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Research paper

Efficient refolding and immobilization of PMMA-tag-fused single-chain Fv antibodies for sensitive immunological detection on a PMMA plate

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ABSTRACT

In this study, we investigated the efficient refolding and site-specific immobilization of single-chain variable fragments (scFvs) genetically fused with a poly(methylmethacrylate)-binding peptide (PMMA-tag). According to the results of an aggregation test of a scFv-PM in the presence of 0.5 M urea, aggregation was hardly detectable at a weak-alkaline pH (8.5) with lower concentrations of NaCl. Consequently, more than 93% recovery of the anti-RNase scFv-PM model was attained, when it was refolded by dialysis against 50 mM TAPS (pH 8.5). These results suggested that the apparent isoelectric point (pI) of a target scFv was decreased to a great extent by the genetic fusion of a PMMA-tag containing 5 acidic amino acids, and, thus, the solubility of the scFv-PM in its semi-denatured form was considerably improved. We also designed alternative peptide-tags composed of plural aspartic acid residues (D5, D10 and D15-tags) to decrease the apparent pI value of the fusion protein. As a consequence, scFv-D5, scFv-D10 and scFv-D15 were also efficiently refolded with yields of more than 95%. It is noteworthy that even scFv-PS-D15, which had both a positively charged polystyrene-binding peptide (PS-tag) and a negatively charged D15-tag, was serially connected at the C-terminal region of scFvs, and also refolded with a yield of 96.1%. These results clearly indicate that controlling the apparent pI value of scFvs by the fusion of oligo-peptides composed of acidic amino acids at the C-terminus resulted in a high degree of recovery via dialysis refolding.

According to the results of a sandwich ELISA using scFv-PMs, scFv-D15 and scFv-PS-D15 as ligands, high antigen-binding signals were detected from both the PMMA and phi-PS plates immobilized with scFv-PMs. Furthermore, the high antigen-binding activity of scFv-PMs was maintained in an adsorption state when it was immobilized on the surface of not only PMMA, but also hydrophilic PS (phi-PS) and polycarbonate (PC). These results strongly suggested that a PMMA-tag introduced at the C-terminus of scFvs preferably recognizes ester and/or carboxyl groups exposed on the surface of plastics.

The scFv-PM developed in the present study has advantages such as being a ligand antibody, compared with whole Ab and the conventional PS-tag-fused scFvs (scFv-PS), and, thus, it is considerably useful in a sandwich ELISA as well as in various immuno-detection and immuno-separation systems.

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1. Introduction

A single-chain Fv (scFv) antibody is an artificial fusion protein with the V_H and V_L domains of whole antibody (whole Ab) genetically linked by a flexible linker $(G_4S)_3$ (Ward, 1993; Owens and Young, 1994; Pini and Bracci, 2000). The molecular sizes of scFvs are approximately one-fifth of that whole Abs. Therefore, we expected that a denser and better-oriented immobilization of scFvs on the surfaces of solid substances would lead to an improvement in sensitivity in various immuno-detection and immune-separation systems such as sandwich ELISA as well as to a reduction in the production cost of a ligand antibody, potentially to a great extent.

Although the cost of producing scFvs using *Escherichia coli* is much lower than that of conventional whole Ab production from either hybridomas or CHO cells, scFvs are mainly recovered as insoluble and inactive aggregates called inclusion bodies, due to the impaired folding of scFv molecules that are over-expressed in *E. coli* cells. Therefore, refolding is necessary for the industrial production of target scFvs in a soluble and active form (Tan et al., 1998; Tsumoto et al., 1998; Cho et al., 2000; Lee et al., 2002; Umetsu et al., 2003; Chen et al., 2006). Furthermore, scFvs have not been utilized as ligand antibodies in immunoassays because the direct attachment of an antigen-binding domain to a plastic surface induces unfavorable conformational change and a decrease in antigen-binding activity.

Our research group has studied the immobilization of proteins utilizing polystyrene-binding peptides (PS-tags) that can recognize the surface structure of hydrophilic polystyrene (phi-PS) (Kumada et al., 2006, 2009c, 2010a). The phi-PS plate is a PS surface oxidized by O_2 plasma irradiation. Phi-PS plates have been widely utilized as a solid support for tissue culture. For ELISA, however, a bare PS or a slightly oxidized-PS (Maxisorp) was utilized. In particular, the immobilization of PS-tag-fused scFvs (scFv-PSs) to phi-PS plates resulted in an enhancement of sensitivity in a sandwich ELISA because the site-specific immobilization of scFvs with high antigen-binding activity was introduced through the PS-tag (Kumada et al., 2009a,b). However, the refolding yield of scFv-PSs using the conventional dialysis method was considerably less than 20%. Thus, the solid-phase refolding that occurred when scFv-PSs were directly immobilized on the surface of a phi-PS plate under semi-denatured conditions was necessary for immobilization and activation. The antigen-binding activities were recovered by washing the surface with PBST (Kumada et al., 2010b, 2011, 2012b, 2013). The solid-phase refolding might seem to be a useful method, while this method was acceptable for approximately 50% of scFv species among 12 different scFvs tested in this group. Therefore, a refolding method is required for a number of scFvs with high versatility.

In recent years, we developed a new affinity peptide that has the ability to attach to the surface of poly(methyl) methacrylate (PMMA-tag). The PMMA-tag (PM-OMP25 peptide: DVEGIGDVDLVNYFEVGATYTFNK) was identified from the amino acid sequence of *E. coli* outer membrane protein F (OMP-F), and consequently, the genetic fusion of a PMMA-tag to the C-terminus of glutathione S-transferase (GST) resulted in a 10-fold higher density and a 5-fold specific activity in the adsorption state, compared with wild-type GST (Kumada et al., 2012a). Therefore, the PMMA-tag is a candidate for an alternative material-binding peptide for the direct immobilization of protein. The aim of this study was to produce and characterize PMMA-tag-fused scFvs (scFv-PMs). We first evaluated the aggregation tendency of scFv-PMs during refolding, and screened conditions that would be suitable for an efficient refolding of various scFv-PMs from 24 different refolding conditions wherein pH and NaCl concentrations were comprehensively changed. In particular, we investigated the influence of isoelectric points (pIs) of tagged scFvs on their refolding yields. Furthermore, the antigen-binding activity of scFv-PMs in an adsorption state on the surfaces of PMMA and other plastics was also evaluated via a sandwich ELISA.

2. Materials and methods

2.1. Materials

Ribonuclease A (RNase) from bovine was purchased from Sigma Aldrich and biotinylated with biotinamidocaproate N-hydroxysuccinimide ester (Nacalai Tesque). Biotinylated RNase (biotin-RNase) was used as a model antigen for the sandwich ELISA. HRP-labeled streptavidin was purchased from VECTOR Laboratories. The gene of an anti-RNase scFv as a model scFv was a gift from Prof. Katakura, Kansai University. Another 11 different scFv genes were originally isolated by this research group. The theoretical isoelectric points (pIs) of scFvs were calculated from the web site (http://web.expasy.org/protparam/). Bulk materials of polystyrene (PS), polypropylene (PP), polycarbonate (PC) and poly(methylmethacrylate) (PMMA) from TOYO Styrene Co. Ltd., Prime Polymer Co. Ltd., Idemitsu Kosan Co. Ltd., Mitsubishi Rayon Co. Ltd. and Mitsui Chemical Co. Ltd., respectively, were used for manufacturing 96 well microtiter plates. Other chemicals were of reagent grade unless specified.

2.2. Microtiter plate

Ninety-six-well microtiter plates made of 5 different plastics, PS, PMMA, PC, PP and PMP, were originally molded from the bulk materials by our research group, and none were treated by any surface modification. Maxisorp™ which has been exclusively utilized as a solid support for immobilization of whole antibodies in a sandwich ELISA was purchased from Thermofisher Scientific. A hydrophilic PS (phi-PS) plate, which is a PS plate treated by O_2 plasma irradiation that has been utilized for tissue culture, was purchased from ACG Technoglass.

2.3. Vector construction for the expression of scFvs with a PMMA-tag and oligo-D-tags

Table 1 shows the amino acid sequences of peptide tags introduced at the C-terminus of scFvs. The single-strand DNAs listed in Table 1 were synthesized for the preparation of expression vectors for each tagged scFv. A pair of single-strand DNAs were heat-denatured and then annealed by cooling to form a double-strand DNA, which was directly ligated with the expression vector pET22 (Merck) that had been predigested with the restriction enzymes *Not* I and *Xho* I. The constructed vectors pET-PM, pET-D5, pET-D10, pET-D15, and pET-PS-D15 were used as shuttle vectors for the expression of each scFv.