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Review

Improving the Stability of Cold-Adapted Enzymes by Immobilization

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Abstract: Cold-adapted enzymes have gained considerable attention as biocatalysts that show high catalytic activity at low temperatures. However, the use of cold-adapted enzymes at ambient temperatures has been hindered by their low thermal stabilities caused by their inherent structural flexibilities. Accordingly, protein engineering and immobilization have been employed to improve the thermal stability of cold-adapted enzymes. Immobilization has been shown to increase the thermal stability of cold-adapted enzymes at the critical temperatures at which denaturation begins. This review summarizes progress in immobilization of cold-adapted enzymes as a strategy to improve their thermal and organic solvent stabilities.

Keywords: cold-adapted enzymes; immobilization; thermal stability; organic solvent stability

1. Introduction

Access to permanently cold regions is not easy, but an increasing number of psychrophilic microorganisms has been deposited into culture collections such as the Polar and Alpine Microbial Collection (PAMC) and are available to researchers [1,2]. Cold-adapted microorganisms are largely categorized into psychrophiles (cold-loving organisms that show optimal growth at less than 15 °C) and psychrotrophs (cold-tolerant organisms that show optimal growth at 20–25 °C) [3,4]. Cold-adapted microorganisms have developed remarkable features enabling survival at low temperatures, such as increased unsaturated fatty acids content in the membrane and increased solute concentrations inside cells [5,6].

Enzymes of cold-adapted microorganisms have evolved mechanisms to avoid the rigidity of protein structures at low temperatures to maintain high catalytic activity via structural modifications, including reduced intramolecular bonds, loop extension, and increased active site accessibility [7–9]. As a result, cold-adapted enzymes exhibit enhanced structural flexibilities compared to their mesophilic counterparts, particularly with more local flexibility around the active site rather than global flexibility [10–12]. The regions distal to the active site have also been shown to affect the catalysis of active sites [13–15]. A lower Gibbs free energy of activation ($\Delta G^{\ddagger}$) resulting from weak substrate binding enables cold-adapted enzymes to perform high catalytic activity at temperatures of approximately 0–30 °C [8,16]. Cold-adapted enzymes exhibit a lower enthalpy (ΔH) and a more negative entropy (ΔS) than their mesophilic counterparts [8]. A larger negative ΔS value indicates that psychrophilic enzymes undergo a larger conformational change, as the enzymes are more flexible without substrate binding [8]. Comparative genome analysis revealed that cold-adapted enzymes exhibit increased glycine content but reduced arginine, proline, and acidic amino acid content [17–19]. Meanwhile, glycine residues provide enhanced flexibility, while arginine and

acidic amino acids residues participate in ionic interactions, and proline residues provide rigidity to protein structures [20–22]. Oligomerization, which is generally used for thermal adaptation of hyperthermophilic enzymes [23], has also been shown to be a strategy for some cold-adapted enzymes such as a GH1 β-glucosidase [24]. Each protein has adopted one or more of these structural modifications to adapt to low temperatures [25].

However, the increased flexibility of cold-adapted enzymes results in weak thermal stabilities at elevated temperatures. The active site appears to be the most heat-labile, especially for multi-domain psychrophilic enzymes [12,26,27]. Trade-offs between stability and activity are the general mechanisms of the cold-adaptation of enzymes [9,28,29].

Santiago et al. reported that, among 92 cold-adapted enzymes reported between 2010 and 2016 in the literature, hydrolases accounted for 91%, followed by oxidoreductases (4%), transferases (2%), isomerases (1%), and ligases (1%) [30]. The majority of these enzymes showed apparent optimum temperatures at 20–45 °C [30]. However, many cold-adapted enzymes do not maintain thermal stability at the apparent optimum temperatures, reflecting their actual temperature of physiological adaptation at much lower temperatures. In contrast, certain enzymes from cold-adapted yeasts, such as lipase A and B from *Candida antarctica* (CalA and CalB), are thermostable. CalA is extremely thermostable with an apparent optimum temperature above 90 °C [31], and CalB shows thermal stability at temperatures up to 60 °C in non-aqueous solutions [32,33].

The temperature adaptation of the catalytic properties has made cold-adapted enzymes promising biocatalysts for industrial applications, and they are now used in the synthesis of heat-labile fine chemicals, as additives in food processing at low temperatures, in detergents for cold-water laundry, and in the bioremediation of contaminated soils and waters in cold regions [34–36]. Thermolability is sometimes preferred, as in the selective thermal inactivation of enzymes by subtle increases in temperature in the food, dairy, and brewing industries, as well as in molecular biology [37]. However, the use of cold-adapted enzymes at ambient temperatures has been hindered by thermolability, despite cold-adapted enzymes exhibiting the potentials for industrial applications. In this review, we summarized the recent progress in improving the stability of cold-adapted enzymes, particularly via immobilization.

2. Protein Engineering to Improve the Stability of Cold-Adapted Enzymes

Many efforts have been made to improve the weak thermal stability of cold-adapted enzymes via protein engineering [38]. Protein engineering such as directed evolution and rational design has traditionally been the most popular tool to improve the activity and stability of cold-adapted enzymes [38]. Directed evolution, which mimics natural evolution, employs error-prone polymerase chain reaction and DNA shuffling [39]. Directed evolution generates a variety of mutations without any structural information [39], but is limited in that a large number of possible variant proteins are generated and that such evolution requires a high throughput screening method [40]. In contrast, rational design uses structural information and is more efficient than directed evolution when structural information is available [41–43].

As hot spots of mutation for activity increase are rather easily identified in active sites, rational design has been used to improve the activity of cold-adapted enzymes [38]. However, for improving thermal stability, hot spots of mutation were not easily identified, so directed evolution has been more successful than rational design [44,45].

Multiple sequence alignment of cold-adapted enzymes with homologous mesophilic and thermophilic enzymes provides valuable insight into the rational design of mutations in cold-adapted enzymes, whereby the corresponding amino acids of thermophilic enzymes are considered for mutagenesis [46]. Mutation of tryptophan 208 in the active site wall of cold-adapted *Pseudomonas mandelii* esterase EstK to tyrosine, in which tyrosine is highly conserved in the corresponding position of hyperthermophilic esterases, conferred catalytic site thermal stability in EstK via a strengthened hydrogen bond [47]. However, the prediction of mutation sites and proper amino acid substitutions remains a challenge [46]. It should be noted that, for cold shock proteins from the mesophile *Bacillus subtilis* and

the thermophile *Bacillus caldolyticus*, which differ by 12 out of 67 amino acid residues, only 2 of these 12 residues were responsible for the difference in thermal stability in the *B. caldolyticus* protein [48].

3. Immobilization of Cold-Adapted Enzymes

Although an increasing number of cold-adapted enzymes has been reported, the majority of studies conducted to date have focused on the isolation, biochemical characterization, and cold-adaptation mechanism [30]. Immobilization of cold-adapted enzymes on a solid matrix is required for their industrial applications as well as removal and recovery of the enzymes for continuous use [49]. In fact, enzyme immobilization is a mature technology that was first commercialized in the 1960s [50]. Enzyme immobilization has been shown to increase (i) stability, (ii) reusability, (iii) activity, (iv) specificity, and (v) handling of the catalyst [49]. Conventional immobilization methods are divided into four categories: adsorption, covalent binding, cross-linking, and entrapment (Figure 1), each of which has advantages and disadvantages [49].

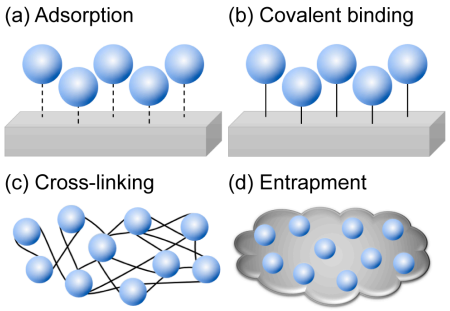

Figure 1. Immobilization of cold-adapted enzymes. (**a**) Adsorption; (**b**) covalent binding; (**c**) cross-linking; (**d**) entrapment.

Many excellent books and review articles on enzyme immobilization are available [49,51–56]. The *C. antarctica* lipases CalA and CalB and lipases from mesophilic yeast *Yarrowia lipolytica* have been extensively used for the development of new immobilization methods [57–59]. Among the enzymes, CalB has received the most attention for biotechnological application and is commercially available as free enzyme or in immobilized form on different carriers, including epoxy-activated macroporous acrylic resin (brand name Novozym 435) [57,59,60]. However, these enzymes from *C. antarctica* are thermostable at temperatures of 60–90 °C [31–33] and thus beyond the scope of this review, even though they originated from cold-adapted yeasts.

When compared with investigations of the immobilization of mesophilic or thermophilic enzymes, few studies of the immobilization of cold-adapted enzymes have been conducted. Several studies have reported the thermal and storage stabilities of immobilized cold-adapted enzymes along with gene cloning and biochemical characterization [61–66]. Although temperature and organic solvents are two major considerations for industrial applications of enzymes, immobilization of cold-adapted enzymes has mainly been evaluated with regard to thermal stability. As cold-adapted enzymes have been immobilized in recent years, more new technologies, such as the use of magnetic nanoparticles [67], single-walled carbon nanotubes (SWCNTs) [68], and graphene oxide [69], have been adopted along with traditionally used matrices such as Sepharose beads. Graphene oxide, a two-dimensional carbon nanosheet with oxygen-containing functional groups (alcohols, epoxides, and carboxylic acids), has recently been used for enzyme immobilization [70]. The degree of oxidation in graphene oxide can be modulated by chemical reduction, and mesophilic enzymes immobilized on chemically reduced graphene oxide showed improved conformations via hydrophobic interactions when compared with those on graphene oxide [71]. Among the immobilization methods, covalent binding has been the most widely used for immobilization of cold-adapted enzymes followed by adsorption and entrapment. Examples of immobilized cold-adapted enzymes are listed in Table 1.

Table 1. Immobilization of cold-adapted enzymes from bacteria.

	Enzyme Name	Species	Support	Chemistry	Comments	Reference
Adsorption	Esterase (EstH)	*Zunongwangia* sp.	Fe_3O_4-cellulose	Hydrogen bonding	48% activity after 30 min at 50 °C	[61]
	β-Galactosidase	*Pseudoalteromonas* sp.	DEAE-Sepharose	Ionic interaction	87%–89% storage stability after 1 week at 4 °C	[62]
	Nucleoside 2′-deoxyribosyltransferase	*Bacillus psychrosaccharolyticus*	PEI-coated agarose	Ionic interaction	Unstable; lost activity within 2 h	[63]
Covalent binding	Pullulanase	*Exiguobacterium* sp.	Epoxy-functionalized silica	Epoxyl group	Maintained thermal stability at 50 °C	[66]
	Esterase (EstK)	*Pseudomonas mandelii*	Graphene oxide	Sulfo-NHS and'EDC	Enhanced thermal stability at 40 °C; catalytic efficiency reduced to 40% of free enzyme	[72]
	β-Galactosidase	*Pseudoalteromonas* sp.	Epoxy-activated Sepharose	Epoxyl group	87%–89% storage stability after 1 week at 4 °C	[62]
	β-Galactosidase	*Pseudoalteromonas* sp.	PEI-coated Sepharose	Glutaraldehyde	98% storage stability after 1 week at 4 °C	[62]
	β-Galactosidase	*Pseudoalteromonas* sp.	Glutaraldehyde-treated chitosan	Glutaraldehyde	Enhanced themal stability at 50 °C; longer shelf life over 12 months	[73]
	Nucleoside 2′-deoxyribosyltransferase	*Bacillus psychrosaccharolyticus*	PEI-coated agarose	Aldehyde-dextran	Operational stability at 37 °C with 75% activity after 30 cycles	[63]
Entrapment	Cellulase	*Pseudoalteromonas* sp.	Sodium alginate beads	Glutaraldehyde cross-linking entrapment	58% activity after seven cycles	[74]
	Pectate lyase	*Bacillus subtilis*	Lipid-functionalized SWCNT	-	Thermal stability at 4–80 °C	[64]
	Laccase	*Pseudomonas putida*	Lipid-functionalized SWCNT	-	Thermal stability at 4–80 °C	[65]

DEAE: diethylaminoethyl; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PEI: polyethylenimine; Sulfo-NHS: hydroxy-2,5-dioxopyrrolidine-3-sulfonicacid sodium salt; SWCNT: single-walled carbon nanotube.

3.1. Adsorption

Physical adsorption, one of the earliest immobilization methods, is still the technique most commonly used to immobilize enzymes [49,75]. In this method, enzymes are reversibly immobilized on a solid support [49]. Enzyme activity is maintained upon adsorption, but enzymes are likely to be detached from the solid support because of the relatively weak interaction with the support [49].

A few cold-adapted enzymes have been immobilized on solid support, including diethylaminoethyl (DEAE)-Sepharose [62] or polyethylenimine (PEI)-coated agarose [63], via ionic interaction. A cold-adapted β-galactosidase, which is preferred for the degradation of lactose during transport and storage of milk at low temperatures, was immobilized on DEAE-Sepharose [62]. This *Pseudoalteromonas* sp. β-galactosidase from Antarctica showed storage stability at 4 °C after 1 week, but no additional thermal properties of the immobilized enzyme were measured at elevated temperatures [62]. A cold-adapted esterase EstH from the marine bacterium *Zunongwangia* sp. was immobilized on cellulose-coated magnetite (Fe_3O_4) via hydrogen bonding with cellulosic sheaths and showed enhanced thermal stability and storage stability [61]. Magnetic nanoparticles have the advantage of easy separation of the enzyme from the reaction mixture by applying magnetic fields [67]. However, magnetic nanoparticles are easily oxidized in air and likely to be clustered together [76].

3.2. Covalent Binding

Enzymes immobilized by covalent binding generally show enhanced stability and high reusability [49]. The enzyme leaching shown in adsorption is significantly reduced in covalent binding during reaction and washing [49]. Cold-adapted enzymes have been covalently immobilized on several solid supports, including agarose [63], chitosan [73], Sepharose [62], silica [66], and graphene oxide [72]. The conformational changes resulting from covalent modifications generally lead to reduced enzymatic activity.

The cold-adapted β-galactosidase (*Pseudoalteromonas* sp.) was also covalently immobilized on Sepharose via coupling to epoxy groups or to the PEI-coated beads via glutaraldehyde [62]. The enzyme bound to the PEI-coated support was more stable than that immobilized on epoxy-functionalized Sepharose as well as DEAE-Sepharose via ionic interaction [62]. Conversely, another cold-adapted *Pseudoalteromonas* sp. β-galactosidase from Antarctica was immobilized on chitosan via covalent binding using glutaraldehyde [73]. The immobilized β-galactosidase, which showed similar profiles with free enzyme but with a 10 °C shift in apparent optimum temperature, was effective at hydrolyzing lactose in milk over a temperature range of 4 to 30 °C [73].

Immobilization lead to overall improvement in the thermal stability of cold-adapted enzymes, especially at critical temperatures, at which the enzymes begin to lose their thermal stability. A psychrophilic pullulanase (*Exiguobacterium* sp.) was shown to lose its activity from 50 °C with almost no activity at 60 °C [66]. Pullulanase immobilized on epoxy-functionalized silica maintained its thermal stability at 50 °C and showed significantly improved thermal stability at 60 °C and 70 °C during 90 min of incubation [66]. Similarly, a cold-adapted *P. mandelii* esterase EstK covalently tethered to graphene oxide exhibited higher thermal stability than that of free enzyme at 20–40 °C, but immobilization did not help improve thermal stability beyond the temperatures [72]. The EstK-graphene oxide complex showed reduced catalytic efficiency of approximately 40% of that of free EstK at 40 °C, owing to distortion of the structure upon covalent attachment [72].

However, direct covalent attachment of nucleoside 2′-deoxyribosyltransferase from *Bacillus psychrosaccharolyticus* (BpNDT), a multimeric protein involved in nucleotide synthesis, onto glyoxyl-agarose resulted in loss of activity, possibly via distortion of the protein structure or subunit dissociation of the enzyme [63]. BpNDT immobilized on PEI-coated agarose via ionic interaction followed by cross-linking with aldehyde-dextran showed reusability at 37 °C for at least 30 cycles with 25% loss of activity [63]. The addition of glycerol (20% working concentration) as a protective agent helped maintain the tertiary structure of BpNDT with improved activity of the recovered enzyme [77]. This strategy could be used for immobilization of multimeric enzymes

preventing subunit dissociation [78]. However, care should be taken to prevent the formation of extra inter-subunit covalent linkage.

Overall, cold-adapted enzymes covalently immobilized on solid support showed improved thermal stability but with reduced catalytic activity when compared with free enzymes.

3.3. Cross-Linking and Entrapment

Cross-linking is used to covalently link enzymes together [79]. Cross-linking of enzyme aggregates has been shown to be effective when a large loading of enzymes is required without support material [79]. Enzymes are precipitated, after which they are cross-linked using agents such as glutaraldehyde or aldehyde dextran [80]. On the other hand, enzymes can also be trapped or encapsulated in a polymer matrix, usually insoluble beads or microspheres [49]. The insoluble carrier may block the active site. However, only a few cold-adapted enzymes were entrapped to date. A cold-adapted cellulase from Antarctic *Pseudoalteromonas* sp. was covalently immobilized in sodium alginate gel beads for the ethanol fermentation of kelp cellulose [74]. The immobilized cellulase showed 58% activity after seven cycles [74]. In another study, a cold-adapted pectate lyase, which catalyzes hydrolysis of the α-1,4 linkage of homogalacturonan, was supplemented with calcium hydroxyapatite nanoparticles as a substitute for cationic activator calcium and entrapped in lipid-functionalized SWCNT [64]. The immobilized pectate lyase showed stability at a wide range of temperatures from 4 to 80 °C, as well as storage stability under repeated freeze-thaw cycles [64].

4. Stability in Organic Solvent

The use of organic solvent in enzymatic reactions is important in industrial applications to increase the solubility of non-water soluble substrates [81,82]. Organic solvents strip off water molecules from the protein surface and penetrate into the enzyme, causing denaturation of the protein structure [83]. The varying hydrophobicity of organic solvents has different effects on catalytic activity of enzymes in each organic solvent [84]. The inherent conformational flexibility of cold-adapted enzymes makes the enzymes susceptible to denaturation by heat and organic solvents [85]. Specifically, heat denatures hydrogen bonding in the protein structure, whereas organic solvents disrupt hydrophobic interactions [85]. Efforts that include the isolation of organic solvent-tolerant enzymes, the modification of enzyme structures, and the modification of the solvent environment [81,82,85] have been made to increase the stability of enzymes in organic solvent. Since the identification of the first organic solvent-tolerant lipase Lip9 from *Pseudomonas aeruginosa* LST-03 with high activity in *n*-decane, *n*-octane, and dimethyl sulfoxide (DMSO) [86], many organic solvent-tolerant enzymes have been cloned [87,88]. Several studies have used directed evolution to improve organic solvent tolerance of enzymes [89–91]. However, rational design was not efficient at improving the stability of enzymes in organic solvents. The mutation of Lip9 on the surface of the protein via directed evolution resulted in a 9- to 11-fold increase in stability in cyclohexane and *n*-decane [92]. However, only a few cold-adapted and organic solvent-tolerant enzymes are known [93–97], and fewer studies have evaluated the effects of immobilization on organic solvent stability.

Recently, the polar organic solvents, methanol and DMSO, were shown to increase the conformational flexibility of the cold-adapted and organic solvent-tolerant lipases PML (*Proteus mirabilis*) and LipS (*P. mandelii*) with increased activity at distinct organic solvent concentrations [98]. Because mesophilic organic solvent-tolerant enzymes have shown no significant changes in the structure in water and pure organic solvent [99–101], the effects of organic solvents on conformational flexibility were considered specific to cold-adapted enzymes displaying inherent conformational flexibility [98]. As a result of increased flexibility in polar organic solvents, both PML and LipS exhibited weaker thermal stability as indicated by reduced thermal denaturation midpoints [98]. Immobilization of cold-adapted enzymes on adequate support in the presence of organic solvents is highly sought after for the application of cold-adapted enzymes in chemical reactions requiring organic solvents or the bioremediation of organic solvent-contaminated sites.

5. Conclusions

An increasing number of cold-adapted enzymes were reported, and their cold-adaptation mechanisms are being characterized; however, the number of immobilized cold-adapted enzymes is much lower than the number of immobilized mesophilic or thermophilic enzymes. Immobilization has been shown to improve thermal stability of cold-adapted enzymes, especially at critical temperatures at which the enzymes begin to unfold. Most studies evaluated the thermal stability or storage stability of immobilized cold-adapted enzymes, but not much information is available regarding how the immobilization affected the conformations of these enzymes. More comparative studies are needed with warmer-temperature enzymes to elucidate the effects of immobilization on the stability–activity relationship. With rapid development in the field of enzyme immobilization, new support materials with enhanced functionality will improve the applications of cold-adapted enzymes at elevated temperatures and in organic solvents. Immobilization of cold-adapted enzymes will be an efficient method when enzymatic reactions at ambient temperatures with continuous use are required.

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