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SHORT COMMUNICATION

Rapid prediction of expression and refolding yields using phage display

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Aggregation limits the recombinant production of many commercially important proteins. We have recently identified mutations that control the aggregation behavior of human antibody variable domains (Dudgeon K., Rouet R., Kokmeijer I., Schofield P., Stolp J., Langley D., Stock D. and Christ D. (2012) *Proc Natl Acad Sci USA,* 109, 10879–10884. This has allowed the generation of a panel of human antibody variable heavy domains with a defined range of aggregation propensities. Here we utilize this unique resource to validate a previously reported heat-denaturation method on phage (Jespers L., Schon O., Famm K. and Winter G. (2004) *Nat Biotechnol,* 22, 1161–1165. Our experiments revealed that the method is not only robust in respect to denaturation conditions on phage, but also highly indicative of solution behavior. In particular, it is an excellent predictor of expression and refolding yields.
Keywords: antibodies/human antibody variable domains/ phage display/protein aggregation

Introduction

Protein aggregation affects the production and formulation of many biopharmaceuticals and limits the availability of soluble protein for biochemical, biophysical and structural analyses (Demarest and Glaser, 2008; Lee *et al.*, 2010; Lowe *et al.*, 2011). Although several high-throughput approaches for the prediction of aggregation propensities have been reported (Chiti *et al.*, 2003; DuBay *et al.*, 2004; Jespers *et al.*, 2004a,b), there has been little independent validation. One of the more promising experimental approaches was developed by Jespers *et al.* (2004a,b) and relies on the heating of proteins displayed on the tip of filamentous M13 bacteriophage, followed by cooling. Although the phage particle itself is resistant to such denaturation cycles, the procedure induces aggregation of the displayed proteins. Aggregation is then detected using a conformation-specific affinity reagent that binds to folded protein, but not to unfolded or aggregated species (such as protein A superantigen, which binds to human antibody variable heavy (V_H3) domains (Jansson *et al.*, 1998)). The method has been predominantly applied to human antibody V_H domains (Jespers *et al.*, 2004a,b; Christ *et al.*, 2006, 2007; Famm *et al.*, 2008; Dudgeon *et al.*, 2009, 2012), which represent a commercially important class of antibody fragments (Holt *et al.*, 2003; Holliger and Hudson, 2005), but also to other protein families (such as human antibody variable light domains) (Dudgeon *et al.*, 2012).

One of the major limitations that had so far prevented independent validation of the method has been the lack of a panel of model proteins with an intermediate range of aggregation propensities. Germline V_H domains readily aggregate when subjected to heating on phage (Jespers *et al.*, 2004a,b). In contrast, model domains (such as HEL4) tend to completely resist aggregation under such conditions. This is not surprising as these model domains are the product of repertoire selections (Jespers *et al.*, 2004a,b) (they are not common in antibody phage display libraries; indeed it has been shown that preselection for aggregation resistance reduces repertoire sizes by several orders of magnitude (Christ *et al.*, 2007)).

We have recently identified mutations that generally increase the aggregation resistance of human V_H domains (charged substitutions, and in particular aspartate, at Kabat positions 28, 30–33 and 35) (Dudgeon *et al.*, 2012). The effects of the mutations are independent of sequence diversity at other positions and mutations can be retrofitted into existing domains. Multiple mutations are required to achieve a high level of aggregation resistance. This has allowed us to generate model domains with a range of intermediate aggregation propensities through stepwise introduction of mutations into a common germline domain (V3–23, DP47).

Here we utilize this panel of model domains to validate the heat-denaturation method on phage (Jespers *et al.*, 2004a,b) and provide detailed comparisons with aggregation behavior in solution.

Material and methods

Generation of mutant antibody variable domains

For phage display studies, segments encoding the human (V3–23/DP47, JH4b) variable domain were cloned into the phage display vector FdMyc. Mutations were introduced using methods originally described by Kunkel *et al.* (1987) and Zoller and Smith (1987). For soluble expression, mutant genes were inserted into the periplasmic expression vector pET12a (Novagen) and protein was expressed in *Escherichia coli* BL21-Gold (Stratagene) at 30°C essentially as previously described (Lee *et al.*, 2007; Rouet *et al.*, 2012). Filtered supernatant was added to protein A resin (GE Healthcare) and the resin was washed with phosphate-buffered saline (PBS). Finally, protein was eluted by adding 0.1 M glycine-HCl pH 2.7, neutralized with 100 mM Tris-HCl pH 8.0 and dialyzed against PBS. The following recently described variants were utilized (numbering according to Kabat): A31D, Y32E, S33D,

31/32DE, 31/33DD, 32/33DD, 31-31DED (Dudgeon *et al.*, 2012). In addition, DP47 wild-type and three previously reported variants were included in the panel: HEL4 (Jespers *et al.*, 2004a,b), S35G (Jespers *et al.*, 2004a,b; Barthelemy *et al.*, 2008) and T28R (Famm *et al.*, 2008).

Heat refolding on phage

Aggregation resistance was analyzed by measuring retention of signal after heating in a phage enzyme-linked immunosorbent assay (ELISA) format essentially as described by Jespers *et al.* (2004a,b). Wells of a Nunc Maxisorp Immunoplate were coated overnight with *Staphylococcus aureus* protein A at a concentration of 5 μg/ml in PBS. The plate was washed with PBS and blocked with 4% (w/v) milk powder diluted in PBS. Single colonies were picked from agar plates and grown overnight (2xTY medium supplemented with 15 μg/ml tetracycline; shaking at 250 rpm at 30°C). Cells were removed by centrifugation and phages were biotinylated directly in the culture supernatant through addition of biotin-PEO4-*N*-hydroxysuccinimide to a final concentration of 50 μM (Pierce). Supernatant was then incubated at 80°C for 10 min, followed by incubation at 4°C for 10 min. Supernatant was added to the blocked ELISA wells for 1 h at room temperature followed by three washes with PBS supplemented with 0.1% Tween-20. Bound phage particles were detected using Extravidin-HRP conjugate (Sigma) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Finally, the level of retained protein A binding after heating was calculated as a percentage of the unheated phage sample.

Heat refolding in solution

Refolding after heat denaturation in solution was determined using size-exclusion chromatography. For this purpose, purified protein at 10 μM in PBS was heated at 80°C for 10 min followed by cooling at 4°C for 10 min. Samples were centrifuged at 16 000 × *g* for 10 min and analyzed on a Superdex-G75 gel-filtration column (Pharmacia) (as below). The recovery of each variant was determined by measuring the area under the curve after heating, expressed as a percentage of the unheated protein sample.

Expression yields

Soluble expression levels of were determined using protein A superantigen ELISA. In brief, the concentration of soluble protein was determined based on standard curves generated using purified sample of each variant. Protein was expressed in *E.coli* BL21-Gold (Stratagene) at 30°C. After 42 h, cells were removed by centrifugation and protein was biotinylated in the culture supernatant by addition of biotin-PEO4-N-hydroxysuccinimide (Pierce; 50 μM final concentration). Supernatant was then added to a 96-well Maxisorp immunoplate (Nunc) coated overnight with 5 μg/ml Protein A (Sigma) and blocked with 4% (w/v) milk powder diluted in PBS. After three washes with PBS supplemented with 0.1% Tween-20, bound antibody domains were detected using Extravidin-HRP conjugate (Sigma) and TMB substrate.

Elution volumes

Gel-filtration elution profiles of purified domains were analyzed on a Superdex-G75 size-exclusion column (Pharmacia) using an AKTA Purifier (GE Healthcare) chromatography system and PBS running buffer.

Results and discussion

Before embarking on detailed studies of solution properties, we initially focused our attention on the conditions of the phage heat-denaturation method itself. Jespers *et al.* (2004a,b) had reported denaturation for 10 min at 80°C and subsequent studies have closely adhered to these conditions (Christ *et al.*, 2006, 2007; Famm and Winter, 2006; Famm *et al.*, 2008; Dudgeon *et al.*, 2009, 2012). However, no specific rationale or data have been provided to support this choice.

We therefore analyzed the influence of both denaturation time and temperature on protein aggregation on phage (Fig. 1). Our experiment revealed a well-defined spread of aggregation resistance among our panel of human V_H domains, ranging from around 2% for the wild-type domain to over 80% for HEL4 (as calculated from retained binding to superantigen after heating). Intriguingly, these values were stable over a wide range of denaturation conditions. In particular, incubation time did not affect aggregation propensities (Fig. 1**A**), even when widely varied (2.5–20 min). Incubation temperature did affect the method only at lower temperatures (Fig. 1**B**), while there was little difference observed between heating at 65 and 80°C. This suggested that temperature had little influence on measurements of aggregation resistance on phage, as long as kept at or above the melting temperature of the domains (~65°C (Famm *et al.*, 2008)). This is in marked contrast to other heat-based directed evolution methods that tend to be strongly influenced by these parameters (Jung *et al.*, 1999; Serrano-Vega *et al.*, 2008; Gong *et al.*, 2009). Taken together, our results indicated that the phage method was remarkably robust with respect to denaturation time and temperature and capable of providing rapid ranking of aggregation propensities.

We next compared biophysical properties of our panel of V_H domains in solution with their properties on phage. For this purpose, purified domains were heated at high protein concentration and aggregation resistance analyzed by measuring recovery on gel filtration (utilizing a Superdex-G75 size-exclusion column which readily separates soluble domains from aggregated species). Comparison of these values against aggregation resistance determined on phage (by measuring retention of superantigen binding) revealed a highly significant correlation (Fig. 2) ($r = 0.95$, $P < 0.0001$). This indicated that the phage heat/cool method is an excellent predictor of resistance to heat-induced aggregation in solution.

In addition to decreasing heat-refoldability, protein aggregation can result in many other undesired biophysical properties, including low expression yields (Lowe *et al.*, 2011). Indeed, many human antibody V_H domains are poorly expressed (Ewert *et al.*, 2003; Jespers *et al.*, 2004a,b; Olichon *et al.*, 2007; Kim *et al.*, 2012; Perchiacca and Tessier, 2012), although it is currently not known whether protein aggregation plays a key role in this. To further investigate the predictive capabilities of the Jespers method, we compared soluble expression yields of the domains (as determined by ELISA capture from culture supernatant) with aggregation resistance on phage. As observed for heat-refoldability, expression levels in solution significantly correlated with the behavior of the domains on phage (Fig. 3) ($r = 0.69$, $P < 0.02$). Indeed, expression levels were increased by up to 10-fold for the most aggregation-resistant domains within our panel. Our results suggest that protein aggregation is a major yield-limiting factor in the expression of human V_H domains.

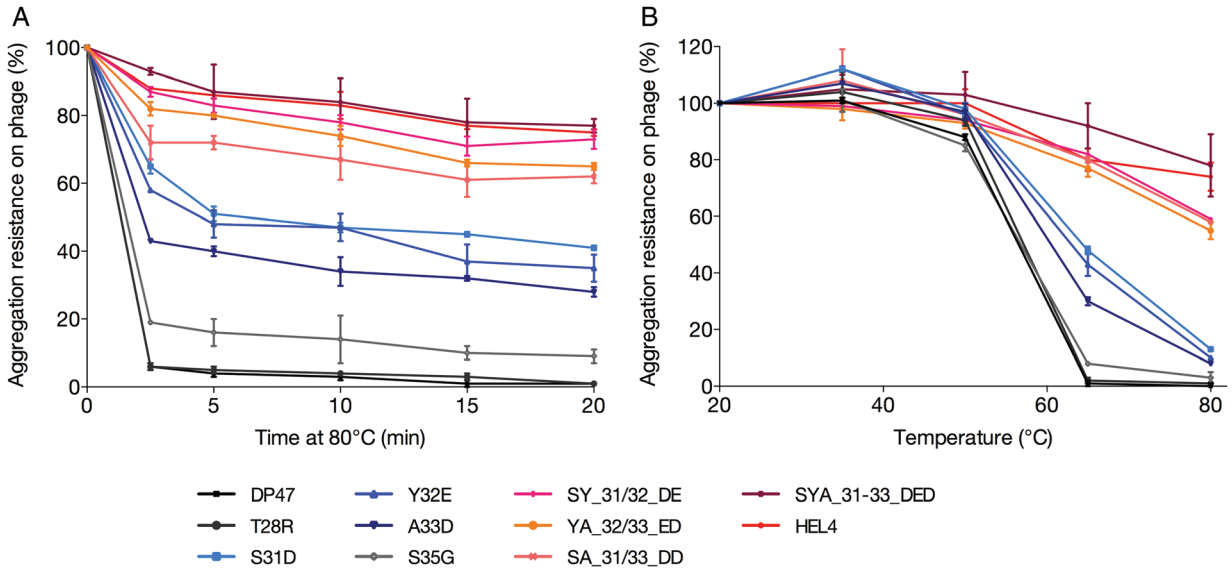

Fig. 1. Influence of incubation temperature and time on protein aggregation on phage. A panel of human antibody V_H domains with a defined range of aggregation propensities (see Material and Methods section) is displayed on phage. Aggregation resistance of the domains is determined by measuring retained binding to protein A superantigen after heating on phage (means, standard deviation shown, $n = 2$). Numbering according to Kabat (Kabat *et al.*, 1992). (**A**) Aggregation resistance after heating for variable incubation times (0, 2.5, 5, 10, 15, 20 min at 80°C for 10 min, followed by cooling at 4°C for 10 min). (**B**) Aggregation resistance after heating at variable temperatures (20, 35, 50, 65 and 80°C for 10 min, followed by cooling at 4°C for 10 min).

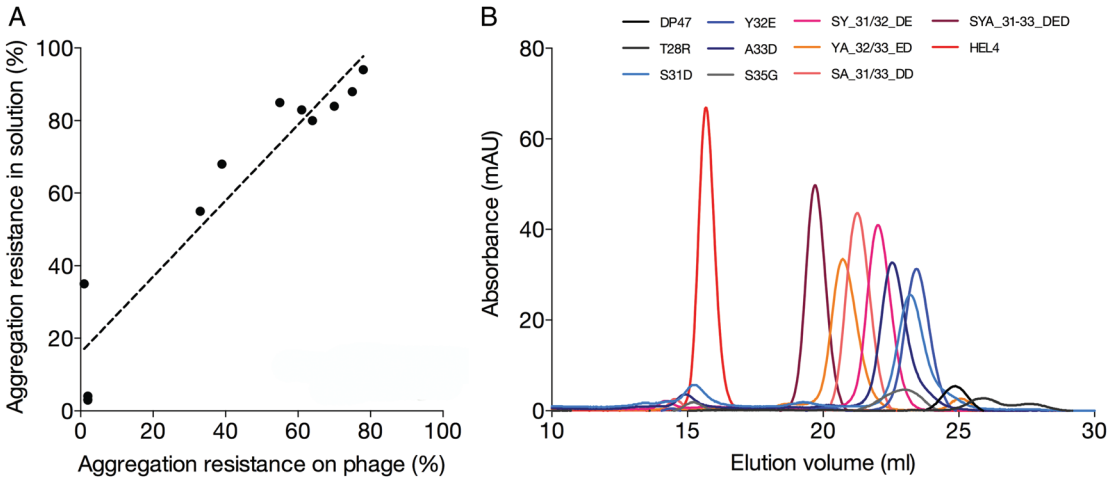

Fig. 2. (**A**) Aggregation resistance in solution and on phage ($r = 0.95$, $P < 0.0001$). (**B**) Aggregation resistance of human antibody V_H domains in solution (as determined by gel-filtration chromatography after heating at a concentration of 10 μM; see Material and Methods section). Aggregation resistance on phage is determined by measuring retained binding to protein A superantigen after heating. All samples were heated for 10 min at 80°C.

As a third solution parameter, we studied behavior on gel filtration in the absence of heating. It has long been known that many proteins, including V_H domains, have a tendency to interact with chromatography matrices. This 'stickiness' interferes with purification and manifests itself through extended elution volumes (Ewert *et al.*, 2003). As we had observed for heat-refoldability and expression yields, a comparative analysis of elution volumes on gel filtration and aggregation resistance on phage revealed a close correlation (Fig. 4) ($r = 0.79$, $P < 0.004$).

The results outlined here demonstrate that the heat-denaturation method on phage is an excellent predictor of solution properties. This is exemplified here by a panel of human V_H domains carrying novel, aggregation-reducing mutations (Dudgeon *et al.*, 2012). In addition to heat refolding yields, the method also predicted expression yields and elution volumes to a surprising degree. Our results further highlight the importance of aggregation resistance in protein production and purification.

In summary, the phage method as originally described by Jespers *et al.* provides a robust readout, largely independent of changes in denaturation conditions. It allows the rapid ranking of large numbers of protein variants and represents a valuable addition to the protein engineering toolkit.

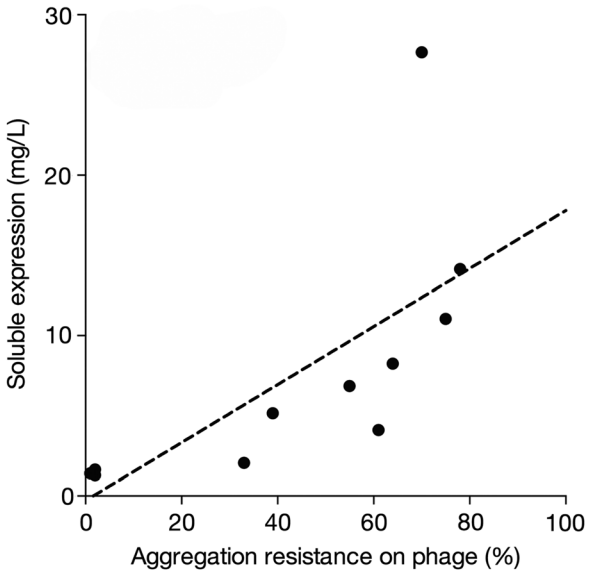

Fig. 3. Expression yield in solution and aggregation resistance on phage ($r = 0.69$, $P < 0.02$). Human antibody V_H domains are expressed and yields are determined by protein A superantigen ELISA (see Material and Methods section). Aggregation resistance on phage is determined by measuring retained binding to protein A superantigen after heating for 10 min at 80°C.

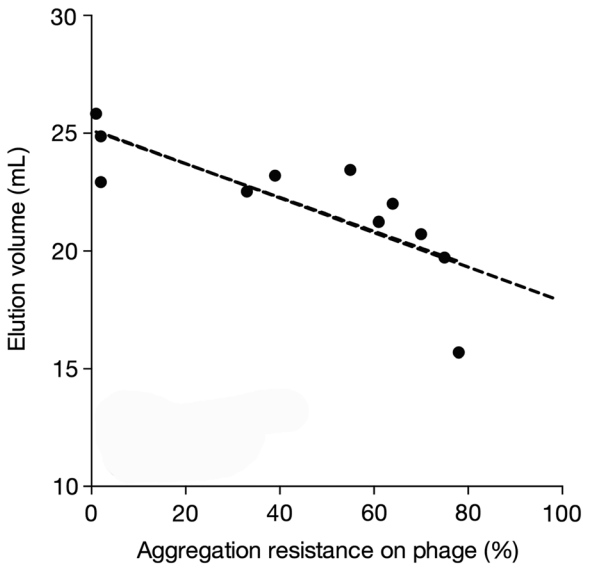

Fig. 4. Elution volume and aggregation resistance on phage ($r = 0.79$, $P < 0.004$). Human antibody V_H domains are expressed and elution volumes are determined by gel-filtration chromatography (see Material and Methods section). Aggregation resistance on phage is determined by measuring retained binding to protein A superantigen after heating for 10 min at 80°C.

Comments

Although the Jespers method allows the rapid and robust ranking of variants, we would caution against extrapolating these results to more diverse sets of proteins, which may well differ in non-aggregation related parameters (such as codon usage).

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