Journal of Structural Biology 175 (2011) 465–468

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Technical Note

Thermal precipitation fluorescence assay for protein stability screening

Junping Fan, Bo Huang, Xianping Wang *, Xuejun C. Zhang

National Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China

ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 22 April 2011
Accepted 5 May 2011
Available online 12 May 2011

Keywords:
In-gel fluorescence
Thermal stability
Membrane protein
High throughput screening

ABSTRACT

A simple and reliable method of protein stability assessment is desirable for high throughput expression screening of recombinant proteins. Here we described an assay termed thermal precipitation fluorescence (TPF) which can be used to compare thermal stabilities of recombinant protein samples directly from cell lysate supernatants. In this assay, target membrane proteins are expressed as recombinant fusions with a green fluorescence protein tag and solubilized with detergent, and the fluorescence signals are used to report the quantity of the fusion proteins in the soluble fraction of the cell lysate. After applying a heat shock, insoluble protein aggregates are removed by centrifugation. Subsequently, the amount of remaining protein in the supernatant is quantified by in-gel fluorescence analysis and compared to samples without a heat shock treatment. Over 60 recombinant membrane proteins from *Escherichia coli* were subject to this screening in the presence and absence of a few commonly used detergents, and the results were analyzed. Because no sophisticated protein purification is required, this TPF technique is suitable to high throughput expression screening of recombinant membrane proteins as well as soluble ones and can be used to prioritize target proteins based on their thermal stabilities for subsequent large scale expression and structural studies.

High throughput cloning and expression screening have become an increasingly accepted technique in structural studies on proteins including membrane proteins (Groisillier et al., 2010; Lundstrom, 2004; Qin et al., 2008). It is widely assumed that a more stable protein would be easier to be purified to a homogeneous form and behave better in subsequent structural studies (Sarkar et al., 2008; Sonoda et al., 2011). In a high throughput protein expression screening pipeline, it is desirable to have a simple yet reliable method to estimate the target protein activity and/or stability so that target proteins that have passed a threshold of expression quantity can be prioritized for further time-consuming processes aimed at three-dimensional structure determination or other analysis. Accordingly, there have been many methods proposed for large scale protein stability screening. For example, a 340-nm light scattering technique is reported to detect membrane protein aggregation (Postis et al., 2008). Thermofluor technique is shown able to evaluate protein stability in different buffer solutions and detergents (Alexandrov et al., 2008; Pantoliano et al., 2001). Size exclusion chromatography (SEC), and an ultracentrifugation dispersity sedimentation method are used to detect aggregations under varied solution conditions (Gutmann et al., 2007; Prive, 2007; Roth et al., 2008; Sonoda et al., 2011). However, these methods usually require purified target proteins and in some cases expensive instruments, making them less convenient to be applied in a high throughput mode. Therefore, high throughput-friendly stability screening techniques remain to be developed.

For a high throughput screening, a target independent stability measurement would have general advantages over target specific activity assays, and protein thermal stability may serve as such a general quality control indicator of the protein samples. Although chemical stability such as resistance to denaturants and biochemical stability such as resistance against proteolytic hydrolysis may also be used as general measurements of protein stability, a thermal stability measurement is often easier to apply than other challenges, experimentally reversible, and thus more suitable to a high throughput research. We designed and tested a novel, GFP-mediated, high throughput compatible, thermal stability assay for assessing the stability of membrane proteins directly from a cell lysate supernatant. Our main approach was to compare fluorescence signals of GFP-target fusion proteins before and after a heat shock treatment and to use the ratio as a measurement of the thermal stability of the target protein. In this method, insoluble protein aggregates formed during a heat shock treatment are removed by centrifugation. Subsequently, the amount of remaining protein in the supernatant is quantified by an in-gel fluorescence analysis (Drew and Gier, 2006).

First, we confirmed that GFP alone is sufficiently stable such that sequentially heating and cooling treatments do not drastically change its fluorescence ability (Fig. S1A). The GFP protein that we used in this experiment was an enhanced GFP previously being used as a reporter of recombinant protein expression (Cabantous

* Corresponding author.
E-mail address: wangxp@moon.ibp.ac.cn (X. Wang).

1047-8477/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2011.05.003

et al., 2005). In a given buffer, the fluorescence of the GFP sample was monitored as temperature increased from 25 °C to 55 °C and reduced back to 25 °C. As expected from thermal destabilization as well as possible quenching effect of rising temperature on GFP, the fluorescence signal decreased when the temperature increased from 25 °C to 55 °C. However, the loss of fluorescence signal was reversible as temperature returned back to 25 °C, and the temperature-fluorescence curve during the temperature reversal overlapped well with that of temperature rising in the range between 25 °C and 55 °C (Fig. S1A). Such an observation maintained true for all 12 buffers that we tested, although the initial fluorescence and the slope of the fluorescence-temperature curve varied from one buffer condition to another. Similarly, in a heat shock assay the GFP sample was heated at a predetermined temperature for a short time (*e.g.* 5 min), and then the sample was incubated at a lower temperature (*e.g.* 4 °C for 5 min) followed by centrifugation to remove insoluble aggregation. The result showed that the fluorescence signal of a purified GFP-alone sample did not change significantly after such a heat shock treatment, while the fluorescence signal from a purified model fusion protein, GST–GFP, dropped dramatically after a heat shock treatment (Fig. S1B). Therefore, we postulated that GFP may not only serve as a reporter of the expression level of the fusion protein but also become a thermal stability indicator of the fusion protein against the heat shock treatment.

To reduce the complication resulted from mixing multiple fluorescent species in the cell lysate, we chose in-gel fluorescence as the backbone technique of our thermal stability assay. Fusion protein samples were prepared from small volume expression, and cell lysate supernatants were analyzed without sophisticated purification. Fluorescence signals were to be measured directly from the band intensity of the full length fusion protein without interference from other contamination, and the ratio of fluorescence signals from full length bands with and without a heat shock treatment would be used as a measurement of the target protein thermal stability (Fig. 1). The technique is thus referred as a thermal precipitation fluorescence (TPF) assay.

There are a few parameters that one may vary during a heat shock-based protein denaturation, including heat shock temperature, duration of the heat treatment, and strength of the centrifugation. The time of the heat treatment should be long enough to allow reproducible sample handling, especially in a high throughput mode where a large number of samples are handled simultaneously. On the other hand, it should be short enough so that one can quickly obtain results without unnecessary waiting. We chose the time range of the heat shock as a few minutes (*e.g.* 2–10 min). Similarly, the heat shock temperature was adjusted such that in-gel fluorescence signals of the target-GFP fusion proteins would show a wide distribution in terms of the ratio between samples with and without a heat shock, theoretically in the range of 0.0–1.0. If the heat shock temperature was too high, most target proteins would be denatured and removed from solution by subsequent centrifugation; but if the heat shock temperature was not high enough, most target proteins would remain the same as untreated samples, and the assay would lose its power to distinguish proteins of different stabilities. Based on our previous studies using a thermofluor technique (Fan et al., 2011), we found that many *Escherichia coli* membrane proteins have melting temperatures around 40–60 °C. Therefore, we started our temperature search in this region.

As a prove-of-principle test, seven *E. coli* membrane proteins of varied expression levels were analyzed with the TPF assay. First, cell cultures of these fusion proteins were lysed with sonication in a Triton X-100 (1% w/v) containing buffer. Two temperatures, *i.e.* 42 °C and 55 °C, and varied time between 1 and 10 min were evaluated. The results showed that after heating at 42 °C for 5 min, 6 of the 7 target proteins maintained their fluorescence intensity ratios above 0.8, suggesting the heat treatment might be insufficient (Fig. 2A). In contrast, after heating at 55 °C for 5 min, fluorescence intensity ratios of many samples became too low (Fig. 2B) such that it might not be suitable for comparing thermal stabilities of target proteins. After numerous trials of temperature–time combinations, we found a heat shock treatment at 48 °C for 5 min would work well for our target *E. coli* membrane proteins which were treated with a Triton-containing lysis buffer (Fig. 2C). Temperature adjustment may be necessary for samples from different sources or treated with different detergents or buffers, but the same principal could be applied to determine the time and temperature parameters. To verify the result from a TPF assay, we performed an alternative thermal denaturation experiment following a previous report (Sonoda et al., 2011). This method requires that the target protein does not contain a GFP-tag. In addition, to minimize noises from contaminated proteins, the target protein sample must be purified before its denaturation half-life time can be measured using the thermofluor technique. Therefore we sub-cloned the target genes into a vector coding no GFP tag and purified the protein samples with immobilized metal affinity chromatography (IMAC). Data obtained from this thermal denaturation experiment are shown in Fig. 2D and S2A. Importantly, there was a clear, although not strict, positive correlation between results from this more conventional thermal stability experiment and our TPF results (Fig. S2B), suggesting that results from the TPF assay reflect intrinsic thermal stabilities of the target proteins.

In a parallel project of high throughput expression screening, we obtained 64 clones of *E. coli* α-helical membrane protein-GFP fusions that produced significant whole cell fluorescence signals in a small volume (1 ml) expression experiment (Fan et al., 2011). As a further application, we performed the TPF assay on these 64 target proteins. Cell lysates in the presence of 0.5% (w/v) DDM were centrifuged, and each supernatant was split into two parts: one part was kept on ice, and the other part was heated

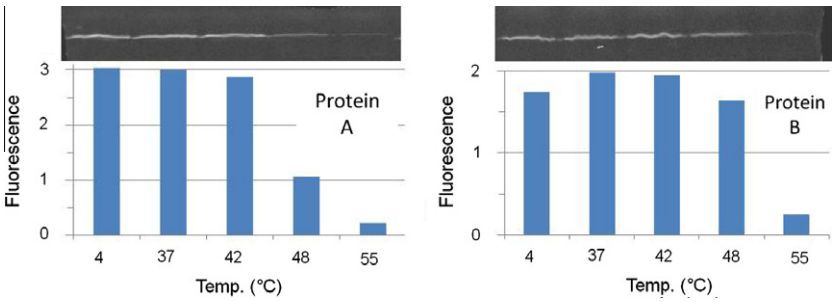

Fig. 1. In-gel fluorescence changes with heat shock treatments at varied temperatures. Cell lysate samples of two recombinant membrane proteins (A and B) in a detergent free buffer were heat shocked at 37, 42, 48, and 55 °C or kept at 4 °C, individually. After centrifugation at 2400*g* for 2 min, the supernatants were subject to an in-gel fluorescence analysis (top), and their fluorescence signals were digitalized (in an arbitrary unit) and illustrated at the bottom.

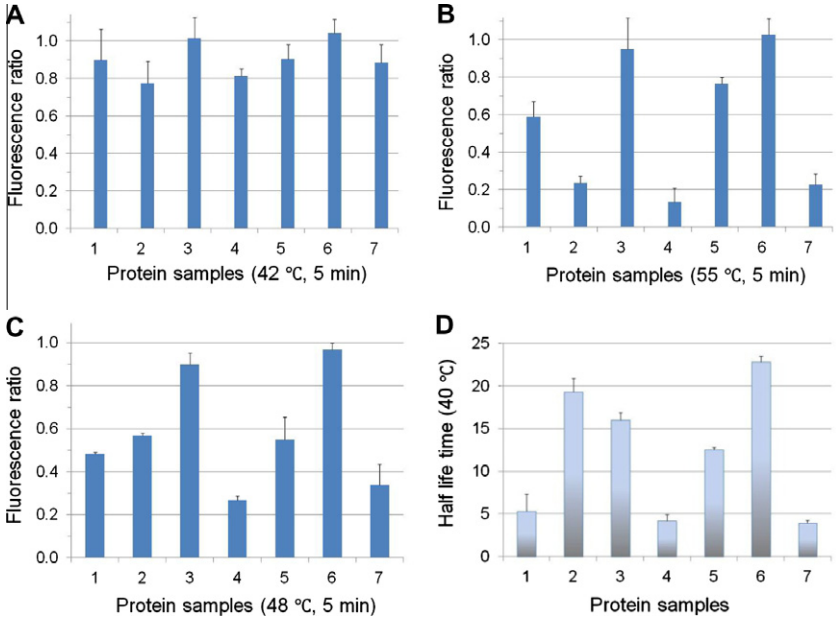

Fig. 2. Determination of heat shock parameters. Seven target proteins of varied expression levels were tested in a thermal precipitation fluorescence (TPF) assay. The results showed that (A) a 42 °C for 5 min treatment was too mild to see significant difference among the 7 samples; (B) a 55 °C for 5 min treatment appeared too harsh to many membrane protein samples; and (C) a 48 °C for 5 min treatment gave a better distribution of the fluorescence ratio. (D) A thermal denaturation half life measurement (Sonoda et al., 2011). The temperature was set to 40 °C. Denaturation half life time was determined by fitting the denaturation curve with an exponential equation (also see the Method section in the supplementary material). All measurements were performed three times, and error bars are shown.

at 55 °C for 5 min followed by incubation on ice for 5 min. After centrifugation (at 13,800*g* for 2 min), in-gel fluorescence signals of the fusion proteins with and without a heat shock treatment were measured, and the ratio of intensities was calculated for each sample pair. Fig. 3A shows a nearly linear distribution of the fluorescence ratios of the 64 fusion proteins, suggesting that the choice of temperature and time combination in heat shock was reasonable for this set of target membrane proteins with DDM as the solubilization detergent. In addition, we studied effects of other detergents on the TPF assay (see Supplementary material). Taken together, our results showed that the TPF assay is applicable in a high throughput mode.

In the current work, our goal is not to obtain detailed thermal stability information such as a melting temperature, unfolding cooperativity or half-life time during a thermal denaturation (Baase et al., 1992b; Ericsson et al., 2006; Sonoda et al., 2011). Instead, we aim to developing a simple, quick assay to prioritize our target proteins in a high throughput pipeline. In the TPF assay, we choose to measure protein thermal stability by following the in-gel fluorescence signals of the fusion protein before and after a heat shock treatment. Heat shock based thermal stability assays are widely used in protein stability studies (Dodevski and Pluckthun, 2011; Sarkar et al., 2008; Tate and Schertler, 2009). In our GFP-based TPF assay, basic assumptions are that heat denatured proteins can be removed by centrifugation and that the fluorescence difference between samples with and without a heat treatment reflects the thermal stability of the target protein. The C-terminal reporter GFP is of high stability such that it resists to thermal denaturation under our experimental condition (Fig. S1A) and remains active under a very harsh chemical condition, an SDS gel (Drew et al., 2008), which would denature most other protein molecules. Therefore, thermal denaturation of the fusion protein is dictated by the stability of the target protein and is thus used to estimate the thermal stability of the latter.

Our result showed that the fluorescence ratio before and after a heat shock treatment is independent of initial in-gel fluorescence signals (Fig. 3B). Therefore, in general one cannot predict the thermal stability of a target protein solely based on its expression level.

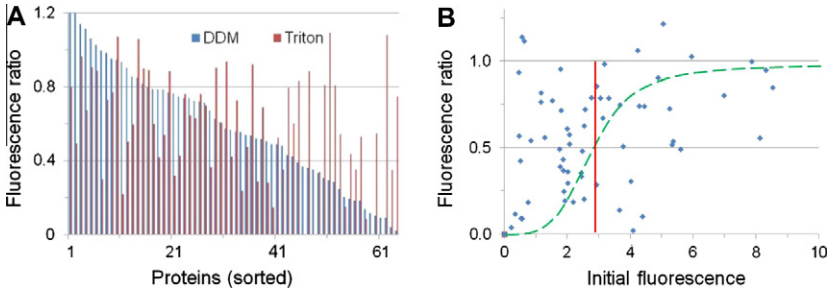

Fig. 3. TPF stability assay of 64 *E. coli* membrane proteins. (A) For each GFP-target fusion protein, two samples were prepared with DDM (blue bars) and Triton X-100 (red bars), respectively. The fluorescence ratios between samples with and without a heat shock treatment (55 °C for 5 min for DDM and 48 °C for 5 min for Triton X-100) were calculated for each sample. And the total results were sorted by the ratio values of the DDM treated samples. (B) The fluorescence ratios are independent of the initial in-gel fluorescence signals. The fluorescence ratios of the 64 samples treated with DDM are plotted against the corresponding initial in-gel fluorescence signals (in an arbitrary unit). The average value of the 64 initial fluorescence reading is represented by a vertical red line, and a hypothetical curve based on an assumption that more stable proteins would express better is colored in green.

Although it is likely to be true that thermal stability contributes positively to the expression of a recombinant protein, it is only one of many factors that determine the overall expression yield. The independence of thermal stability of a target protein from its yield further supports the necessity of using a thermal stability based method to prioritize candidates in a high throughput expression screening pipeline.

Although our method will not replace more detailed stability measurement such as thermofluor (Ericsson et al., 2006; Sonoda et al., 2011) and circular dichroism (Baase et al., 1992a) techniques and may not accurately predict target protein stability towards other types of denaturation challenges, this TPF assay does not require sophisticated instrument, is relatively easy to perform, and can be accommodated into many laboratory setups as well as high throughput expression screening pipelines. As reported before, GFP fluorescence is a simple and effective method to screen for detergents that effectively extract the target membrane protein from the cell membrane (Drew et al., 2005; Sonoda et al., 2011). By using different detergents during sample preparation, one may apply the TPF method to obtain compatibility information of a group of target membrane proteins with multiple detergents (Fig. 3A); and such information is likely to be useful for detergent selection and exchange during membrane protein purification and crystallization. Moreover, the TPF assay can also be used in high throughput expression screening for soluble proteins where detergent consideration can be omitted.

Acknowledgment

This work was supported by the MOST (China) "973" Project grants 2009CB918803 and 2011CB910301.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2011.05.003.

References

Alexandrov, A.I., Mileni, M., Chien, E.Y., Hanson, M.A., Stevens, R.C., 2008. Microscale fluorescent thermal stability assay for membrane proteins. Structure 16, 351–359.

Baase, W.A., Eriksson, A.E., Zhang, X., Heinz, D.W., Sauer, U., Blaber, M., Baldwin, E.P., Wozniak, J.A., Matthews, B.W., 1992a. Dissection of protein structure and folding by directed mutagenesis. Faraday Discuss., 173–181.

Baase, W.A., Eriksson, A.E., Zhang, X.J., Heinz, D.W., Sauer, U., Blaber, M., Baldwin, E.P., Wozniak, J.A., Matthews, B.W., 1992b. Dissection of protein structure and folding by directed mutagenesis. Faraday Discuss., 173–181.

Cabantous, S., Pedelacq, J.D., Mark, B.L., Naranjo, C., Terwilliger, T.C., Waldo, G.S., 2005. Recent advances in GFP folding reporter and split-GFP solubility reporter technologies. Application to improving the folding and solubility of recalcitrant proteins from *Mycobacterium tuberculosis*. J. Struct. Funct. Genomics 6, 113–119.

Dodevski, I., Pluckthun, A., 2011. Evolution of three human GPCRs for higher expression and stability. J. Mol. Biol. 408, 599–615.

Drew, D., Gier, J., 2006. Optimization of membrane protein overexpression and purification using GFP fusions. Nat. Methods 3, 303.

Drew, D., Newstead, S., Sonoda, Y., Kim, H., von Heijne, G., Iwata, S., 2008. GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. Nat. Protoc. 3, 784–798.

Drew, D., Slotboom, D.J., Friso, G., Reda, T., Genevaux, P., Rapp, M., Meindl-Beinker, N.M., Lambert, W., Lerch, M., Daley, D.O., et al., 2005. A scalable, GFP-based pipeline for membrane protein overexpression screening and purification. Protein Sci. 14, 2011–2017.

Ericsson, U.B., Hallberg, B.M., Detitta, G.T., Dekker, N., Nordlund, P., 2006. Thermofluor-based high-throughput stability optimization of proteins for structural studies. Anal. Biochem. 357, 289–298.

Fan, J., Heng, J., Dai, S., Shaw, N., Zhou, B., Huang, B., He, Z., Wang, Y., Jiang, T., Li, X., et al., 2011. An efficient strategy for high throughput screening of recombinant integral membrane protein expression and stability. Protein Expr. Purif. 78, 6–13.

Groisillier, A., Herve, C., Jeudy, A., Rebuffet, E., Pluchon, P.F., Chevolot, Y., Flament, D., Geslin, C., Morgado, I.M., Power, D., et al., 2010. MARINE-EXPRESS: taking advantage of high throughput cloning and expression strategies for the post-genomic analysis of marine organisms. Microb. Cell Fact. 9, 45.

Gutmann, D.A., Mizohata, E., Newstead, S., Ferrandon, S., Postis, V., Xia, X., Henderson, P.J., van Veen, H.W., Byrne, B., 2007. A high-throughput method for membrane protein solubility screening: the ultracentrifugation dispersity sedimentation assay. Protein Sci. 16, 1422–1428.

Lundstrom, K., 2004. Structural genomics on membrane proteins: mini review. Comb. Chem. High Throughput Screen. 7, 431–439.

Pantoliano, M.W., Petrella, E.C., Kwasnoski, J.D., Lobanov, V.S., Myslik, J., Graf, E., Carver, T., Asel, E., Springer, B.A., Lane, P., et al., 2001. High-density miniaturized thermal shift assays as a general strategy for drug discovery. J. Biomol. Screen. 6, 429–440.

Postis, V.L., Deacon, S.E., Roach, P.C., Wright, G.S., Xia, X., Ingram, J.C., Hadden, J.M., Henderson, P.J., Phillips, S.E., McPherson, M.J., et al., 2008. A high-throughput assay of membrane protein stability. Mol. Membr. Biol. 25, 617–624.

Prive, G.G., 2007. Detergents for the stabilization and crystallization of membrane proteins. Methods 41, 388–397.

Qin, H., Hu, J., Hua, Y., Challa, S.V., Cross, T.A., Gao, F.P., 2008. Construction of a series of vectors for high throughput cloning and expression screening of membrane proteins from *Mycobacterium tuberculosis*. BMC Biotechnol. 8, 51.

Roth, C.B., Hanson, M.A., Stevens, R.C., 2008. Stabilization of the human beta2-adrenergic receptor TM4–TM3–TM5 helix interface by mutagenesis of Glu122(341), a critical residue in GPCR structure. J. Mol. Biol. 376, 1305–1319.

Sarkar, C.A., Dodevski, I., Kenig, M., Dudli, S., Mohr, A., Hermans, E., Pluckthun, A., 2008. Directed evolution of a G protein-coupled receptor for expression, stability, and binding selectivity. Proc. Natl. Acad. Sci. USA 105, 14808–14813.

Sonoda, Y., Newstead, S., Hu, N.J., Alguel, Y., Nji, E., Beis, K., Yashiro, S., Lee, C., Leung, J., Cameron, A.D., et al., 2011. Benchmarking membrane protein detergent stability for improving throughput of high-resolution X-ray structures. Structure 19, 17–25.

Tate, C.G., Schertler, G.F., 2009. Engineering G protein-coupled receptors to facilitate their structure determination. Curr. Opin. Struct. Biol. 19, 386–395.