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Review Article

The “cold revolution”. Present and future applications of cold-active enzymes and ice-binding proteins

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ABSTRACT

Psychrophilic organisms adapted to cold environments produce molecules of relevance for biotechnological application, in particular enzymes active at low temperatures and ice-binding proteins that control the growth of ice crystals. The use of cold-active enzymes supports low temperature processes that preserve heat labile compounds and can result, in some circumstances, in energy saving. Among the several possible applications in biotransformations, this paper focuses on reactions of relevance for the food industry and in molecular biology, representative of different market segments. Ice-binding proteins reduce tissues damage provoked by ice crystals and are therefore of relevance for frozen foods and for the cryopreservation of organs and tissues in the biomedical sector.

Introduction

Arctic and Antarctic living organisms have raised considerable interest in the scientific community in order to understand how they can survive and thrive at temperatures near or even below the freezing point of water. Knowledge about the biochemical and physiological adaptive mechanisms allowing life in the cold has been reviewed in [1–3]. There are many challenges that organisms inhabiting low-temperature environments, so-called “psychrophiles”, must face, including membrane rigidity and low rate of chemical reactions, as well as possible damage caused by freezing [2,4]. To survive in these extreme environments, cold-adapted organisms have evolved adaptive changes including the production of cold-active enzymes (CAEs) [5–7], and proteins that control the growth of ice crystals, known as “ice-binding proteins” (IBPs) [8,9]. Biotechnologists have also discovered the potential of these molecules and started to design novel processes to exploit them [10]. Here, we explore the potential and present applications of CAEs and IBPs.

The optimal temperature for CAEs activity (T_{opt}) is usually close to 20–30 °C, but for some enzymes it may be higher and approach the T_{opt} values of thermophilic ones [1]. This is unsurprising since the real hallmark of cold activity is the ability to retain a significant fraction of activity at low temperature rather than the absolute T_{opt} value (Fig. 1). Such a property depends on the high flexibility of structural regions critically important for catalytic activity, resulting in the reduction of the activation energy [6,7]. Often, although not always, conformational flexibility is reflected in low protein thermostability [6,11]. Because of their inherent low activation energy and high activity at low temperatures, CAEs can help to reduce energy consumption and the environmental impact of biotransformation reactions [12]. Moreover, operating temperatures are permissive for heat-labile and perishable substrates and raw materials. Not least, the possibility of inactivating CAEs by moderate heating can also be advantageous whenever the catalyst has to be removed at the end of a process [10]. Thus, CAEs can be used to re-design existing processes based on mesophilic enzymes or to develop new ones, promoting an up-coming “cold revolution” in different fields [10,13].

Avoiding freezing, rather than resisting it, is one of the most unusual strategies of cold adaptation developed by bony fish, insects, yeasts, bacteria, grasses and algae that inhabit cold environments. It relies on the inhibition of the growth of ice crystals by ice-binding proteins [8,9,14,15]. IBPs display two major activities, both of them mediated by their ability to bind to ice crystals. The adsorption of IBPs to the ice surface reduces the freezing point and slightly increases the melting point of water [8,9]. The difference between the two values is defined as the thermal hysteresis (TH) gap (Fig. 2A) [16], the width of which is strictly dependent both on the concentration of a given IBP and its specific features [8,9]. The second activity performed by IBPs is related

Abbreviations: AFP, antifreeze protein; AFGP, antifreeze glycoprotein; AP, alkaline phosphatase; CAE, cold-active enzyme; CPA, cryoprotective agent; IBP, ice-binding protein; IRI, ice recrystallization inhibition; TH, thermal hysteresis; UDG, uracil-DNA N-glycosylase

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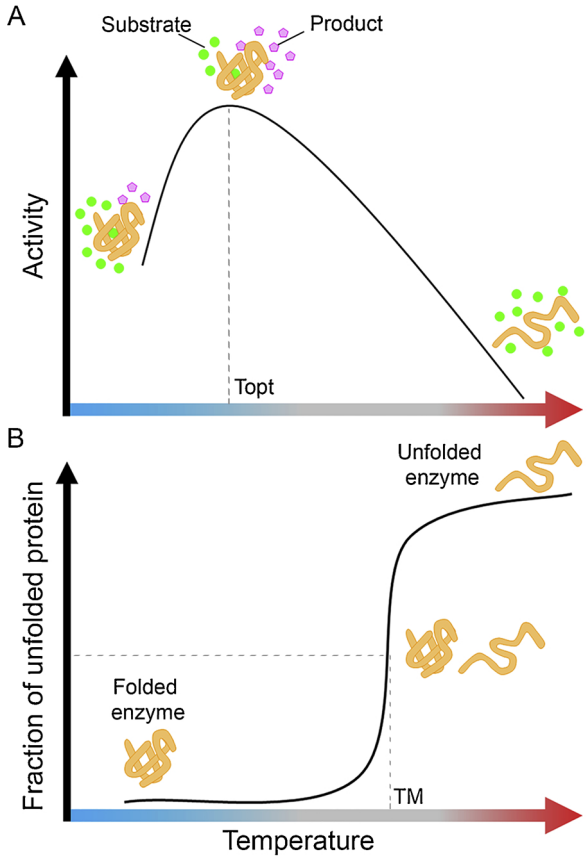

Fig. 1. Activity and heat-driven unfolding of psychrophilic enzymes. (A) Temperature dependence of a generic cold active enzymes (CAE). Generally, CAEs exhibit optimal temperature for catalysis (T_{opt}) in the range from 20 to 30 °C and maintain relatively high activity at low temperature. (B) The inactivation of psychrophilic enzymes usually anticipates the loss of protein structure (temperature of melting), suggesting that the thermolability concerns first their active sites.

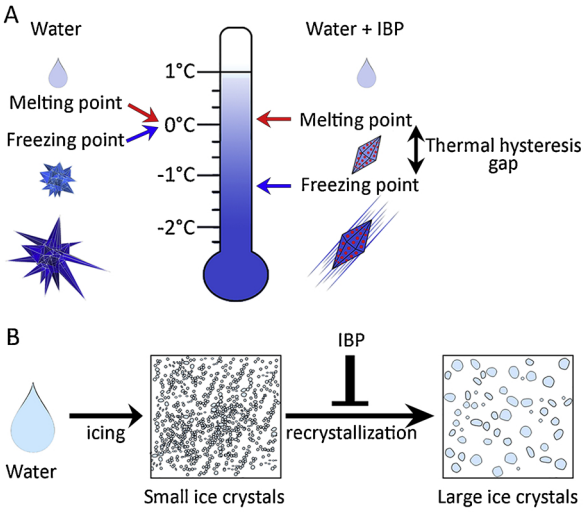

Fig. 2. Activity of ice-binding proteins. (A) Thermal hysteresis. In the absence of IBPs (left), water melting and freezing points are very close to an equilibrium temperature. When present, IBPs (red dots, right side) bind to ice crystals and increase the difference between the melting and freezing points (thermal hysteresis gap). (B) Ice recrystallization inhibition. In the absence of IBPs, large ice crystals grow at the expenses of smaller ones (ice recrystallization). IBPs stabilize small ice crystals, inhibiting the recrystallization process. Panel B is reproduced from [15] with permission.

to ice recrystallization, which occurs by the spontaneous coalescence of small ice crystals into larger ones (Fig. 2B) [17]. Adsorption of IBPs onto small ice crystals results in their stabilization and inhibition of the recrystallization (IRI) process [18]. All IBPs display both TH and IRI activities to different extents, giving rise to distinct biological roles [8,9,15]. Based on TH activity, IBPs are classified as moderate (TH activity in the range 0–2 °C) and hyperactive (TH activity in the range 2–13 °C) [8,9]. As for IRI activity, IBPs are grouped as 'ineffective', 'effective' and 'very effective'. The latter are active at nanomolar concentrations [19], and are produced by some fishes (antifreeze glycoproteins - AFGP) and bacteria [19–21]. Indeed, IRI is of utmost relevance in all freezing processes involving living cells and food products, since large ice crystals damage cell membranes and impair cell viability and food quality [22,23]. This review focuses on the IRI activity of IBPs, and its relevance for the food industry and cryopreservation.

Cold-adapted enzymes

Two important slices of the industrial enzymes market are 'food enzymes', whose volume is steadily increasing, with an expected global demand of over $3.6 billion by 2024 [24], and enzymes used in molecular biology. The market for molecular biology kits, reagents and enzymes was $5.69 billion in 2016 and is expected to reach $13.60 billion by 2022 [25]. Here, we consider the state of the art of CAEs in the food industry [26,27] and molecular biology as representatives of low- and high-value added sectors, respectively.

"Cold biocatalysis" in food processing

Enzymes are widely used in the food industry to prepare beverages, dairy, baking and brewing products [28]. Different processes based on the transformation of heat-labile products can take advantage of low temperature to avoid food spoilage and alterations of flavor and nutritional value [29]. To date the industrial application of *bona fide* cold-active enzymes is still in its infancy. Instead, the food industry employs mesophilic enzymes with low T_{opt}. Nevertheless, a number of CAEs described and protected by patents are ready for exploitation.

Alpha-amylases (EC 3.2.1.1). Cold-active amylases may be of interest for baking to improve bread softness and prevent stalling [30–32], since they can be easily inactivated during cooking [33]. A patent developed with Novozymes (Bagsvaerd, Denmark) concerns a *Bacillus licheniformis* enzyme whose specific activity was improved in the temperature range from 10 to 60 °C by protein engineering [34]. A second patent developed with the industrial partner ColdZYMES ApS, Greenland, describes a system for the heterologous expression of a *Clostridium* α-amylase retaining activity at temperatures lower than 10 °C [35].

β-D-Galactosidases (EC 3.2.1.23) hydrolyze lactose into glucose and galactose and catalyze the transgalactosylation of lactose, which is used in the synthesis of galacto-oligosaccharides [36,37]. β-galactosidases are used in the dairy industry to produce lactose-free products. Lactose hydrolysis is of benefit in lactose intolerance and increases milk sweetness [38]. Moreover, in the production of ice cream, the treatment of milk (or whey) with β-D-galactosidases avoids the formation of lactose crystals and the undesired 'sandiness' of the texture. Several industrial β-D-galactosidases are produced by mesophilic microorganisms and have temperature optima in the range 30–60 °C [39]. To control spoilage, lactose hydrolysis is usually carried out at 30–40 °C for 4 h or at 5–10 °C for 24 h [40]. The use of cold-active β-galactosidases

Table 1
Patented cold-active or thermolabile enzymes. Hypertext links to the patents are reported in **Table S1**.

Enzyme	Organisms	Patent number
Alpha-amylase	*Bacillus licheniformis*	US6673589
	Clostridium perfringens	US20170044510A1
β-D-Galactosidase	*Pseudoalteromonas haloplanktis*	US6727084
Protease	*Rhizomucor miehei*	US4591565
	Rhizomucor pusillus	US6149950
	Pseudoalteromonas SM9913	CN102224938B
	Flavobacterium balustinum	US6200793
	Leucosporodium antarcticum PI12 strain	US8623996
Alkaline phosphatase	*Pandalus borealis*	WO2002031157A8
	Colwellia psychrerythraea	US20120142061A1
		CN106754823A
Uracil-DNA N-Glycosylase	*Gadus morhua*	US7037703
	Psychrobacter sp. HJ147	US7723093B2
	Marine bacterium BMTU 3346	WO1997020922A1
	Psychrophilic marine bacterium	WO2017162754A1
Nuclease	*Pandalus borealis*	US20020042052A1
	Vibrio salmonicida	WO2013121228A1
	Shewanella sp. strain Ac10	WO2006095769A1

for this reaction could contribute to improving the flexibility of the process and to cost reduction, by allowing sugar hydrolysis to occur during refrigerated shipping and storage (4 to 8 °C) [41]. Enzymes from mesophilic *Kluyveromyces* sp. retain 10–20% activity at low temperature and are therefore the most frequently exploited for milk processing [41–43]. Patented cold active β-D-galactosidases are reported in Table 1.

Proteases (EC 3.4.) find wide application in food processing, including brewing, bakery, dairy, meat tenderization and the production of hydrolysates from meat, fish, gelatin and soy [28]. Presently, the commonest enzymatic meat tenderizers are cysteine proteases, such as bromelain, papain, actinidin and ficin from fruits. They are thermostable and most remain active upon heating at 70 °C. The inherent thermolability of CAEs is a desirable feature of meat tenderizers, since it would allow enzyme inactivation at cooking temperatures. Table 1 reports a list of patented thermolabile proteases from mesophilic as well as from psychrophilic microorganisms. Among proteases from psychrophilic sources, that from *Pseudoalteromonas* strain SM9913 might be used in tenderizing collagen-rich meat [44], while that from *Flavobacterium balustinum* promises wider applications, due to its optimal temperature of 40 °C and high thermolability (full inactivation at 50 °C in ca. 10 min) [45].

Proteases are applied in cheese manufacture for the hydrolysis of milk proteins (milk coagulation) [28]. Inactivation at the end of the process prevents undesired long-lasting proteolytic activity in the curd and in cheese, and allows processing of the whey after curd separation. The commonly used rennet endoproteases undergo denaturation on brief heating or pasteurization (60 °C for 20 min or 71.5 °C for 15 s) [46]. The demand for novel proteases as alternatives to rennet, and possibly easy to inactivate, is increasing [28]. However, most microbial enzymes derive from mesophilic organisms and are rather heat stable [47]. Therefore, several studies have been aimed at increasing the thermal sensitivity of the aspartic proteases from *R. miehei* and *R. pusillus* [48]. That described in patent [46] uses chemical treatment to induce thermal lability.

Pectinases (EC 3.2.1.15) account for ca. 40% of all food processing enzymes [49,50] and are employed to clarify and reduce the viscosity of fruit juices [51], in the extraction and purification of natural oils [52], and in wine-, coffee- and tea-making [53,54]. Well-established industrial processes are based on thermostable enzymes, most of which are active at 35–60 °C and inactive below 10 °C [55]. Low temperature processes may be of advantage to limit contamination [56,57], preserve volatile aromatic compounds [58] and increase storage capacity [59]. The need for cold-active enzymes is exemplified by the process of wine-making, where pectinases are added to improve the efficiency of juice extraction and the release of aroma and polyphenols [60]. Here, the fermentation temperature is 10–15 °C, much lower than the optimal temperature of mesophilic enzymes. Publications on cold-active pectinases have been reviewed [61].

"Cold biocatalysis" in molecular biology

CAEs are the standard catalysts in several molecular biology techniques (Table 2) due to their thermolability, which is fundamental for some sequential enzymatic reactions where the increase of temperature allows termination of their activity used in the first reaction steps. Moreover, several *in vitro* reactions require low temperatures, for which CAEs are more suitable than the mesophilic or thermophilic counterparts.

Alkaline Phosphatases (APs, EC 3.1.3.1) catalyse the dephosphorylation of the 5′ end of linearized DNA fragments. They are used to perform DNA 5′ end-labelling and to prevent dsDNA self-ligation of plasmid vectors, thus increasing cloning efficiency [62]. Although commercially available and broadly used, the mesophilic calf intestinal AP [63] has some drawbacks related to its heat-stability, which imposes harsh or time-consuming procedures for inactivation or removal before subsequent cloning steps (e.g. ligation). In contrast, cold-active APs can be easily inactivated by mild or short heating treatments. To date, APs

Table 2
Commercially available cold-active enzymes for molecular biology applications.

Enzyme	Organisms	Products
Alkaline phosphatase	*Pandalus borealis*	Shrimp AP (New England Biolabs)
		Shrimp AP (Thermo Fisher Scientific)
		Shrimp AP (Takara-Clontech)
		Shrimp AP (ArcticZymes)
		Shrimp AP (Jena Bioscience)
	N.A.	FastAP Thermosensitive AP (Thermo Fisher Scientific)
	Alteromonas undina P2	Thermolabile AP (SibEnzyme)
	Antarctic bacterium TAB 5	Antarctic Phosphatase (New England Biolabs)
Uracil-DNA N-Glycosylase	*Gadus morhua*	Cod-UDG (ArcticZymes)
		UDG, heat-labile (Thermo Fisher Scientific)
	Marine bacterium BMTU 3346	UDG, heat-labile (Merck)
	Psychrophilic marine bacterium	Antarctic Thermolabile UDG (New England Biolabs)
Nuclease	*Shewanella sp.* strain Ac10	Cryonase (Takara-Clontech)
	Pandalus borealis	dsDNase (Thermo Fisher Scientific)
	N.A.	dsDNase (ArcticZymes)
	N.A.	HL- dsDNase (ArcticZymes)
	N.A.	HL-ExoI (ArcticZymes)
Protease	Arctic marine microbial	1 ArcticZymes Proteinase (ArcticZymes)

from three different psychrophilic organisms are commercially available (Table 2).

Uracil-DNA N-Glycosylases (UDGs, EC 3.2.2.27) recognize and remove uracil from DNA. Since 1990, they have been used to avoid PCR carryover of DNA contaminants, i.e. the accumulation of PCR products in laboratory environments [64], a major source of false-positive results in diagnostic PCRs, including the loop-mediated isothermal PCR [65,66]. The UDG-based protocol involves the use of uracil instead of thymine, which results in the amplification of UDG-sensitive DNA products. PCR mixtures are pre-treated with UDGs to selectively degrade carryover contaminants but not template DNA that contains thymine [58]. It is important to avoid any UDG activity during the following steps of amplification, when the desired DNA products, also containing uracil, are synthesized. This motivates the use of thermolabile UDGs, which can be easily inactivated. The enzymes currently used are listed in Table 2.

Nucleases (EC 3.1.21). Double-strand specific DNases can be used to decontaminate PCR master mixes and to remove genomic DNA in RNA preparations. Another application involves ssDNA-specific enzymes, such as *Exo*I, which hydrolyse the target nucleic acid from its 3′ end. Any subsequent procedure step requires the inactivation of nucleases, which are therefore preferably thermolabile. Examples of heat labile nucleases are reported in Table 2.

Proteases (EC 3.4.) are used to remove protein contaminants from nucleic acid preparations [62]. The most popular is Proteinase K, which retains relatively high activity at 20 °C and is stable up to 95 °C [67]. A heat-labile protease is a valuable alternative, since its temperature of inactivation is compatible with RNA integrity and conformation of dsDNA. One such enzyme is commercially available (Table 1).

DNA ligases (EC 6.5.1.1) catalyse the formation of phosphodiester bonds, joining DNA fragments with protruding or blunt ends [62]. Currently, DNA ligases from T4 and T7 bacteriophages, *Chlorella* virus and *E. coli* are the most used. For DNA with protruding ends, the optimal ligation temperature is a compromise between the ligase T_{opt} and the optimal temperature for annealing short DNA protruding ends, usually very low [62]. For this reason, the reaction efficiency can be increased by low temperatures (4–8 °C) and by extending the incubation time over several hours (usually overnight) [62]. A novel DNA ligase from the psychrophilic *Pseudoalteromonas haloplanktis* proved to be active up to 4 °C [68], but is not commercially available.

Ice-binding proteins

The market of IBPs was estimated at $2.7 million in 2017 and is expected to reach $10 million by 2023 [69]. Although application of IBPs can be envisaged in the fields of medicine, cosmetics and food, a recent report suggests that the driving force for the IBP market is the medical industry (e.g. cryopreservation) [69]. Below, we describe some applications of IBPs in the food and medical fields, the major sectors in which these proteins are used.

Applications of IBPs in the frozen food industry

Freezing is broadly used to preserve food from microbial decomposition and is obtained by icing residual food moisture. The kinetics of the process plays a fundamental role in maintaining frozen food quality and texture [64]; the faster the icing process, the smaller the intracellular ice crystals and the milder the damage to cellular structures. In addition to preserving the nutritional value, taste and texture of fresh products, frozen foods also have the advantage of shorter preparation and cooking times compared to fresh products. A major challenge is to maintain the cold chain, aimed at keeping optimal storage temperature during production, storage and distribution. Inappropriate refrigeration during distribution is one of the main causes of food waste that in the U.S. is estimated to reach~ 12% [70]. Moreover, thermal fluctuations may cause freeze-and-thaw cycles, inducing ice recrystallization that can greatly affect the quality and shelf life of food products [71,72]. For instance, the formation of large ice crystals alters the cream texture and smoothness of ice creams [73,74]. In frozen fish and meats, the formation of intracellular large ice crystals damages the membranes favouring drip and consequent loss of nutrients during thawing [71,72]. Such drawbacks call for the exploitation of inhibitors of ice recrystallization, such as IBPs.

IBPs are produced by several organisms including winter flounder, carrots and cabbages [71,75]. Among IBPs, that from the ocean pout, an arctic fish, was approved by the U.S. Food and Drug Administration and the European Food Safety Authority as a non-allergenic and non-toxic food additive [76].

Generally, when IBPs are used as food additives they are called “ice structuring proteins” [8]. In some ice creams, IBPs are added to avoid the granular texture induced by ice recrystallization [73,77]. Moreover, in the “helical popsicle” made by a frozen core coated with a fruit gelatine, the addition of IBPs in the core favours the removability of popsicle from the package thus preventing it to break in small pieces [78]. A recent study shows that the small ice crystals formed in the presence of IBPs can aggregate into a 3D network, thus inducing a greater hardness and roughness of ice cream texture, which may be desirable in some frozen dessert, but not in ice creams [79]. This study suggests that the mere presence of IBPs in ice creams is not enough to preserve their smoothly and creamy texture. Most probably these latter features depend on other factors (*i.e.* stabilizers, air and fat), which contribute to the complex multiphase structure of ice creams.

Bread dough is highly perishable, and since the 1960s freezing has been used to increase its shelf life [22,75]. However, frozen dough presents texture alterations and low CO_2 content, which cause poor bread quality [80–82]. The process developed to avoid these problems includes the use of stronger flours with higher content of gluten, the addition of freeze-tolerant yeasts and of IBPs [81]. Several studies reported that added IBPs enhance the amount of total gas produced after thawing of frozen sweet dough [83] and dough softness during freezing [81].

In other cases, IBPs have been physically added in food before freezing [71]. For instance, IBPs were infiltrated into blench vegetables [84] or injected into animals or fishes [85]. The injection of antifreeze glycoproteins (AFGP) 24 h before lamb slaughter was shown to induce the formation of small ice crystals and to reduce dripping of water during thawing [86].

Applications of IBPs in cryopreservation

Cryopreservation is the storage of biological materials in liquid nitrogen (−196 °C) [87,88]. The main challenge is to preserve cell viability after thawing [89,90]. Generally, dispersed cells are cryopreserved in freezing solutions using two methods: slow-freezing [87] and vitrification [91]. In the slow-freezing approach, cells are frozen at a slow cooling rate (1 °C/min) and stored; these steps are followed by a fast thawing process [92]. In contrast, vitrification requires an ultrafast cooling rate, in order to avoid ice formation, and high amounts of cryoprotectants [23,91]. The main risk for both methods is the formation of large ice crystals inducing dehydration and rupture of cell membranes [87]. Besides the chilling injury, a critical role is played by the toxicity of chemical cryoprotectant [93,94] agents (CPAs) such as glycerol and dimethyl sulfoxide. Both molecules can penetrate into cells, reducing the intracellular concentration of electrolytes and preventing *de facto* dehydration and cell lysis, disrupting intracellular signalling [95] and driving protein unfolding [96]. In this scenario, the IRI activity of IBPs might play a key role in the development of new, less cytotoxic cryoprotectants [23,97].

The application of IBPs or AFGPs to mammalian cells and tissues is described in a few patents [98,99] and widely in literature, and has been reviewed in [23]. Several studies describe the addition of IBPs to freezing medium coupled with lowering the amount of conventional

CPA. The addition of IBPs to biological materials enhances cell viability after thawing, regardless of freezing method and storage temperature [23]. In addition, microorganisms with high biotechnological potential, such as commercial microalgae, can be cryopreserved by the addition of IBPs [100].

Conclusions and future perspectives

Some features of CAEs make them suitable for improving existing biotechnological processes through the replacement of mesophilic/thermophilic enzymes. Moreover, a deeper exploitation of CAEs can pave the way for the design of novel transformation processes fully based on psychrophilic catalysts and their combined cold activity and thermolability. Nevertheless, such a “cold revolution” has just began. Indeed, it emerges that several low-temperature processes do not employ CAEs as such. The main bottlenecks can be recognized in low CAE activity and/or stability under environmental or process conditions, including immobilization and re-use procedures, which overall reflect on the costs of the whole cold process [101]. This is particularly true in low-value added sectors, such as food processing, employing massive amounts of low-cost enzymes. The industrial success of CAEs and their competitiveness towards mesophilic and thermophilic enzymes will therefore depend on the ease of production and on their structural robustness, either intrinsic or conferred by protein engineering. In this case, the main bottleneck could be the availability of a suitable specific catalyst, a limit which in turn reflects our poor knowledge of cold ecosystems [102].

The use of IBPs in the cryopreservation of cells, tissue and organs is promising and can contribute to the effectiveness of transport and storage phases. Moreover, the development of "safe" CPAs could be a driving force for organ and tissue banking [103,104]. Unfortunately, in some cases, for example in the preservation of red blood cells [105] and cardiomyocytes [106,107], the addition of IBPs was observed to induce cell damage. Overall, one of the major limitations in the use of IBPs in cryopreservation is still the need for high IBP concentrations that could cause the formation of needle-like ice crystals and damage cell membranes [107–109].

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2019.09.003.

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