqueous solutions [36]. T_{melt} is the calculated melting point value and m is the molality of sucrose. As can be seen in Fig. 4a, the measured values were below the expected ones and

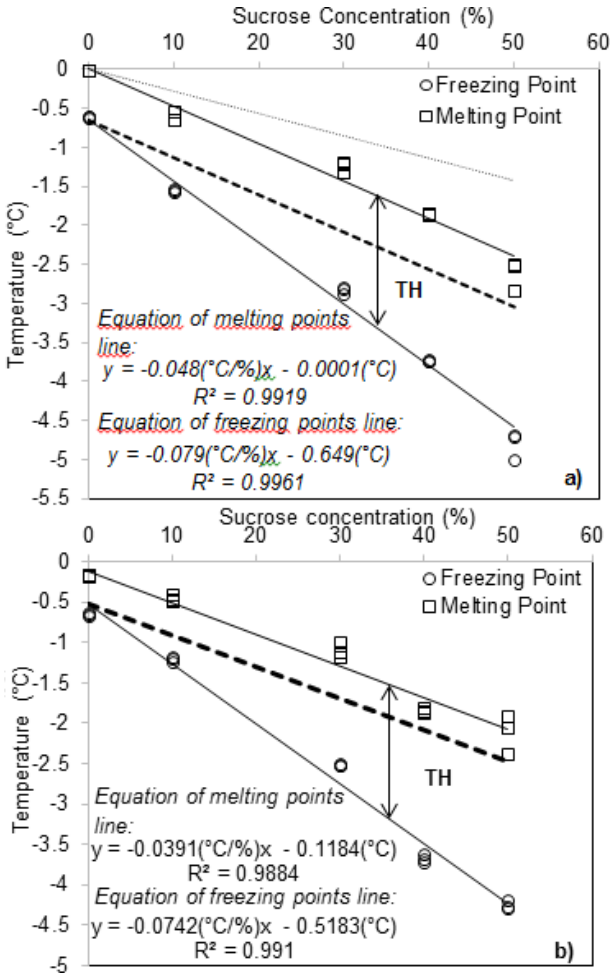

Figure 4. Effect of increasing sucrose concentration (% w/v) on the melting and freezing values in the presence of 10 mg mL^{-1} (a) AFP I and (b) AFGP. The measurements were repeated three times and each point represents a measurement. The difference between the melting and freezing points lines is TH. The thick dashed lines represent the plots of the expected freezing points. The thin dashed line in Fig. 4a is a plot of the melting point depression equation for a sucrose solution.

the difference between both values became larger with increasing sucrose concentration.

Fig. 5 indicates that as the sucrose concentration rises, the TH values increase linearly. This is similar for both AFP I and AFGP. The TH values start in pure water with 0.59 °C and 0.48 °C in the presence of AFP I and AFGP, respectively, and rise up to approximately 2.0 °C with higher sucrose concentration. While the AFP I is approximately 3.7 times more active in the presence of a 50 % sucrose concentration than in the absence of sucrose, the AFGP is 4.5 times more active. This rise in antifreeze activity of AFPs with increase in sucrose concentration was already described by Sidebottom et al. who found that the TH activity of *Lolium* AFP was 0.1 °C in water and 0.45 °C in a 30 % sucrose solution [35]. But they did not elucidate a mechanism of action of sucrose on TH activity.

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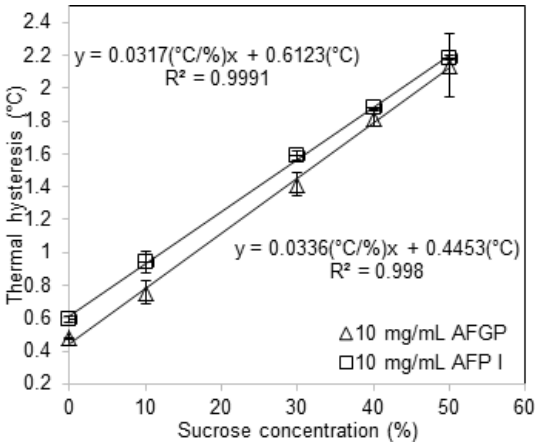

Figure 5. Effect of increasing sucrose concentration on the thermal hysteresis activity of AFP I and AFGP while their concentration remains constant at 10 mg mL^{-1}.

Vorontsov et al. [37] reported that the presence of buffer components results in the enhancement effect on the total depression of the freezing point of AFP aqueous solutions. Therefore, they preferred to perform their experiments in water instead of a saline buffer without focusing on the main reason of this effect. Li et al. [38] investigated the effects of various low-molecular-weight solutes on TH activity of *D. canadensis* AFP (DAFP-4) and found that the TH activity of DAFP-4 highly depends on the concentration of these solutes and that it rises with increasing solute concentration. TH activity was determined to be approximately six times higher in the presence of citrate compared to the its absence. They also added sucrose and a similar tendency was observed. They could not suggest a mechanism due to the differences seen in trends in the efficacy of solutes [36].

Even though the mechanism is still unknown, some ideas have been suggested to explain this enhancement effect. Kristiansen et al. investigated the impact of the presence of salts with RiAFP and the impact of the concentration change of both AFP and salts on TH activity. They found that the TH activity of RiAFP rose with increase in salt concentrations. They also examined the relation between the dependency of TH activity on the presence of salts and solubility of AFP. They suggested that the presence of salts in a solution resulted in a decrease in the solubility of proteins, thus inducing more available AFP molecules for adsorption to the ice surface. This enhancement effect of salts on the antifreeze activity of AFP is elucidated by the salting-out effect [39].

Evans et al. [40] hypothesized that the positive dependency of TH activity and salt concentrations comes from the influence of salt on the hydration shell of AF(G)Ps. They concluded that also salting-in effects may lead to an increase of TH. In our case, sucrose can function in the same way as the salts in the salting-out theory. The added sucrose binds water which is not anymore available for the solvation of the AF(G)P. This may increase the affinity of the AF(G)P molecules to the ice surface and promote a salting-out-like effect. Sucrose cannot function as a salting-in agent as it is not charged. But as the protein concentration in the solutions is relatively low, it is still questionable if this effect can have the observed impact. It could influence the AF(G)P concentration on a local basis taking into account the water-AFP-ice (WAI) theory.

It was concluded in literature that a WAI interphase between the ice phase and water phase is created in the presence of AFP and AFP molecules concentrate in the WAI. This promotes the local colligative effect on the freezing-point depression [41]. This mechanism was considered for AFP+solute solutions and it was suggested that the higher local solute concentration results in local colligative melting at much lower temperatures [42]. The concentration of AFP in the WAI could be further enhanced due to the salting-out-like effect described above. There are other further investigations on the enhancement of TH due to the addition of ions [43] or other proteins [44, 45]. But the explanations given are specifically based on the nature of the added ions or proteins which are not comparable with the addition of sucrose as an uncharged carbohydrate.

3.2 Ice Crystal Morphology

The unique ice crystal morphologies are obtained as a result of interaction of ice with a substantial number of AFPs [46]. For instance, while shapes like lemons or ellipsoids of revolution were observed in the presence of the hyperactive TmAFP from *Tenebrio molitor* [47], the presence of moderate AFPs, type I, II, and III AFPs led to the ice cystals growing in the *c*-axis direction and resulted in bipyramidal shapes [48]. The schematic representation of the formation steps of a hexagonal bipyramidal ice crystal in the presence of moderate AFPs is illustrated in Fig. 6 adopted from Rahman et al. [13].

Firstly, the single ice crystal has a circular shape (Fig. 6_1) in the absence of AFPs. In the presence of AFPs, the six prism planes of an ice crystal are observed with the binding of AFP (Fig. 6_2). The ice crystal has three planes, i.e., basal, pyramidal, and prism, and the morphology can be represented differently depending on the growth rate of these three planes. If the growth rates of the basal and prism planes are higher than that of the pyramidal plane, the shape seen in Fig. 6_2 is observed [49]. The two different planes, namely, the basal plane and the

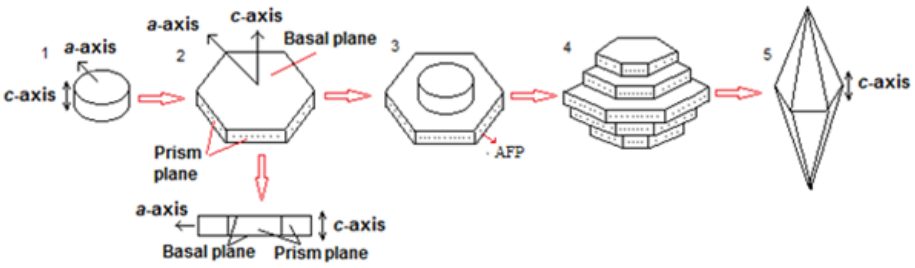

Figure 6. Schematic representation of a potential interaction between the single ice crystal and moderate AFPs.

prism plane of ice crystals, are illustrated in Fig. 6_2. The growth along the basal plane is called the *a*-axis growth while the growth along the prism plane is named the *c*-axis growth. A new disk addition takes place on the hexagonal plate based on the moderately active AFP-binding to specific planes of ice crystal (Fig. 6_3). Therefore, the ice crystal grows in directions parallel to the *c*-axis (Fig. 6_4) [17]. As the process continues, the shape of the ice crystal is transformed into the shape of a hexagonal bipyramidal (Fig. 6_5) [13].

The effect of both AFP I and AFGP on the morphology of ice crystals was examined to assess the antifreeze activity of these AFPs. The images of bursting ice crystals in the presence of various concentrations of AFGP are presented in Fig. 7.

Fig. 7(a-b)$_1$ show a single ice crystal grown in the presence of AFGP. When the temperature was reduced, AFGP bound to specific planes of the ice crystal. Also new disks continuously joined the basal plane and therefore the ice crystal grew along the *c*-axis (Figs. 7(a-b)$_2$). These single crystals grew with a very slow rate at the beginning of cooling. The hexagonal bipyramidal shape was observed at the end of this process (Figs. 7(a-b)$_2$ and 7(a-b)$_3$). This modified shape of ice crystals is attributed to the binding of AFGP to the ice crystal as explained above [13].

In just a few seconds, the rapid elongation was observed from the tips of the bipyramids as seen in Figs. 7(a-b)$_4$. These images represent the burst growth at which the temperature was detected to be the nonequilibrium freezing point, and at this point the maximum growth rates were calculated. The morphology of crystals is comparable to other results in the literature in which the same morphology was observed for native AFGP8. Although the same shape modification was seen in the morphology of ice crystals grown in the presence of native AFGP8, the ice crystals can grow into different morphologies depending on the type and concentration of some other AFGPs with no TH [50].

Knight and DeVries stated that the inhibition dynamically of the basal plane growth by AFGPs was based on their attachment to nearby prism planes and the ice crystal grew along the *c*-axis. The spicule growth was observed below the freezing point [19]. The oil bubbles, (round objects, seen besides the single ice crystals) are due to the observation of the crystals on the immersion oil drop as explained in Sect. 2 and have no effect on the measurements.

Figs. 8(a-b) illustrates the bursting of the ice crystals in the presence of AFP I. Initially, new molecules attached to the single ice crystal (Figs. 8(a-b)$_1$) at a very low rate and as expected, the growth of crystals happened in a similar way along the *c*-axis as seen in Figs. 8(a-b)$_2$. This attachment carried on as the temperature drop continued, and the crystals started growing at a higher rate (Figs. 8(a-b)$_3$). The bursting of the crystals occurred as displayed in Figs. 8(a-b)$_4$ at which the maximum growth rate was determined. It was observed that while the bipyramidal shapes were obtained at concentrations lower than 5 mg mL^{-1} AFP I, for high concentrations, as seen in Fig. 8, the ice crystals had not the morphology seen in the presence of AFGP. The distinct hexagonal bipyramidal shape was not obtained. Unfaceted growth was detected in the presence of AFP I.

The shape modification of crystals was examined in various concentrations of sucrose in the presence of AFPs. The effect of sucrose concentration on ice shape in the presence of AFGP is illustrated in Fig. 9. The ice crystals develop shapes in the *c*-axis direction in a manner similar to that observed in a 40 % sucrose solution in the presence of 10 mg mL^{-1} AFGP. The hexagonal bipyramid morphology was obtained in different sucrose solutions as well. The change in sucrose concentration has no impact on the crystal shape. This indicates that there is no effect of sucrose concentration on ice morphology while the ice shape is strictly dependent on the type of AFPs.

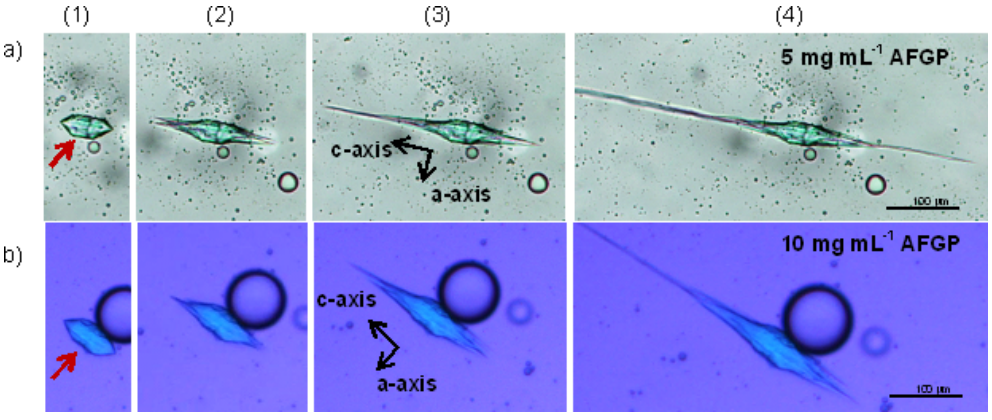

Figure 7. Change in ice shape during the single ice crystal growth (1-4) in the presence of 5 mg mL^{-1} AFGP (a) and 10 mg mL^{-1} AFGP (b) in a 40 % sucrose solution. The single ice crystals, (a-b)$_1$ grow along the *c*-axis with binding of AFGP to specific planes (a-b)$_2$. The growth from both tips of the crystals continues as the temperature decreases (a-b)$_3$. The growth happens at the maximum rate and the bursting of crystals takes place swiftly (a-b)$_4$. The red arrow indicates the single ice crystal, the black arrow the direction of the axis. Round objects are oil bubbles from the preparation in different levels and have no influence on the ice crystals.

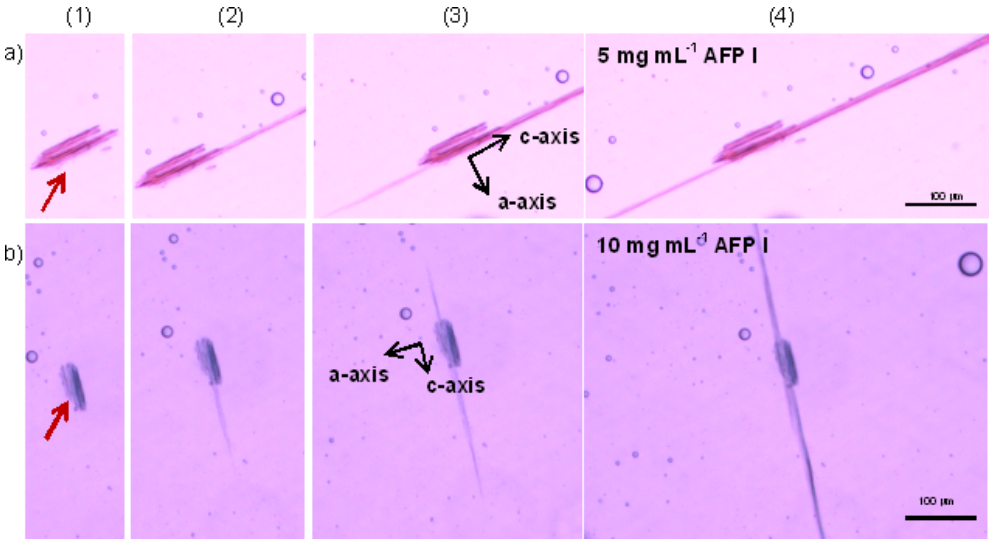

Figure 8. Change in ice shape during the single ice crystal growth (1-4) in the presence of 5 mg mL^{-1} AFP I (a) and 10 mg mL^{-1} AFP I (b) in a 40 % sucrose solution. The attachment of new molecules to the single ice crystal, $(a\text{-}b)_1$, is seen in $(a\text{-}b)_2$ images and the growth rate increases with decrease in temperature. The growth continues along the *c*-axis, $(a\text{-}b)_3$ then the crystals burst $(a\text{-}b)_4$. The red arrow indicates the single ice crystal, the black arrow the direction of the axis. Round objects are oil bubbles from the preparation in different levels and have no influence on the ice crystals.

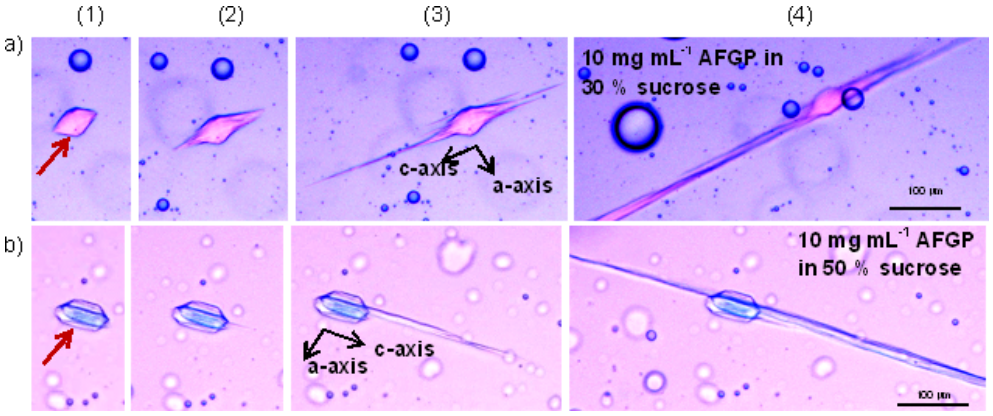

Figure 9. Change in ice shape during the single ice crystal growth (1-4) in a 30 % sucrose solution (a) and in a 50 % sucrose solution (b) in the presence of 10 mg mL^{-1} AFGP. $(a\text{-}b)_1$ illustrates the single ice crystals obtained in various sucrose concentrations. AFPs adhere to specific planes of crystals $(a\text{-}b)_2$ and therefore the crystals grow from the bipyramidal tips $(a\text{-}b)_3$ and the crystals undergo uncontrolled growth $(a\text{-}b)_4$. The red arrow indicates the single ice crystal, the black arrow the direction of the axis. Round objects are oil bubbles from the preparation in different levels and have no influence on the ice crystals.

3.3 Bursting Rate

In addition to these evaluations, the effect of AFPs on the bursting rate of ice crystals was assessed. The dependence of the *c*-axis bursting rates of ice crystals on the concentration of AFPs and sucrose concentration is demonstrated in Figs. 10a and 10b, respectively.

As expected, the presence of both AFPs alters the bursting rate of ice in a manner which is consistent with the literature [10]. As displayed in Fig. 10a, the bursting rate is very low at a low concentration of both AFPs and rises with increase in concentration. This value continues to grow with increase in concentration up to 10 mg mL^{-1} for AFGP and up to 5 mg mL^{-1} for AFP I. This result indicated for AFGP is in alignment with the result of the study of Knight and DeVries [19]. They suggested that the growth rate of spicules depends on the concentration of AFGP and rises with increasing concentration. They performed their experiments up to 9.5 mg mL^{-1} and the same dependency is obvious in the results for AFGP in this study. The maximum values of the bursting rates are seven to eight

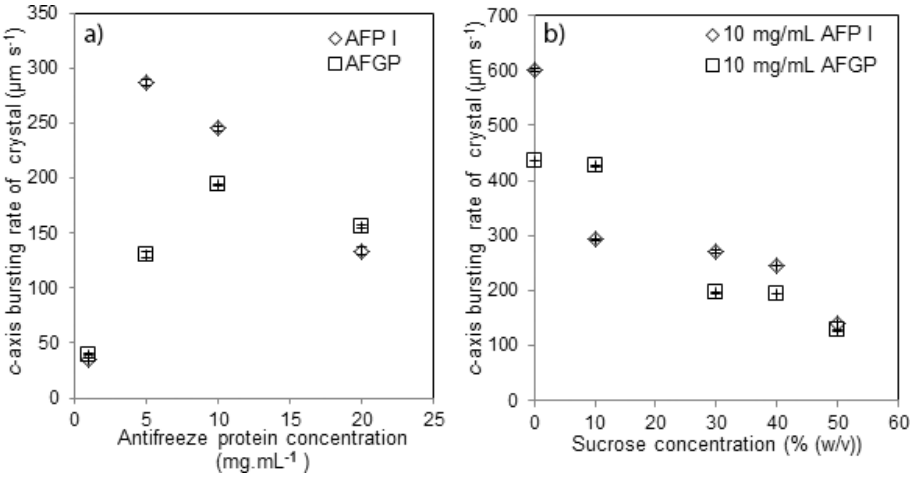

Figure 10. Effect of (a) AFP I and AFGP concentrations in a 40 % sucrose solution on the bursting rate of ice crystals along the *c*-axis, and the effect of (b) sucrose concentration (% w/v) in the presence of 10 mg mL^{-1} AFP I and AFGP concentration on the bursting rate of ice crystals along the *c*-axis.

times higher than the minimum values obtained at lowest concentrations.

This increment in growth rate with increasing AF(G)P concentration may be explained by the ability of AF(G)Ps to reduce the temperature at which an ice crystal bursts. With lower temperature, the level of supercooling increases and the growth rate rises. However, at higher concentrations, the bursting rate declines. This increase and then decrease in the *c*-axis bursting rates can be explained by the binding of AFPs to specific planes of ice crystals.

Chakrabartty and Hew [10] examined the effect of peptides on the *a*-axis and *c*-axis growth rate of ice crystals. They found that while the *a*-axis growth rate of ice crystals decreased continuously with higher concentration of antifreeze peptides, the *c*-axis growth rate of ice crystals rose at low concentration and then decreased with higher concentration. The promotion effect seen in the *c*-axis growth rate at low AFPs concentrations was attributed to the preferential binding of AFPs to the planes perpendicular to the *a*-axes, the prism faces of an ice crystal. Therefore, the *a*-axis growth rate of the ice crystal declines with higher concentration of AFPs. During this process, the *c*-axis growth rate increases. After binding to the these specific planes, if there are more available AFP molecules which do not find a possible surface for binding to the planes perpendicular to the *a*-axes, most likely they bind to the planes perpendicular to the *c*-axis, the basal planes of the ice crystal. This causes a reduction of the *c*-axis growth rate of the ice crystal after a specific concentration [10].

Fig. 10b indicates that the bursting rate of ice crystals notably decreases with higher sucrose concentration in the presence of both AFP I and AFGP. This tendency can be explained by a possible mechanism of action of sucrose explained in Sect. 3.1. The presence of sucrose results in diminishing the amount of freezable water content [42]. Moreover, another possible explanation is related to the viscosity of the solution. The viscosity of the solution rises with higher sucrose concentration which can have an effect on the decreasing growth rate of the ice crystal.

4 Conclusion

TH activities of both AFP I and AFGP were determined in sucrose solutions. Their activities strictly depend on the concentrations of both proteins and sucrose. The results indicate that TH activities of both AFP I and AFGP rise with higher AF(G)P and sucrose concentrations. The TH activity of AFP I was only slightly higher than that of AFGP. The sucrose molecules promote the depression of the freezing points of both AFP I and AFGP. Even if there are other possibilities it is assumed that this effect is most likely based on a salting-out-like effect.

In all cases, the ice crystals grow in the *c*-axis direction. The hexagonal shape of ice crystals transforms into a hexagonal bipyramidal form in the presence of AFP I while the unfaceted growth is observed in the presence of AFGP. It was also revealed that the *c*-axis bursting rate is linked to concentrations of AF(G)P and sucrose. It increases up to a specific concentration, which is related to the direct interaction of the AFPs with ice, and then the *c*-axis bursting rate decreases. The *c*-axis bursting rate dramatically declines with higher sucrose concentration. The results of this study let conclude that AFP I and AFGP exhibit a high TH activity in sucrose solutions and thus, in the presence of these AFPs, the ice growth can be controlled.

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Symbols

m	[mol kg^{-1}]	molality
T_f	[°C]	nonequilibrium freezing temperature
T_m	[°C]	melting temperature

Abbreviations

AFGP	antifreeze glycoprotein
AFP	antifreeze protein
AFP I	type I antifreeze protein
IBP	ice-binding protein

IR ice recrystallization
ISP ice-structuring protein
TH thermal hysteresis

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