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ORIGINAL ARTICLE

Role of ice structuring proteins on freezing–thawing cycles of pasta sauces

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Abstract The freezing of the food is one of the most important technological developments for the storage of food in terms of quality and safety. The aim of this work was to study the role of an ice structuring protein (ISP) on freezing–thawing cycles of different solutions and commercial Italian pasta sauces. Ice structuring proteins were related to the modification of the structure of ice. The results showed that the freezing time of an aqueous solution containing the protein was reduced to about 20% with respect to a pure water solution. The same effect was demonstrated in sugar-containing solutions and in lipid-containing sauces. The study proved a specific role of ISP during thawing, inducing a time decrease similar to that of freezing and even more important in the case of tomato-based sauces. This work demonstrated the role of ISP in the freezing–thawing process, showing a significant reduction of processing in the freezing and thawing phase by adding the protein to pure water and different sugar-, salt- and lipid-containing solutions and commercial sauces, with considerable benefits for the food industry in terms of costs and food quality.

Keywords Antifreeze protein · Ice structuring protein · Freezing · Thawing · Food

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Introduction

The freezing of food is one of the most important technological steps for its storage, both in terms of food quality and safety. Moreover, freezing is one of the treatments that less modifies the organoleptic and nutritional characteristics of a food, keeping a good rate cost-benefit.

As summarized by Kiani and Sun (2011), freezing process involved three stages: (1) cooling the product to its freezing point (pre-cooling or chilling stage); (2) removing the latent heat of crystallization (phase transition stage); (3) cooling the product to the final storage temperature (tempering stage).

The freezing procedure is directly connected to the control of crystal size, whose nucleation and growth are important parameters to be controlled in frozen food: excessive crystal sizes may induce food tissue damage and therefore food texture modification. On the other hand, rapid freezing promotes nucleation at the expense of accretion, thus better preserving the texture of food (Hew and Yang 1992; Lillford and Holt 1994; Persson and Londahl 1993; Teramoto and Fuchigami 2000).

Improvement of the freezing process through the control of crystallization of water is an important subject of research. Some examples of new methodologies to control the crystallization of water and enhance the freezing process by reducing the ice crystal size, or restricting water crystallization, include ultrasound assisted freezing, high pressure freezing, ice nucleating proteins, antifreeze proteins, magnetic resonance freezing and microwave assisted freezing (Kaale and Eikevika 2014; Kiani and Sun 2011).

In foods, water is found in two forms: free water, that is normally in liquid state with dissolved solutes, freezing at a temperature dependent on the quantity of these solutes, and bound water, that is linked through electrostatic forces to

proteins, sugars, and starches, and is usually present in small percentage. Bound water has a lower freezing point than free water (Feeney and Yeh 1996, 1998). The presence of free water allows enzymatic and chemical reactions, even mediated by pathogenic microorganisms, which can lead to deterioration of the food.

Thawing happens mostly keeping food at room temperature (Fellows 2000) although microwave applications are also very common to speed up the overall process.

The family of ice structuring proteins (ISPs) (Arai and Watanabe 1986; Davies and Hew 1990; Harrison et al. 1987; Regand and Goff 2006) includes anti-freeze proteins (AFPs) and ice nucleation proteins (INPs) that can be directly added to food and interact with ice, therefore influencing ice crystal size and crystal structure within the food (Li and Sun 2002).

Anti-Freeze Proteins (AFP) have been found in a wide range of organisms, such as fishes, insects, plants, and micro-organisms, all of which have the ability to stand below-zero temperatures without being frozen (Griffith and Vanya Ewart 1995; Jingkun and Tung-Ching 1995; Jingkun et al. 1997; Payne et al. 1994; Venketesh and Dayananda 2008; Yang et al. 1988). These proteins have different structures or compositions, according to the organism that synthesizes them, but they ultimately all rely on the same principle: AFPs modify the formation of ice crystals, inhibiting their growth and lowering their formation temperature, by creating a gap between the melting point in a colligative way and the freezing point of ice in a non-colligative way (Crevel et al. 2002). This results in freezing point depression, with the stabilization of the super-cooled state over a certain temperature range depending on the AFP concentration in water, whose effect can be verified by the thermal hysteresis of a freezing–melting cycle (Petzold and Aguilera 2009; Wen and Laursen 1993).

AFPs have a potential use in industrial, medical, and agricultural applications in different fields, such as food technology, preservation of cell lines, organs, cryosurgery, and cold hardy transgenic plants and animals. This scientific interest has turned up into a number of studies detailing techniques to extract AFP from different organisms (A/F Protein Inc., USA, producing proteins from ocean pout and wolfish; Ice Biotech Inc., Canada, producing proteins obtained from wheat grass juice) or to produce AFP from genetically modified micro-organisms (e.g. patent EP 1 573 017 B1, published on 14/09/2005 by Unilever, for the production of AFP type III HPLC-12, by means of genetically modified yeasts).

It has been reported that these proteins improve the freezing process when they are introduced in food, by inhibiting the growth of ice nuclei and recrystallization during freezing, though their practical application in food industry is still lacking (Li and Sun 2002). In our study, we used an ice structuring protein from cold acclimated wheat grass juice. The proteins extracted by cold acclimated plants have some different peculiarity from ISPs deriving from other organisms, the most important being a much weaker thermal hysteresis activity (Griffith and Yaish 2004). This article describes the freezing and the thawing of synthetic solutions and some real sample (different sauces) containing ISPs. These solutions are different from each other for their components ($C_{12}H_{22}O_{11}$ sucrose, NaCl sodium chloride, emulsion with soya lecithin increasing concentration). In this work, we want to exploit the peculiarity of ISPs from plants in the food field. The new challenge of this work was particularly to establish the role of ISPs in thawing, which is a crucial step in food consumption. The reduction of freezing and thawing time would be a huge benefit for food industry, in terms of costs and food quality (Li and Sun 2002; Virtanen et al. 1997; Xua et al. 2009).

Materials and methods

In this work, the behaviour of an antifreeze protein in different solutions, starting from water and from distilled water three industrial pasta sauces. The protein were extracted from cold-acclimated wheat grass juice (spray dried wheat grass juice stabilized by skimmed milk powder; protein equal or greater than 10%, serial number 2H/OECA/09/30, by Microstar, Zhuhai Biotech Ldt., http://www.msbiotech.com/index.html), and termed as CAWJP. This type of plant protein was characterized by many authors (Chun et al. 1998; Griffith et al. 2003, 2005; Griffith and Yaish 2004).

The solutions used were listed below (all percentages are referred as weight of CAWJP—i.e. spray dried wheat grass juice stabilized by skimmed milk powder—over weight of liquid):

- Distilled water.
- A—$C_{12}H_{22}O_{11}$ (sucrose) 20% in distilled water.
- B—NaCl (sodium chloride) 5% in distilled water.
- C—NaCl 1% in distilled water.
- D—emulsion containing sucrose 1%, sodium chloride 1%, olive oil 5%, soy lecithin 2% in distilled water.
- E—emulsion containing sucrose 1%, sodium chloride 1%, olive oil 5%, soy lecithin 4% in distilled water.
- F—commercial tomato sauce.
- G—commercial "Arrabbiata" sauce (composition: tomatoes, red chili peppers, garlic).
- H—commercial "Alfredo" sauce (composition: butter, heavy cream, Parmesan cheese, garlic, fresh parsley).

The content of CAWJP was 0% (baseline scenario), 0.015, 0.2 and 0.3%. These solutions were stirred in 20 ml sealed glass bottles. The liquid volume used in tests was 10 ml. The needle-shaped, thermocouple {type K [Chromel (Ni–Cr) (+)/Alumel (Ni–Al) (−)], TERSID S.p.A. Milano} was inserted through a hole in the lid fitting exactly its diameter, and placed in the middle of each bottle inside the liquid. The samples were placed in a refrigerator (IGNIS) to carry out the freezing tests, with a set point of −18 °C. The thawing tests were carried out by placing the frozen sample at room temperature. The solutions reserved 240 min to achieve −18 °C in freezer and 180 min to thaw to room temperature. The data were collected by a data logger (HD 32.8.16 TERSID S.p.A. Milano), with an acquisition interval of 15 s. The error in the temperature measurement was related to the accuracy of the K thermocouple, which was observed to be ±0.75%.

The bottle positioning ensured the same thermal history of each sample inside the refrigerator (with a deviation from the mean value comparable to the one given by the thermocouples' noise, being >1 °C). In fact, the latent heat released by sample did not effect the neighboring sample. Each test was repeated at least three times, to check the reproducibility.

The morphology of ice crystals during freezing and thawing cycle was measured by optical microscopy. A Peltier cell (MICROLA, Torino), equipped with heat exchanger, was placed on the plate of the optical microscope (LEICA) and the height of the lens was adjusted in order to get the right focus. An aluminium slice was placed in contact with the Peltier cell, and the temperature measurement was made between the upper side of the Peltier cell and the slice. Droplets of different solutions were put on the slice and Peltier cell was set to the target temperature of −5 °C from ambient temperature. The temperature was monitored and controlled by software. The software allowed the interface between temperature and time for an easy understanding of the time of freezing. The evolution at varying temperature was recorded over time.

Results and discussion

The effects of an ISP extracted from cold acclimated wheat grass on freezing–thawing cycles of some simple and complex samples were investigated. A key calculation in the design of a freezing process was determination of freezing time. Three distinct periods were noticed at different locations within a food undergoing freezing: pre-freezing, phase change and post freezing. To investigate these important points, the change in temperature during freezing process of pure water, in comparison with some solutions and emulsions, with or without the presence of ISP were determined.

Figure 1 shows the freezing profiles of water and of the solution A, in the absence and presence of CAWJP: during the pre-freezing phase, as expected, the temperature of pure water decreased to the freezing point as long as sensible heat was removed. A small degree of supercooling was noticeable, after which the nucleation phase started (the temperature rose to 0 °C). The temperature remained at the freezing point until the phase change is completed and all the latent heat was removed. Then the post freezing phase occurred with a further removal of sensible heat.

In pure water, the different freezing phases were well defined; conversely, in the solution A, the three phases were not so different, due to the presence of solutes in the solution.

The presence of the ISP in water changed the profile of freezing: hence, the protein was able to reduce the freezing time in comparison with pure water. The peculiarity of the protein in shifting the freezing point was even more evident for solutions.

The choice of the proper percentage of cold acclimated wheat grass juice was protein-specific. In this work the percentages were intended as referred to the percentage of juice. The first important comparison was the behaviour of ISP in distilled water. In water, the protein induced a considerable reduction in the freezing time, and this decrease can be attributed to the protein's ability to modify the ice structure. The reduction of the freezing time was about 20% as compared to pure water: this was calculated as the duration of the freezing cycle (portion of the curve from initial freezing to the steady state at the set point temperature), with respect to the one obtained during the reference experiment with pure water. In the case of the solution A, the first phase was similar to water, but the temperature at which nucleation occurred was

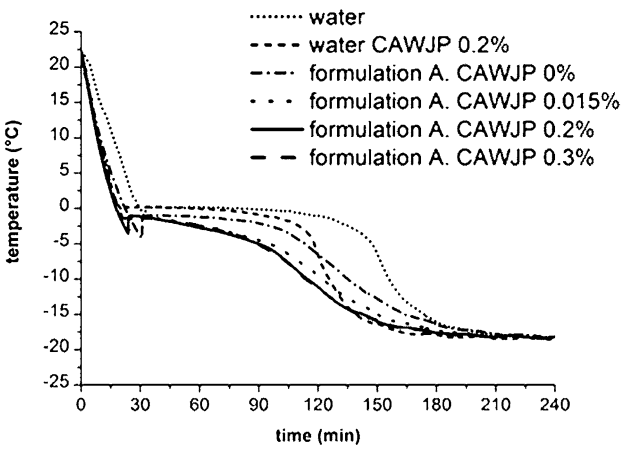

Fig. 1 Temperature-time plot of freezing cycle of CAWJP (0, 0.015, 0.2, 0.3%) in sample A (comparison with water and CAWJP 0.2 in water)

lower than that of water. This deviation in the temperature–time profile was the result of the effect of the solute concentration during freezing. Hence, as water present in the food converted into ice, the remaining water become more concentrated with solutes and the freezing point was lowered. Sucrose has a colligative effect, which depressed the freezing point. At the endpoint temperature, the solutions may still have some water present as liquid (in foods frozen to −18 °C, up to 10% water can be in the liquid state). The effect of ISP in decreasing the freezing time was about 12%, and it was more evident mainly at a ISP concentration of 0.2–0.3%: in these cases, the freezing profile is shifted to shorter times, being about 20% shorter with respect to the solution A without protein.

As far as subcooling was concerned, it was an important aspect but it mainly depended on external factors (such as the presence of vibrations during the freezing test in freezer), that allow or not the occurrence of this phenomenon. This consideration was valid also for the subsequent tests under different conditions.

Based on this first set of tests, it was decided to study the behaviour of protein in solutions of different composition, using solutions containing sodium chloride in different percentage (B with 5% of salt, C with 1% of salt). The percentage of CAWJP chosen for this set of tests was 0.2%, being the ISP amount that had the better effect to concentration ratio in the previous tests.

A 5% concentration of salt was an excessive amount to reach protein folding, which lost its quaternary structure and its native folding. In such conditions, it was assumed that the contribution of the ionic force was higher than the properties induced by the protein. For this reason, the data referred to this experiment were not shown due to their little significance for this application.

As detailed in Fig. 2b, the freezing profiles of C and water were similar, both experiments being carried out with a 0.2% protein content. In the solutions with sugar, the effect of the protein was more relevant than that containing sodium chloride (Fig. 2a). In fact, the reduction of the freezing time in presence of ISP was around 20% for sugar containing solutions.

In order to approach the composition of the solutions to be studied to that of a real sauce, and observe the behaviour of the protein under conditions of greater complexity, olive oil and soy lecithin was added to the solutions containing either sugar or salt (solution D with 2% soy lecithin, solution E with 4% soy lecithin). Under working conditions, ISPs continued to show a reduction of the freezing time (Fig. 2c, referred to sample D with 2% soy lecithin). In the case of a percentage of soy lecithin equal to 4% (sample E), the behaviour was similar to that of 2% (not shown). This demonstrated that an increase in the amount of lecithin did not imply a consequent increase of the protein effect, which remained similar to that obtained for a 2% concentration of lecithin.

The aim of the second part of the study was to use the knowledge coming from the preliminary tests for studying sauce samples with different compositions. Different types

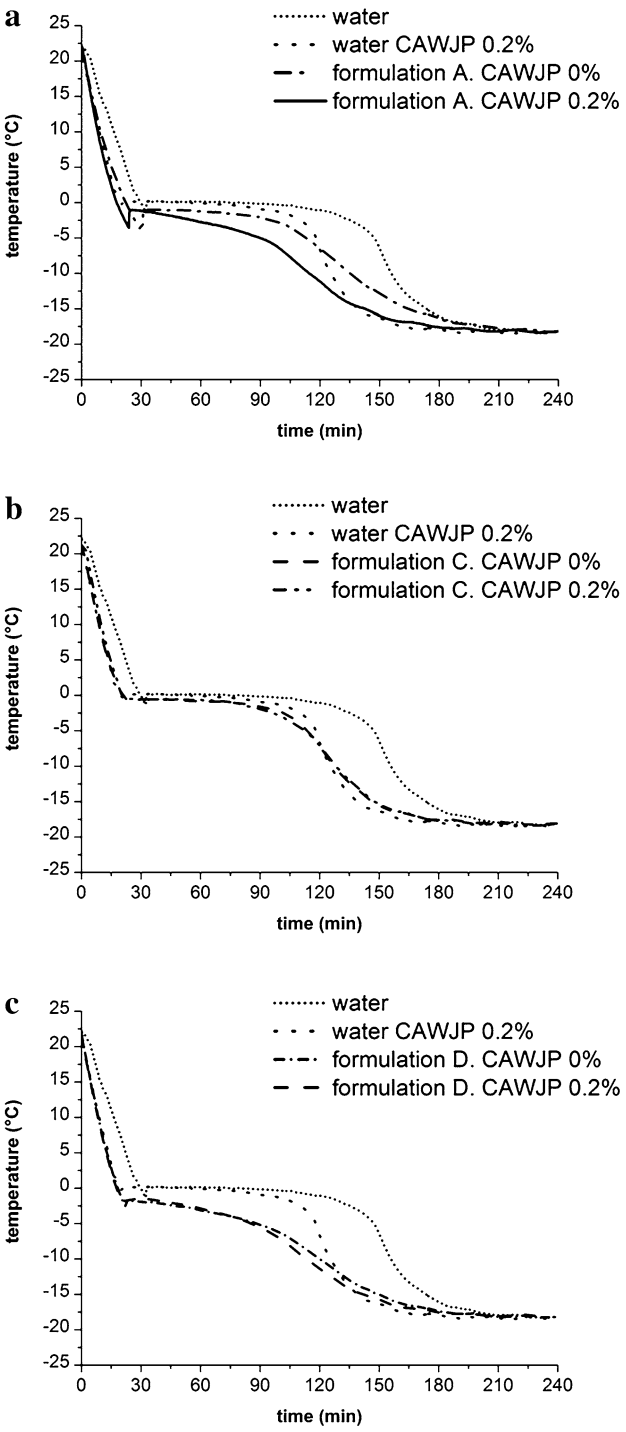

Fig. 2 Temperature-time plot of freezing cycle of CAWJP (0, 0.2%) in sample A (**a**), in sample C (**b**) and in sample D (**c**) (comparison with water and CAWJP 0.2% in water)

of sauces were used for these tests: the first one was a simple tomato sauce and the second was a red hot chili peppers and tomato sauce (Arrabbiata sauce) and the last was a cream and cheese sauce (Alfredo sauce). The first and second ones were aqueous sauces because they were based on tomatoes, while the cream and cheese sauce was constituted by lipids. With these samples, we compared the behaviour of ISPs using two approaches: the temperature–time profile and the observation of the phenomenon by microscope, with a Peltier cell.

Figure 3 shows the temperature–time plots freezing cycle of tomato, Arrabbiata and Alfredo sauce. The freezing reduction time was referred to the sample without protein. The tomato sauce showed a decrease of about 12% with respect to the base case without ISPs, as well as the Arrabbiata one. These data were similar composition of the two sauces.

The decrease of the freezing time in Alfredo sauce was quite surprising in the light of the preliminary tests (Fig. 3c): the comparison between the sauce and the one with the protein led to a freezing time reduction of 17%, while the corresponding comparison between the aqueous solution containing the protein and the sauce containing the protein showed a 37% reduction.

Previous studies underline that these types of proteins were localized in the apoplast (Ding et al. 2014; Smallwood et al. 1999). Studies carried out on the sequencing of m-RNA encoding the amino acids of this protein showed that this protein was highly hydrophilic (Atıcı and Nalbantoğlu 2003; Hassas-Roudsari and Goff 2012). Probably the presence of skimmed milk powder (with a minimum amount of fat) in the extract promoted the interaction with the sample, and then the subsequent interaction of the protein for the modification of the structure of the ice crystals during the freezing.

The morphology of freezing cycle in the two most relevant sauces, Arrabbiata and Alfredo sauces, was compared (see Fig. SM1, reported in Supplementary Material). Comparing the Arrabbiata sauce with and without ISP, the time of freezing falls when the protein is incorporated in the sauce (Figs. 3b, SM1a). The decrease in freezing time during this type of tests was more evident and not comparable in absolute with the fall in freezing–thawing cycle, but confirmed the trend of a 30% reduction for Arrabbiata sauce and 50% for Alfredo sauce. As shown in Fig. SM1a, at the time t = 360 s, the sample G with the 0.2% CAWJP was entirely frozen, while the sample without protein did not completely froze. The pockets shown at t = 60 s were due to solidification of lipids contained into the sauce, although at t = 120 s the formation of some water crystals can be appreciated. Similar behaviour was observed for sample H with the 0.2% CAWJP; in addition, the gap time of freezing was more noticeable (Fig. SM1b).

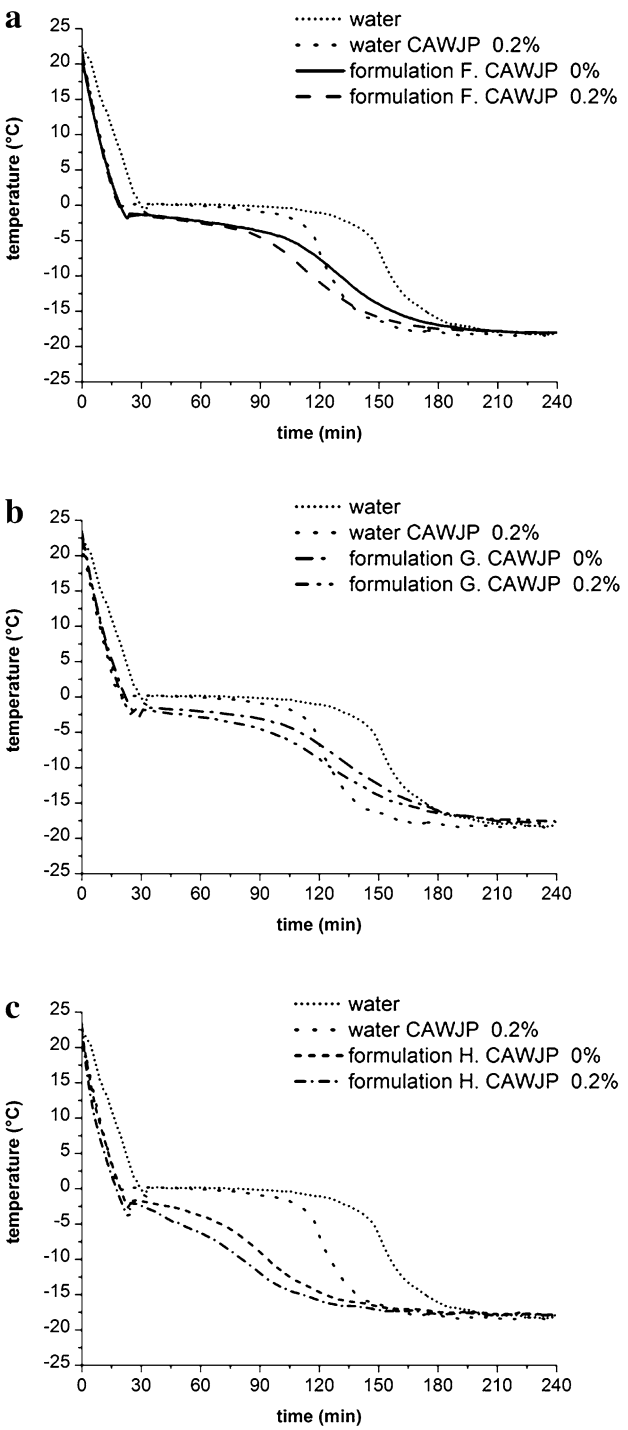

Fig. 3 Temperature-time plot of freezing cycle of CAWJP (0, 0.2%) in sample F (**a**), in sample G (**b**) and in sample H (**c**) (comparison with water and CAWJP 0.2% in water)

According to previous works (Regand and Goff 2006), the pattern of morphology was also very different. The sauces with ISP displayed a pattern that appeared more compact and with a smaller crystallization area. This was a consequence of the ISP nature, which was able to depress

the freezing point of aqueous solutions below the melting point, thus inhibiting ice re-crystallisation and suppressing or modifying ice crystal growth (Rui et al. 2009).

In order to investigate the complete freezing–thawing cycle, the same tests were carried out for thawing with all the samples. A crucial issue of the study was to evaluate the role of ISP in thawing process. As it can be seen in Fig. 4a–c, the comparison among the three solutions with sugar, salt and emulsion with soy lecithin (all with protein) with water with and without ISPs as reference scenarios, underlined the specific role of ISP in thawing. In the case of water, the protein changed the profile of thawing: initially the temperature increased linearly, which was due to the specific heat of water at constant pressure. Until the ice underwent melting, the temperature remained constant at 0 °C. The thawing time depended on the latent heat of fusion. Subsequently the temperature rose again, with a slope that was different from the one initially assumed, due to the different value of the specific heat of liquid water. The ISP modified completely the thawing profile, with a decrease in the thawing time.

The presence of ISPs in water decreased the thawing time by around 20%, very close to the freezing reduction time. The same trend was observed for the direct comparison between the sample A with and without ISP. In Fig. 4b one can notice that the presence of salt seemed to hinder the effect of the protein.

In case of more complex solutions, the protein had a very important role in the reduction of thawing time. The percentage of the time reduction in the direct comparison between sample D with CAWJP 0% and with CAWJP 0.2% (Fig. 4c) was about 37%. Furthermore, if one compares the water CAWJP 0.2% and the sample D with CAWJP 0.2%, the thawing halved in the latter case.

In Fig. 5, the comparison between the sauces evidenced the decrease in the thawing time when the sauces were prepared with 0.2% CAWJP. The decrease in thawing time was high for all sauces, 34% for the tomato sauce, and 20% for both Arrabbiata and Alfredo sauces. Some experiments devoted to taste evaluation revealed that the organoleptic properties were maintained after the freezing–thawing cycle in the presence of ISPs.

The protein plays a role in the reduction of crystals during the cycle freezing–thawing. The thawing of Arrabbiata and Alfredo sauce with and without ISP were compared (Fig. SM2, reported in Supplementary Material). The presence of ISP reduced the thawing time (about 30% from direct comparison); this was explained by comparing screenshots D and D′ in sample G. The complete thawing occurs in 60 s for the sauce without ISP and 40 s for the sauce with ISP.

In Alfredo sauce the situation was similar: the thawing time was reduced by half in the presence of ISP protein.

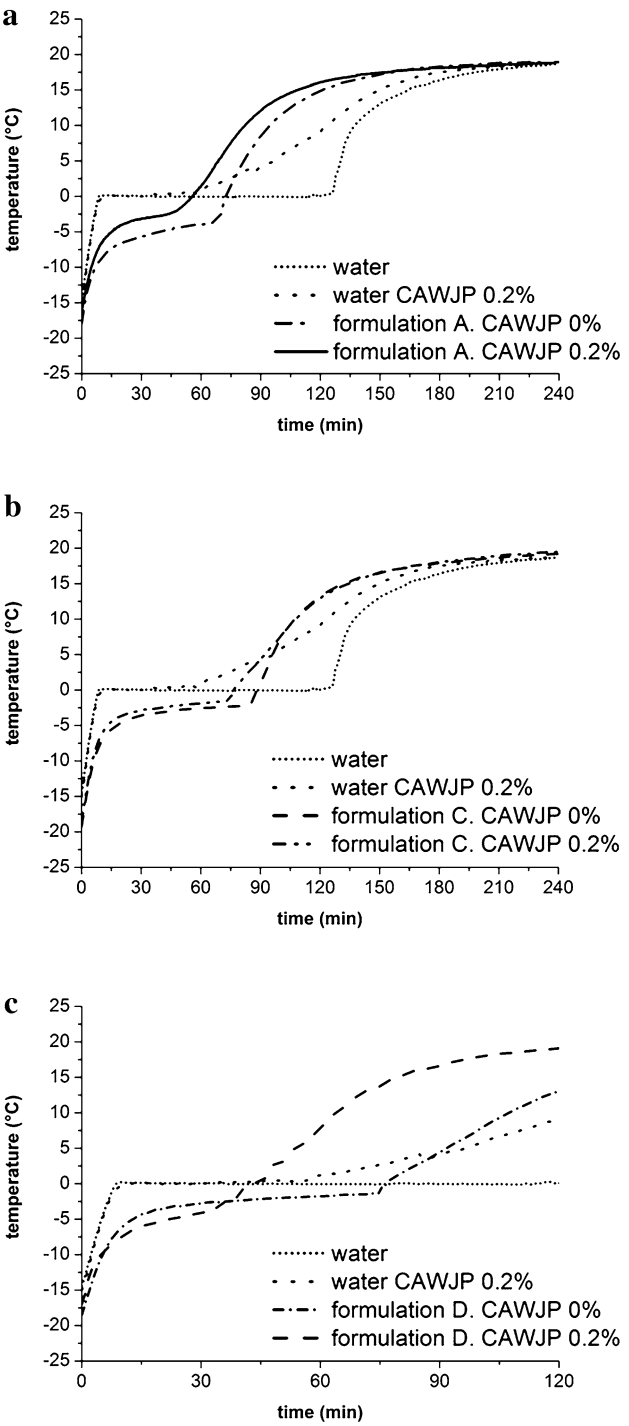

Fig. 4 Temperature-time plot of thawing cycle of CAWJP (0, 0.2%) in sample A (**a**), in sample C (**b**) and in sample D (**c**) (comparison with water and CAWJP 0.2% in water)

These experiments showed role of ISP not only in freezing but also in thawing for the reorganization of the ice structure.

For sake of completeness, we put in evidence that these last results seem to be in contrast with the conclusions reported by Regand and Goff 2005. They found that

AWWE inhibited ice recrystallization during cycled recrystallization with long periods at low frequency. This did not give any information about the nucleation rate and growth of very small crystals (probably not visible with optical microscopy). It was hypothesized that AWWE inhibited the crystal growth only after that they reached a critical size, because of the kinetics of diffusion and adsorption of the ISP to the ice crystal interface (Regand and Goff 2005). It could be assumed that, if the diffusion of water molecules toward the larger crystals is prevented by the AWWE, H_2O molecules can diffuse only towards the smaller crystals, which therefore grew up more rapidly up to the critical size. The process proceeded in this way until the liquid phase was completely frozen, in a shorter time compared to the pure solution.

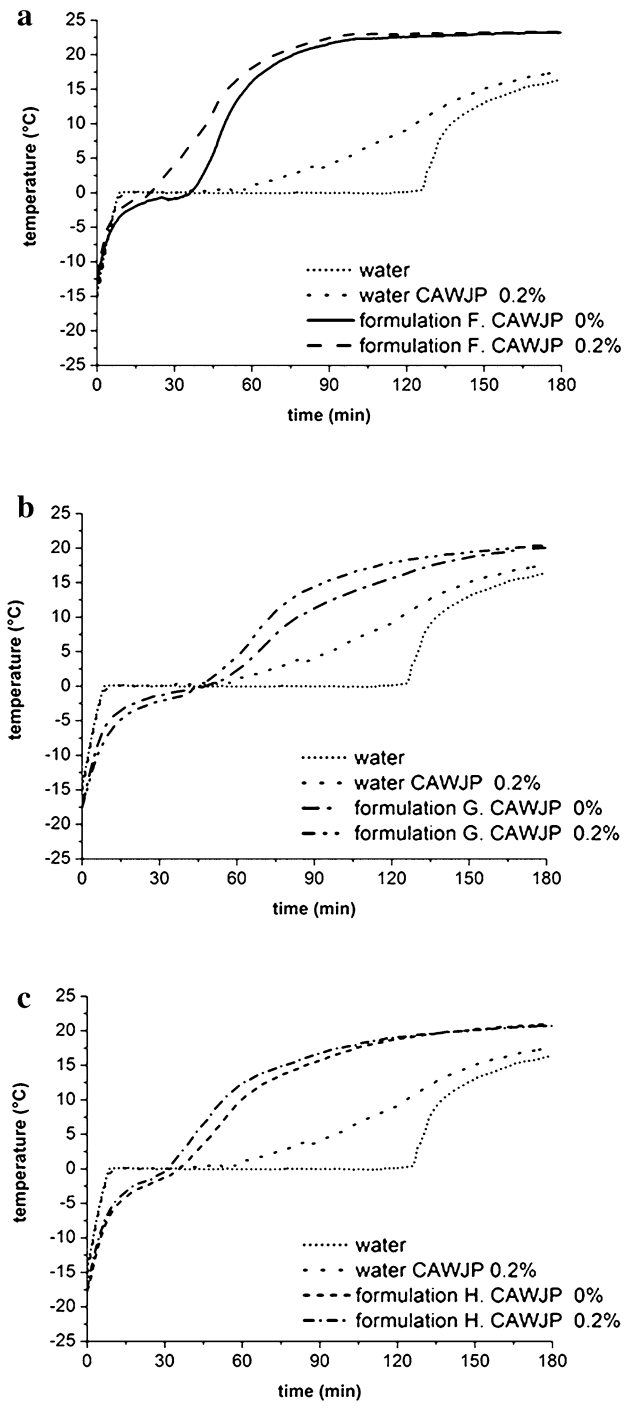

Fig. 5 Temperature-time plot of thawing cycle of CAWJP (0, 0.2%) in sample F (**a**), in sample G (**b**) and in sample H (**c**) (comparison with water and CAWJP 0.2% in water)

Conclusion

This work demonstrated that the ice structuring protein (ISP) plays an important role in the freezing and thawing phases in simple and complex food systems.

This was proved for water with ISP (freezing time reduction 20%) and for solutions with sugar and salt. In complex solutions, the ISP also caused a reduction in freezing time (12% with respect to the ISP-free solution). The decrease in freezing time was significant mainly for commercial sauces. Tomatoes, Arrabbiata and Alfredo sauces in the presence of IPS can be frozen in <2 h, with significant decrease in freezing time for Alfredo sauce (17%), Arrabbiata and tomato sauce (12%).

The decrease in thawing time for solutions were similar to the freezing one. For the commercial sauces, the effect on thawing time was even enhanced: in water-based sauces (tomato and Arrabbiata) the thawing time reduction was about 20–34%, while in the Alfredo sauce the reduction was around 20%.

The use of juice containing protein was therefore promising for the food industry. Although the cost of such extracts containing this type of protein was expensive, the reduction in processing times in the freezing phase and thawing phase, maybe useful for industries.

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