Thermochimica Acta, 212 (1992) 105–113
Elsevier Science Publishers B.V., Amsterdam

Anomalous behavior of ice in solutions of ice-binding arabinoxylans [1]

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(Received 24 October 1991; accepted 5 March 1992)

Abstract

Arabinoxylans produced by rye seed interact with ice, reducing the rate of crystallization and changing the patterns of crystallization. These polysaccharides were found to be concentrated approximately fourfold in ice, compared with the crude extract, when separated centrifugally from a partially frozen solution. Cryomicroscopic and DSC examinations revealed that incubation of the frozen sample for up to 1 h elevated the melting point of these partially purified polymers by as much as 0.8 K. A possible explanation is that these polymers produce a long-range order in water which alters its bulk properties, perhaps of the type seen in gels, gums and clays and variously called "vicinal", "anomalous" or "biological". The relationship between this ordering of water and the restructuring of ice in the frozen plant tissue which occasioned this study, is, however, conjectural.

INTRODUCTION

All plants which overwinter in temperate climates are able to survive surprisingly large freezing stresses. In principle, the stress may exceed 10 atm $°C^{-1}$ below the freezing point, but plants have found means to ameliorate this. How this feat is accomplished is little understood [1]. "Orthodox" seeds routinely dry to such an extent that the saccharides present in them form glasses whose transition temperatures are above ambient: the high concentrations eliminate freezable water and the high viscosities produce quiescence by restricting biochemical activity [2]. This is not true of the herbaceous plants from which these seeds come, whose water contents remain high even when their tissues are frozen and whose glass transition temperatures (−30 to −40°C) are well below the killing temperature (−10 to −15°C). One consistent feature of cold adaptation is

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[1] Presented at the 20th NATAS Conference, Minneapolis, MN, 20–26 September, 1991.

the accumulation in the plant cells of large amounts of saccharides, often exceeding 40% of the solute weight of the tissue.

Considerable information has been gained on certain polysaccharides which modify the rate and patterns of ice formation in cereal crops. By far the best studied is an arabinoxylan in rye [3], which increases in content during the cold "hardening" process from negligible to about 0.4% of tissue solute. Because it is active in very small quantities, it is most easily interpreted as an interfacial poison of ice, as are the "antifreeze" glycoproteins in fish [4]. Unlike these glycoproteins which promote supercooling even in the presence of ice but fail abruptly at about −2°C, the arabinoxylans change the morphology of ice without preventing its formation and are still active at lower freezing temperatures. The interaction of this material with ice has been shown to be a slow process, perhaps requiring days for full development [3]. Our standard differential scanning calorimeter does not have adequate stability to study this material properly during freezing, but can be used to measure its melting properties. As a consequence, the studies reported in this paper have not expanded knowledge of the modification of ice formation, but instead have shown a possible relationship to a larger and better studied field: these partially purified arabinoxylan solutions elevate the melting temperature of ice, a well-characterized behavior associated with a long-range ordering of water in clays [5], some gels [6] and small cavities [7].

EXPERIMENTAL

The arabinoxylans of rye are present also in the seed at perhaps one hundredfold the amount, whence we retrieved them because of their ready availability. While complete purification requires considerable effort [8], a crude extract showing all the ice-binding properties of the pure substance can be simply made by grinding 50 g of rye seed in 400 ml of 80% (v/v) ethanol at 50°C to remove the highly soluble salts and monosaccharides, drying the precipitate and taking the supernatant in Milli-Q (Millipore Corp.) water. The resulting solution had a 2% concentration by refractometry (as sucrose), measured on an Abbé type refractometer (Bausch and Lomb). According to published figures [8], this is approximately half arabinoxylan and half polyglucose (starch). The oligomers which bind to ice were further purified about fourfold (7.5% by refractometry) by freezing the sample at −20°C in a centrifuge tube, centrifuging at room temperature at 7000***g*** for 10 min and retrieving the ice plug from the melted liquid. The extract had a freezing point depression of 0.84°C (451 mosm), equivalent to 8.2% sucrose by refractometry. Small samples were sealed in 10 μl "Robotics" sample pans and differential scanning calorimetry (DSC) was performed with a DSC-4 (Perkin-Elmer). Heating thermograms were made in the usual way. For thermograms made during cooling, to reveal the

presence of ice remaining in the sample, the frozen sample was held for several minutes at a temperature near its melting point and then cooled at 1°C min^{-1} or less until an exotherm appeared. The holding temperature was increased in 0.1°C increments until the sample supercooled to the temperature of heterogeneous nucleation, indicating that no ice remained in the sample at the holding temperature. Freezing point determinations were made with an Osmette A osmometer (Precision Systems). The cryomicroscope consisted of a temperature-programmable microscope stage (Linkam, Carshalton Beeches, Surrey, England) on an interferometric microscope with long working distance lenses (Carl Zeiss, Jena).

Because the ice–water transition was the focal point of these studies, all thermometers were calibrated at the ice point. This dictated different methods for the different instruments. Calibration of the osmometer was fairly objective: a sample of saline of known concentration was supercooled by a standardized amount, ice growth initiated, and the value to which the latent heat of freezing elevated the sample temperature was assigned a value based on the known freezing point depression of the solution. For the cryomicroscope, the temperature value was somewhat arbitrary because there is a gradient of 1–2 K across the sample from the center of the 2 mm opening for observation to the heating mantle which surrounds it. In this case, the ice point was defined as the temperature at which the ice in equilibrium with pure water in the center of the sample neither advances nor retreats. Because the heat transfer is poor at the melting temperature, determination takes hours. For the DSC, standard methods of calibration [9] are inappropriate for aqueous solutions; the assignment of temperature is arbitrary because of the temperature gradient within the sample, particularly when the heat sink has been cooled in liquid nitrogen. Our laboratory maintains a chronological file of thermograms of the melting of pure water which is updated whenever the instrument is recalibrated [10] and which becomes an element in the interpretation of results. The relevant thermograms are presented in Fig. 2.

RESULTS

Samples of 10 μl of the purified extract were placed on the cryomicroscope stage in order to study the pattern of ice formation. It was noted in one sample which had been held frozen for about 1 h that the melting point had increased by about 1°C. This finding was verified using DSC. Figure 1 compares two melts of a solution of the freeze-purified polymer. Holding the sample at −10°C for 60 min elevated the melting temperature by 130 mK or more. Though this was less than the value seen by cryomicroscopy, it was still significant. To evaluate the effect of recrystallization within the sample on the heat flow, and thus on the apparent temperature of melting, the experiments were repeated using 5 μl samples of deionized

water (Fig. 2). An aging period of 60 min at −3°C did elevate the apparent melting temperature, but only by 60–80 mK.

Because aqueous solutions have a high heat of melting and a poor thermal conductivity compared to typical DSC samples, and because any remaining ice in the melt floats away from the heat source, a considerable temperature gradient exists within the DSC sample; melts of pure ice show good values for the enthalpy of melting, but as Fig. 2 shows, the expected sharp peak is broadened to over 1.5 K even at the lowest practicable heating rates [10]. As an alternative definition of the ice point in the DSC, we determined when the last ice had vanished from the sample. Samples of 2–3 μl of deionized water were frozen in a sample pan and held for about 10 min at a series of temperatures near the melting point, after which the sample was cooled (Fig. 3). Incremental increase in incubation temperature produced an incremental increase in the amount of ice formed during subsequent cooling, provided that some ice remained in the sample pan. When all the ice had melted, the sample supercooled to its heterogeneous nucleation temperature, about −15°C, whereupon all the ice formed at once. In Fig. 3, the melting point for the last ice in pure water can be specified as measuring between +0.3 and +0.4°C on the DSC scale.

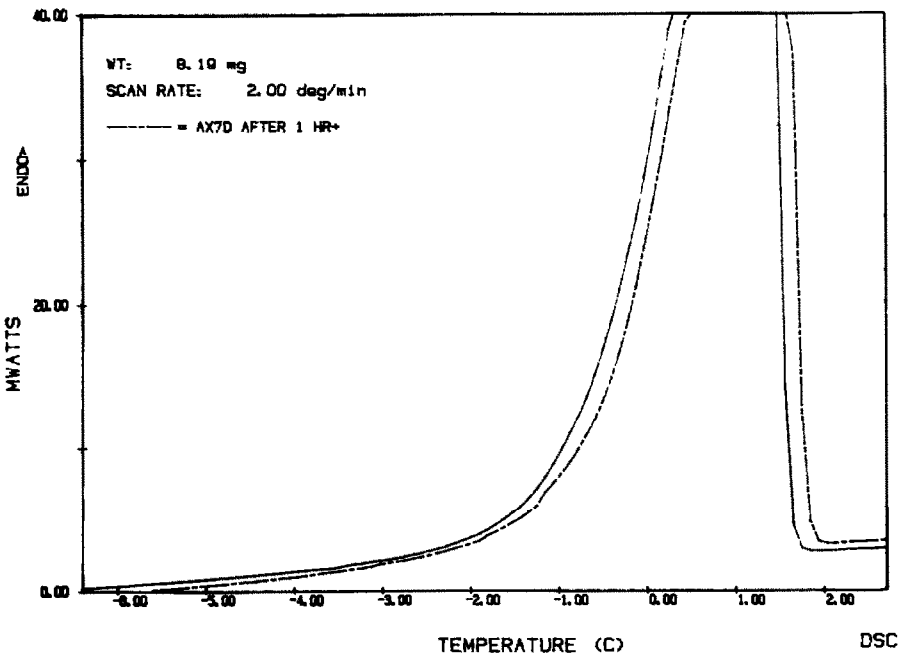

Fig. 1. The effect of frozen storage on the melting temperature of freeze-purified arabinoxylan extract. Incubation at −10°C for 60 min increases the melting temperature by about 0.25°C above that seen in the same sample frozen and warmed immediately.

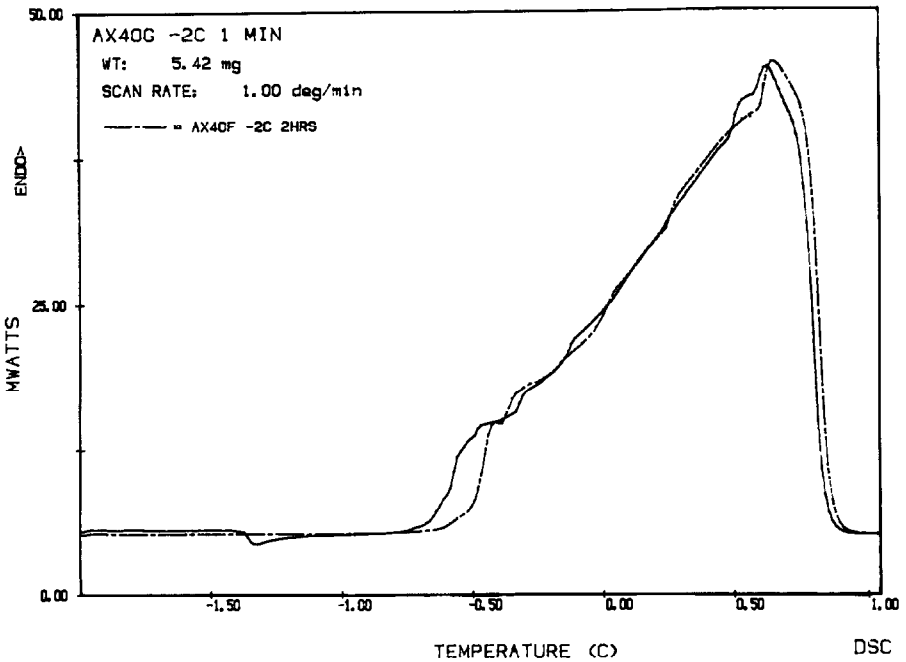

Fig. 2. The effect of frozen storage on the melting temperature of deionized water (Milli-Q). Storage for 60 min elevates the apparent melting temperature by only 60–80 mK, probably the result of changes in heat flow though the sample (cf. Fig. 1).

Figure 4 shows the analogous experiment performed on purified extract. The results are strikingly different from those in Fig. 3. The last ice crystal disappears between +0.3 and +0.4°C, as in the deionized water, but the sample cooled to about −0.5°C before any additional ice formed. Taking calibration differences into account, it is apparent that ice is present and stable up to 0.8 K above the freezing temperature (recall that the freezing point of this material is about −0.84°C by osmometry). Thus the sample cools until it has reached its freezing point before the seeds of ice grow, and the same amount of ice forms during all cooling experiments after the sample is held at or above this temperature. Only when the holding temperature is below the freezing point does significantly less ice form during subsequent cooling, resembling the pattern seen in pure water in Fig. 2.

So that samples having the same freezing point depression could be compared, solutions of sucrose, urea are KCl isosmotic with the polymer extract were prepared and tested in the same manner. All of these showed some tendency for ice to superheat, though less than the oligosaccharide, and the behavior was a mixture of the two shown in Figs. 3 and 4. Figure 5 illustrates an experiment with sucrose, in which superheating approached

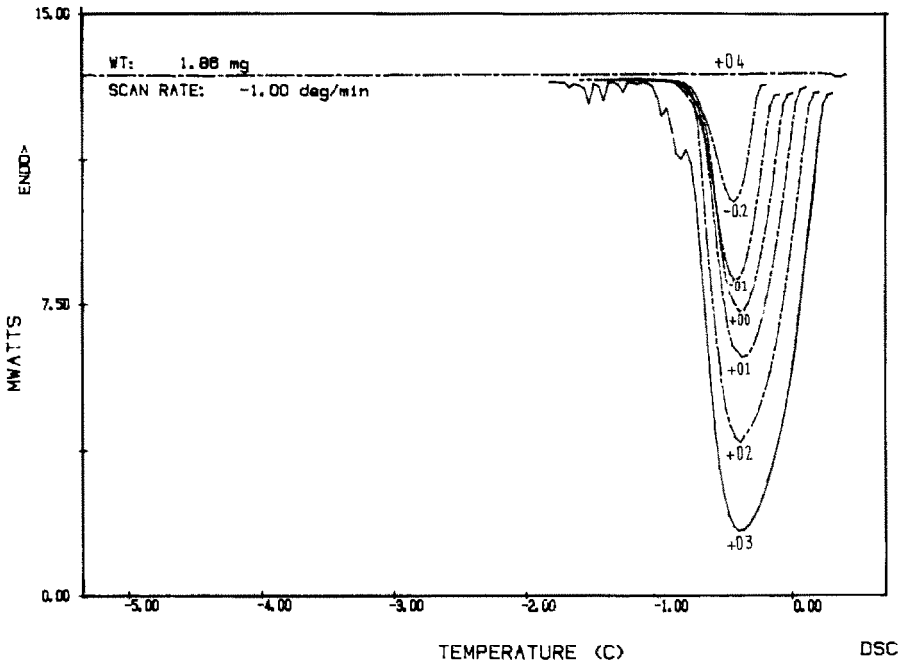

Fig. 3. A sample of deionized water held at a series of temperatures near its melting point and then cooled. When ice is present, the thawed portion begins to freeze immediately upon cooling. When no ice remains (+0.4°C), the sample supercools until heterogeneously nucleated at about −15°C. If these curves are integrated, a temperature profile of the sample can be derived.

0.4 K. Incubation had no observed effect. Unexpectedly, superheating was also observed in urea and KCl solutions but only amounted to about 0.2 K.

DISCUSSION

There is clear experimental evidence [11] that these saccharides have been evolved in cereal grasses subject to cold stress in order to reduce the damaging effect of ice upon living cells. It is therefore not surprising that they might also have an effect on the melting of ice. Nor is the appearance of this phenomenon in sucrose, an oligosaccharide with a degree of polymerization of two, unexpected. The 0.2 K superheating in urea and KCl, however, may be the result of systematic error in the instrument or sample preparation. A possibility we have not entirely ruled out is that the small remaining ice islands are surrounded by a melt of pure water rather than by a solution which does not mix readily. But the viscosity cannot be very high 30 K above the glass transition temperature, and while a difference of 0.2 K might be explained this way, a difference of 1 K is more difficult to justify.

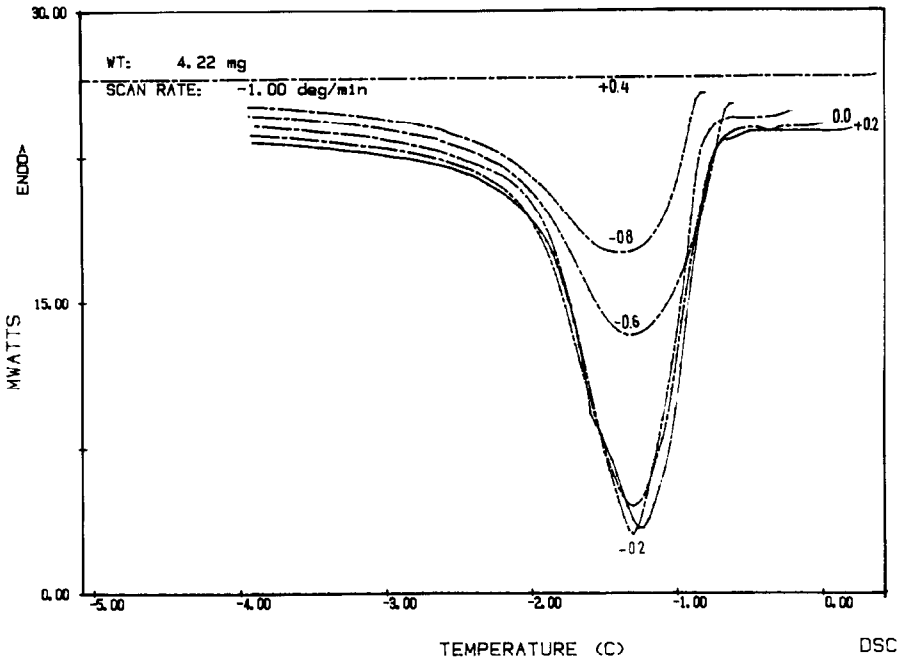

Fig. 4. The effect of incubation temperature on the amount of ice formed during cooling in arabinoxylan solutions. After storage above the freezing temperature of about −0.8°C, the amount of ice formed during subsequent cooling does not vary; greater superheating merely increases the discrepancy between the melting of the final ice crystals and the freezing at the bulk freezing point (cf. Fig. 3).

The "antifreeze" polypeptides from fish also exhibit superheating [12], but it should be emphasized that the "antifreeze" and "antimelt" behavior of both compounds is distinguishable. The fish antifreeze glycoproteins act by attaching to the growing ice interface and poisoning it. This is not overcome until the material has been supercooled over a degree; within that limit, supercooling persists indefinitely.

The simplest interpretation of the published work is that the arabinoxylans act in a similar manner, though our experiments were not subtle enough to probe it. Nonetheless, their effect on the superheating of ice, while it probably has origins in an interfacial interaction, can be more reasonably treated as a bulk property. The polymer is intercalated into the ice in substantial amounts and it is apparent that considerable structuring is required for the superheating effect to manifest itself. While our equipment is not sensitive enough to examine the kinetics, the rearrangements within the ice require longer than 10 min, but seem relatively stable after 1 h. The interaction between the polymer in solution and the advancing ice interface may be similar but the consequences will be quite different when the polymer is part of a crystalline lattice. This makes it difficult to relate

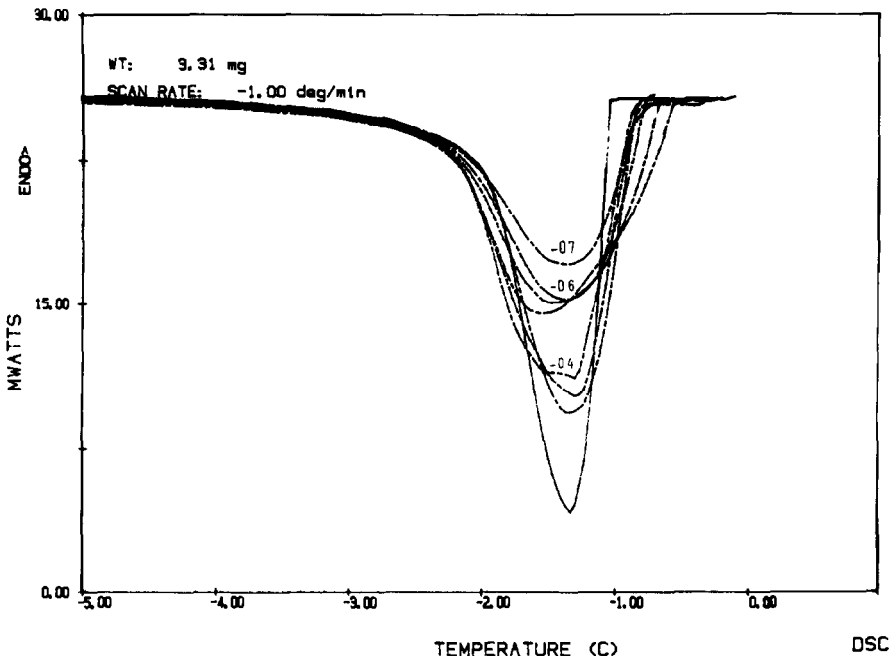

Fig. 5. The effect of incubation temperature on the amount of ice formed during cooling in a sucrose solution isosmotic with the arabinoxylan solution. There is some tendency for the ice to superheat, but the amount of ice formed during subsequent cooling is not constant above the freezing point (cf. Figs. 3 and 4).

the phenomena we report here to the contribution this material makes to cold tolerance in rye grass.

The most exciting consequence of these results is that they relate these polymers to a growing family of compounds shown to impart long-range order to water, an ordering variously called "vicinal", "anomalous" or "biological". Such order, with a decay constant of 0.3–0.4 nm [13], has been postulated in biological systems for 30 years [14], though solid evidence has emerged in only the past few years. The associated properties of this "vicinal" water [15] are a reduced specific gravity of about 0.96 [5] and an ion selectivity [6], whose appearance in the arabinoxylans may serve as a test of this hypothesis. The ion selectivity is currently being tested by membrane filtration and the polymer appears to have an affinity for potassium.

ACKNOWLEDGMENTS

We thank Dr. Harold T. Meryman for his continued support, criticism and enthusiasm. This work was supported in part by NIH Grants BSRG 2507 RR 05737 and GM-17959-18.

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