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Induced fit of passenger proteins fused to Archaea maltose binding proteins ☆

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Abstract

Maltose binding proteins (MBPs) are used as carriers for improving the solubility of passenger proteins. Our results indicate that the higher solubility of the fusions correlates with their elevated heat stability. Fusions of the otherwise thermo-sensitive GFP with MBPs from Archaea, but not GST-GFP and *Escherichia coli* MBP-GFP, maintained their fluorescence and structure after 10 min incubation at 100 °C and could be purified by heating the bacteria lysate, with yields even higher than those obtained using metal affinity chromatography. Furthermore, only correctly folded proteins could stand the heating treatment and, therefore, the heat-purification method can be used as a quality control step to select homogeneous monodispersed material whereas soluble aggregates are removed by precipitation.

Keywords: Maltose binding protein; Thermostability; Fusion proteins; Protein aggregation; Melting temperature

The solubility of heterologous recombinant proteins expressed in *Escherichia coli* is often scarce. Proteins the folding and stabilization of which rely on the presence of eukaryotic-specific membranes or post-translational modifications have no theoretical chance of being expressed soluble in bacteria. In other cases, the folding machinery is the limiting factor whereas the high rate of recombinant expression can result in metabolic disorders that lead to misfolding of potentially stable constructs.

Several biotechnological improvements have been introduced over the years to improve the yields of recombinant proteins, like the control of the expression rate using low temperatures and inducer concentrations [1] or the increase of the chaperone concentration obtained either by stimulating the heat-shock response [2,3] or by recombinant co-expression [4,5]. However, fusions of the target protein to suitable partners remain the most frequently used strategy to increase the yields of soluble proteins [6]. Despite some efforts, a comprehensive large-scale comparison of the solubilizing effect of the different tags is still missing because the abundant available data [7–10] have not been collected using the same standards.

Even more scarce information is available about the mechanisms by which the carrier proteins influence the solubility of the passengers. Recent results indicate that it is passive [11] but it remains to understand why the fusion to one carrier is more efficient in combination with a specific protein whereas another protein is better solubilized when fused to a different partner. Consequently, a pragmatic approach suggests, for any protein, the systematic fusion to a large number of different carriers for an exhaustive comparison and choice of the optimal candidate [10].

An interesting result made using variable regions of mouse antibodies relates the capacity to tolerate extensive heating to the solubility of the same protein expressed recombinantly in bacteria [12]. This fact suggests that the

☆ *Abbreviations:* MBP, maltose binding protein; IMAC, immobilized metal affinity chromatography; GST, glutathione *S*-transferase; DLS, dynamic light scattering; T_m, melting temperature; CD, circular dichroism.

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mechanisms controlling the protein (re)-folding under heat and overexpression stress are similar. We already demonstrated that fusions of thermo-sensitive passenger proteins and thermostable carriers could be recovered soluble after heat treatment but did not investigate the thermo-tolerance features [13]. The carrier could either passively keep in solution the denatured passenger during the heating and allow its independent refolding or stabilize the whole fusion protein. In a previous work, thermostable carriers maltose binding proteins (MBPs) resulted more efficient as solubilizing partners for a set of proteins than glutathione *S*-transferase (GST) [14]. The data presented in this paper indicate that the higher thermostability of the MBP carriers in comparison to GST can improve the conformational order of the passenger protein and that heat tolerance can be used for the selective recovery of monodispersed proteins.

Materials and methods

Protein expression and purification. GFP-fusions [14] were transformed into BL21 (DE3) pLysS cells induced with IPTG when the OD_{600} was 0.5 and cultured at 20 °C for 16 h, at 37 °C for 3 h, and at 42 °C for 2 h, respectively. Small-scale culture for expression screening and large-scale cultures followed by metal affinity purification (IMAC) were performed as described in [10].

The total lysate was ultracentrifuged to separate the pellet from the supernatant that underwent heat purification [13]. Samples were heated 15 min at 70 °C in a water bath, cooled down for 15 min on ice, and centrifuged to separate the precipitated proteins from the soluble fraction. This was concentrated and buffer exchanged using either a Vivaspin 500 cartridge (Vivascience) or a HiTrap column (GE Healthcare).

Maltose affinity purification was performed using amylase resin from New England Biolab and the manufacturer's instructions.

Protein aggregation and biophysical characterization. Protein aggregation was estimated using three independent parameters. Dynamic light scattering (DLS) was measured at 20 °C using a Dynapro-801 instrument (Protein solutions). The gel filtration index was calculated as the ratio between the amount of monodispersed and aggregated protein separated by size exclusion chromatography [15] using a Sepharose 12 column connected to a FPLC system (Amersham). The aggregation index measured at the fluorimeter (Aminco Brown—Luminescence Spectrometer) is the ratio between the signal at 280 (light scattering due to aggregation) and 340 nm (absorbance of aromatic residues) [16]. The same instrument was used to record the GFP fluorescence of the samples at 510 nm using an excitation wavelength of 450 nm and a slit width of 5 mm.

Circular dichroism (CD) was measured using a Jasco J-710 instrument equipped with J-700 software and quartz Suprasil precision cells (Hellma). The spectra indicative of the protein secondary structure were recorded between 194–200 and 250 nm. The melting temperature (T_m) of the fusion proteins was calculated as the intersection point between the curve of ellipticity and the middle ellipticity of the two values of constant ellipticity (native and unfolded state). The curve of ellipticity was recovered after having chosen the wavelength with the highest difference of ellipticity between the spectra of the folded and denatured state. Then the temperature was progressively increased at a rate of 30 °C/h from 15 to 90 °C and the ellipticity values recorded at every increase of 0.2 °C.

Results

GFP-fusions with MBPs are more aggregation-resistant than the fusion GFP-GST

In a previous paper, it was shown that a set of proteins expressed at 37 °C as fusions with different MBPs was extremely more soluble than fused to GST [14]. However, a comparative expression screening performed at 20 °C using 1.5 ml of bacterial culture did not show significant differences between the yields of soluble GST-GFP and soluble MBP-GFPs (Fig. 1A). The yield results were confirmed after large-scale expression and purification. GFP-fusions with MBP from *E. coli* and *Pyrococcus furiosus*, as well as with GST, produced 6–7 mg/L of soluble protein, whereas the fusion with MBP from *Thermococcus litoralis* yielded 10.8 mg/L (Table 1). No degradation products were observed after SDS–PAGE using the purified proteins from large-scale purification (Fig. 1B), indicating that the harsh conditions used in the small-scale screening and not an intrinsic instability of the constructs explain the break-down pattern of Fig. 1A.

The presence of soluble aggregates was quantified using the aggregation index [16], the gel filtration index [15], and by DLS. Low values of the aggregation index indicate high

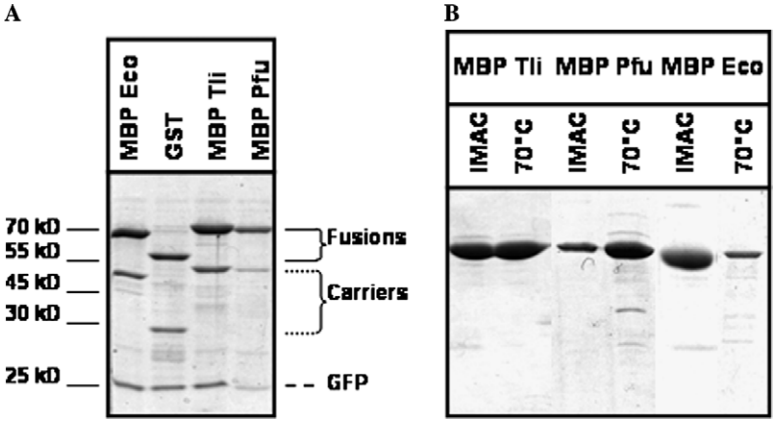

Fig. 1. Purification strategies for proteins fused to MBPs and GST. (A) Small-scale IMAC purification of recombinant GFP fused to GST and *E. coli*, *T. litoralis*, and *P. furiosus* MBP, respectively. (B) GFP-fusion constructs with MBPs from *E. coli*, *T. litoralis*, and *P. furiosus* purified from large-scale cultures either by IMAC or by heating the lysate at 70 °C for 15 min.

Table 1
Yields and physical characterization of fusion proteins between GFP and MBP or GST

Fusion carrier	Yields ($mg_{protein}/L_{culture}$)	Aggregation index	Gel filtration index	Melting temperature (°C)
MBP *Eco* (20 °C)	5.85	0.08	3.4	60.1
MBP *Eco* (70 °C)	0.75	2.02	—	—
MBP *Pfu* (20 °C)	5.60	0.11	2.5	>100
MBP *Pfu*				
(70 °C)	7.35	0.19	2.3	
(100 °C)		0.98		
MBP *Tli* (20 °C)	10.80	0.08	3.2	>100
MBP *Tli*				
(70 °C)	12.10	0.11	2.9	
(100 °C)		0.21		
GST (20 °C)	5.25	0.95	1.4	58.2

The constructs were purified by IMAC or by heating the supernatant after lysate centrifugation (15 min at 70 °C) from cultures grown at 20 °C. The aggregation state of the purified proteins was analyzed using the aggregation index (Ads. 280 nm/340 nm) and the gel filtration index (monomeric protein/aggregate protein as estimated by size exclusion chromatography). *Tli* and *Pfu* MBPs were incubated 10 min at 100 °C whereas the melting temperature was calculated after heat-dependent denaturation of the proteins in a CD spectrophotometer.

monodispersity and all the MBP-GFP fusions scored significantly better than the GST-GFP (Table 1). The gel filtration index data confirmed that soluble MBP-fusions were less aggregated than GST-GFP (Table 1). The mass of the protein species present in solutions was determined by DLS as well and these data further confirmed the presence of soluble aggregates exclusively in the GST-GFP sample.

Heating purification of GFP-fusions with thermostable MBPs has negligible effect on the protein structure

The thermo-tolerance of the different GFP constructs was tested by heating the soluble fraction recovered after cell lysis 15 min at 70 °C [13]. No soluble GST-GFP was detected in the soluble fraction after the heat treatment whereas, according to the aggregation index value (Table 1) some soluble aggregates of *Eco* MBP-GFP were recovered in the soluble fraction (Fig. 1B).

In contrast, the fusions with the thermostable *Tli* and *Pfu* MBPs were still soluble and both the fluorescence (data not shown) and the yields after heating were the same or higher than after IMAC (Table 1). The heat-purified samples contained only a minimal amount of contaminant proteins (Fig. 1B) and both aggregation indexes indicated that the fusions were monodispersed (Table 1). DLS analysis confirmed the results and *Pfu* MBP bound to amylose resin with the same efficiency after being purified either by IMAC or by heating treatment (data not shown).

The secondary structure of the two thermostable constructs was analyzed by CD and the IMAC-purified samples were used as a control. The spectra of both the heat-purified constructs showed that the heat treatment did not significantly modify the protein structures (Fig. 2). Moreover, even an incubation of 10 min at 100 °C did not induce the aggregation nor quench the fluorescence of the purified *Tli* MBP-GFP protein (Table 1).

Thermostable proteins from Archaea can conserve their native folding at temperature higher than 100 °C and

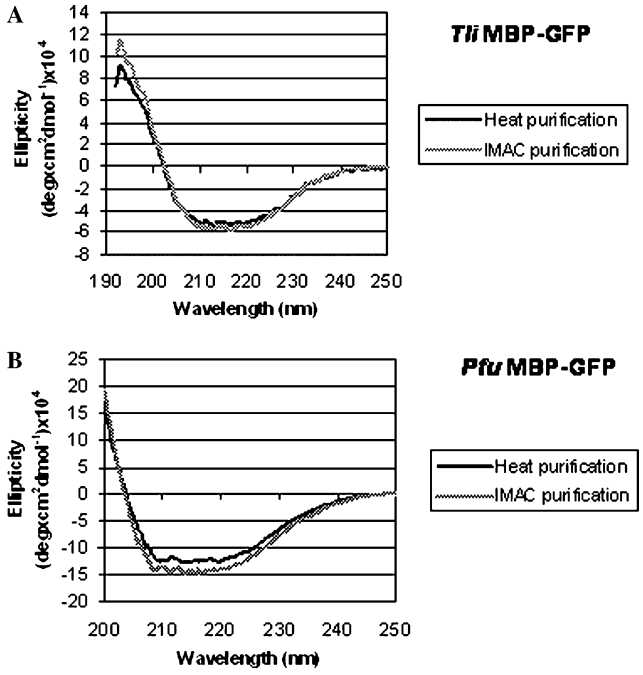

Fig. 2. CD spectra of the thermostable GFP-MBP constructs purified by IMAC and heating treatment. (A) Comparison of the CD spectra of the fusion protein between GFP and MBP from *T. litoralis* purified by IMAC or by heating the lysate at 70 °C for 15 min. (B) Comparison of the CD spectra of the fusion protein between GFP and MBP from *P. furiosus* purified by IMAC or by heating treatment.

improve the thermostability of the passengers to which are fused [13]. Two alternatives are possible: either the passenger protein is denatured but kept in solution and has the chance to refold once moved back to permissive temperature or the whole fusion is stabilized. We measured the T_m of the four GFP-fusions and found that the value of *Eco* MBP-GFP was only slightly higher than that of GST-GFP but the fusions of *Tli* and *Pfu* MBP-GFPs did not melt at the temperature (70 °C) used for their selective purification. Surprisingly, they remained folded and preserved their fluorescence even at 100 °C (Table 1 and Fig. 3).

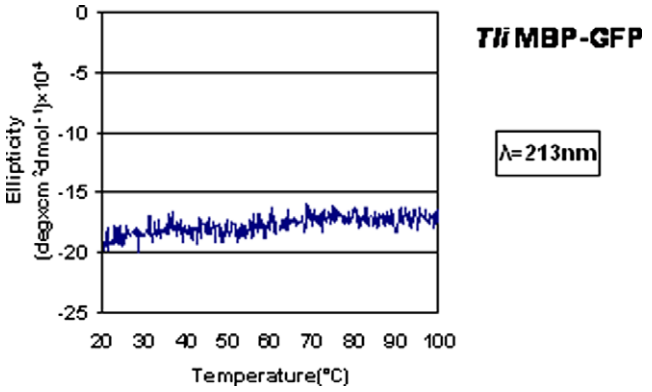

Fig. 3. Denaturation spectrum of *Tli* MBP-GFP. Protein sample was progressively heated and its ellipticity at 213 nm recorded between 20 and 100 °C.

Heating purification applied to fusions with thermostable MBP enables direct protein quality control

It may be expected that during their growth aggregate seeds reach a critical mass and precipitate. Independent indexes for aggregation evaluation [15] have previously shown that soluble GST-GFP aggregates have lower complexity of precipitated aggregates but it was not possible to identify a critical value at which precipitation begins. The data of Table 1 indicate that all the soluble GFP-fusions had a relative low aggregation index, even in the case of the heat-purified *Eco* MBP-GFP. Most of the heated *Eco* MBP-GFP precipitated during the heating and it could be expected that this portion was represented by larger aggregates. We could not purify the heat-precipitated *Eco* MBP-GFP from the bulk of the bacterial proteins to analyze its aggregation structure and, therefore, designed a new experiment for testing the hypothesis that only monodispersed protein and aggregates of low complexity remain soluble after heat treatment.

Bacteria transformed with the thermostable *Tli* MBP-GFP were grown at 42 °C after recombinant expression induction. Bacteria suffer severe stress under such a temperature and cell functions become inefficient. After lysis and IMAC purification the yield of soluble fusion protein was lower than 5% of the yield obtained when the bacteria were grown at 20 °C (Table 2). The aggregation index clearly showed that the fusion protein formed large soluble aggregates and these precipitated when heated at 70 °C (Table 2). Therefore, low aggregation complexity seems to be crucial for maintaining the solubility of a construct during heating and, consequently, heating purification can be envisaged for selectively recovering scarcely aggregated proteins, namely it represents a quality protocol to remove the soluble aggregates that are produced when instable passengers are fused to highly soluble carriers [15,16].

Table 2
Yields and aggregation conditions of GFP-MBP (*T. litoralis*) grown at 42 °C

	Yield IMAC ($mg_{protein}/L_{culture}$)	Aggregation index	Yield heating ($mg_{protein}/L_{culture}$)
GFP-MBP *Tli* (42 °C)	0.38	5.3	0

The protein was purified by IMAC or by using the heating protocol and its aggregation index (Ads. 280 nm/340 nm) calculated.

Discussion

At non-restrictive temperature (20 °C) the yields of soluble GST and MBP-fusions with GFP were similar, whereas MBP-fusions were more soluble when the cells were grown at 37 °C. However, soluble protein does not mean useful, native folded protein. The existence of soluble aggregates of both GST and MBP fusion proteins has been well documented [15,16] and, in particular, the progressive aggregation of GST-GFP that leads to amyloid fibril formation [15,17] has been described. Since the GFP passenger is common to all the constructs analyzed in this work, a particular abundance of amyloidogenic stretches in the carriers [18] could explain the specific development of aggregates from some expression products. We compared the sequences of GST, MBP *Eco*, and *Pfu* and found that there is no correlation between number of amyloidogenic stretches in the carrier and propensity to the aggregation of the fusion protein (Table 3).

As an alternative, the aggregation of the fusion constructs could rather depend on the stability of the common passenger protein (GFP) but only in the case in which the preservation of its native folding (or refolding) would be positively influenced by the intrinsic stability of the carrier. "Induced fit" has been described as a consequence of interactions between different classes of macro-molecules [19,20] and protein fusions could represent a related case. We used several independent methods to measure protein aggregation and all showed that the fusions with MBPs assured higher monodispersity. At least in the case of Archaea MBPs, these proteins do not merely keep the unfolded/misfolded passenger in solution during the heat treatment but are able to "pass" their stability to the whole fusion construct. An example of induced fit is given by *Tli* MBP-GFP that, heated at 100 °C, did not denature and was still less aggregated than GST-GFP grown at 20 °C and purified at 4 °C.

A previous paper correlated efficient refolding after heat denaturation and high soluble yields after recombinant expression [12]. Interestingly, all the thermostable MBP-

Table 3
Identification of 6-residue amyloidogenic stretches

Proteins	
MBP *Eco*	112–117 ALSLIY; 113–118 LSLIYN; 190–195 AGLTFL; 337–342 SAFWYA
MBP *Pfu*	114–119 LGQFIA; 137–142 LSVFFQ; 164–169 AGLLYY
GST	160–165 DVVLYM

Sequences recognized by a specific software [18] as potentially involved in the formation of amyloid structures were identified in MBP from *E. coli* and *P. furiosus*, and in GST from *Schistosoma japonicum*.

fusions are less aggregated and assure higher yields of monodispersed recombinant protein than the thermo-sensitive GST-GFP. Therefore, our data confirm that the same mechanisms that enable higher thermotolerance are involved in the folding improvement during recombinant expression. Good performances of other thermostable proteins used as stabilizing carriers have been documented [13] and we can hypothesize that the previously reported chaperone-like effect of MBPs on the passenger proteins [21] could result from their improved thermostability.

Another interesting result is that only correctly folded MBP-fusions conserve their stability at high temperature whereas partially aggregated proteins precipitate when heated, as shown in the case of *Tli* MBP-GFP cultured at non-restrictive and restrictive temperatures. Therefore, it becomes possible to use a simple physical method to discriminate not only between thermo-sensitive and heat-tolerant constructs but even between monodispersed proteins and soluble aggregates.

In conclusion, in the case of thermostable fusion proteins, heat tolerance can be used for simultaneous purification and protein quality control [22], and it can be envisaged to use heat tolerance for fast screening of construct potentially soluble and monodispersed once expressed in a recombinant system. Crystallographic analysis would be now necessary to analyze the fine structure of heat-treated proteins.

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