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재조합 대장균을 이용한 아플라톡신 B_1에 특이적인 친화력 증대 단쇄가변분절의 생산에 관한 연구

Production of a single-chain variable fragment with improved affinity to aflatoxin B_1 using recombinant *Escherichia coli*

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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

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ABSTRACT

Aflatoxin B_1 (AFB_1) is one of the most toxic mycotoxins present in foods and feed. AFB_1 produced by *Aspergillus* sp. possesses hepatotoxic, teratogenic and mutagenic properties and causes toxic hepatitis, hemorrhage, immunosuppression and hepatic carcinoma. For effective immunological detection of AFB_1 at low concentrations, the development of high affinity antibody for AFB_1 is required. Immunological detection of low molecular weight toxins such as AFB_1 using a single-chain variable fragment (scFv) is a potentially novel and safe method of diagnosing fungal infection and food contamination. In previous studies, the single-chain variable fragment against AFB_1 (scFv-WT) was constructed from its monoclonal antibody gene and expressed in recombinant *Escherichia coli*.

The single-chain variable fragment containing 6 mutations (scFv-M37) compared to the scFv-WT was isolated from an artificial mutagenic library using yeast surface display combined with fluorescence-activated cell sorting, which showed a 9-fold higher affinity than its wild type. But it was not identified which mutation brought positive effects on an affinity improvement since the 6 mutations in the scFv-M37 were generated by random mutagenesis using error-prone PCR. In this study, the effect of the 6 mutated residues on the affinity

improvement was characterized by construction of individual back mutant scFvs for the 6 mutated residues and analyzed by using surface plasmon resonance. Notably, a back mutation of V_H-A110T (scFv-BM3) in the FR-IV of the heavy chain resulted in a 3.2-fold affinity enhancement compared to the scFv-M37, which was attributed to decreasing the dissociation rate constant (k_d) in the interaction between AFB_1 and the back-mutated scFv. Thus, the back-mutated scFv (scFv-BM3) has a 31-fold higher affinity than its parental wild type scFv. Biophysical analyses using circular dichroism and gel filtration revealed that the back-mutation of V_H-A110T caused a subtle conformational change of the scFv toward tighter binding to AFB_1. This fine-tuning approach via back mutation of residues in the randomly mutated scFvs can be applied to other antibodies for additional affinity improvement.

To apply the scFv-BM3 to immunological detection of AFB_1, periplasmic expression in *E. coli* was attempted to produce a functional form of scFv-BM3, but most scFv-BM3 proteins accumulated as inactive aggregates in the cells. However, it was found that the scFv-BM3 secreted into the culture medium had binding activity to AFB_1. Expression conditions of the scFv-BM3 were further manipulated to enhance secretion into the culture medium. This extracellular secretion of functional scFv-BM3 was significantly improved by

supplementation with Triton X-100 and optimization of expression conditions. The scFv-BM3 purified from the culture medium exhibited a typical anti-parallel β-sheet structure and adopted a proper conformation to bind AFB_1 with high affinity and specificity in various biophysical and biochemical analyses. This extracellular production method can be applied to the production of other functional antibody fragments such as scFv and Fab using *E. coli*.

In summary, an affinity improvement of the single-chain variable fragment against aflatoxin B_1 was achieved via the back-mutation of the randomly mutated scFv. A simple and efficient method to produce active form of the functional antibody fragment was demonstrated using recombinant *E. coli* by extracellular secretion.

Keywords : single-chain variable fragment, aflatoxin B_1, recombinant *Escherichia coli*, back mutation, affinity improvement, extracellular secretion

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Introduction

1.1. Mycotoxins

A mycotoxin is a toxic secondary metabolite produced by filamentous fungi and capable of causing disease and death in both humans and other animals (Bennett et al., 2003). The term mycotoxin is usually reserved for the toxic chemical products produced by fungi that readily colonize crops (Turner et al., 2009). One mold species may produce many different mycotoxins, and several species may produce the same mycotoxin (Robbins et al., 2000). It is estimated that about 450 mycotoxins exist and about 20 classes of them are known to cause health concerns in both humans and animals. Major food commodities affected by mycotoxins are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, and some derived products such as beer, wine and fruit juices. Mycotoxins also enter the human food chain by consuming meat or other animal products such as milk and cheese which fed with contaminated feed. The most important mycotoxins are aflatoxins, ochratoxins, citrinins, ergot alkaloids, patulins, and fusarium toxins (Fig. 1-1).

Aflatoxins are a type of mycotoxins produced by *Aspergillus* species of fungi such as *A. flavus* and *A. parasiticus* (Martins et al., 2001). The

umbrella term aflatoxin refers to four different types of mycotoxins produced, which are B_1, B_2, G_1, and G_2 (Yin et al., 2008). Aflatoxin B_1, the most toxic, is a potent carcinogen and has been directly correlated to adverse health effects such as liver cancer in many animal species (Martins et al., 2001). Aflatoxins are largely associated with commodities produced in the tropics and subtropics such as cotton, peanuts, spices, pistachios and maize (Martins et al., 2001; Yin et al., 2008).

Ochratoxin is a mycotoxin that comes in three secondary metabolite forms, A, B, and C. All are produced by *Penicillium* and *Aspergillus* species. The three forms differ in that Ochratoxin B (OTB) is a nonchlorinated form of Ochratoxin A (OTA) and that Ochratoxin C (OTC) is an ethyl ester form Ochratoxin A (Bayman et al., 2006). *A. ochraceus* is found as a contaminant of a wide range of commodities including beer and wine. *A. carbonarius* is the main species found on vine fruit, which releases its toxin during the juice making process (Mateo et al., 2007). OTA has been labeled as a carcinogen and a nephrotoxin, and has been linked to tumors in the human urinary tract, although research in humans is limited by confounding factors (Bayman et al., 2006; Mateo et al., 2007).

Citrinin is a toxin that was first isolated from *P. citrinum*, but has been identified in over a dozen species of *P.* and several species of *Aspergillus*. Some of these species are used to produce human foodstuffs such as cheese (*P. camemberti*), sake, miso, and soy sauce (*A. oryzae*). Citrinin is associated with yellowed rice disease in Japan and acts as a nephrotoxin in all animal species tested. Although it is associated with many human foods (wheat, rice, corn, barley, oats, rye, and food colored with Monascus pigment) its full significance for human health is unknown. Citrinin can also act synergistically with Ochratoxin A to depress RNA synthesis in murine kidneys (Bennett et al., 2003).

Ergot alkaloids are compounds produced as a toxic mixture of alkaloids in the sclerotia of species of *Claviceps*, which are common pathogens of various grass species. Ingestion of ergot sclerotia from infected cereals, commonly in the form of bread produced from contaminated flour, cause ergotism, the human disease historically known as St. Anthony's Fire. There are two forms of ergotism: gangrenous affecting blood supply to extremities and convulsive affecting the central nervous system. Modern methods of grain cleaning have significantly reduced ergotism as a human disease; however, it is still an important

veterinary problem. Ergot alkaloids have been used pharmaceutically (Bennett et al., 2003).

Patulin is a toxin produced by *P. expansum*, *Aspergillus*, *Penicillium*, and *Paecilomyces* fungal species. *P. expansum* is especially associated with a range of moldy fruits and vegetables, in particular rotting apples and figs (Moss et al. 2008; Trucksess et al., 2008). It is destroyed by a fermentation process and so is not found in apple beverages such as cider. Although patulin has not been shown to be carcinogenic, it has been reported to damage the immune system in animals (Moss et al., 2008). In 2004, the European Community sets limits to the concentrations of patulin in food products. They currently stand at 50 µg/kg in all fruit juice concentrations, at 25 µg/kg in solid apple products used for direct consumption, and at 10 µg/kg for children's apple products including apple juice (Moss et al., 2008; Trucksess et al., 2008).

Fusarium toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize (Cornely, 2008; Schaafsma et al., 2007). They include a range of mycotoxins; fumonisins, which affect the nervous systems of horses and may cause cancer in rodents; trichothecenes, which are most

strongly associated with chronic and fatal toxic effects in animals and humans; and zearalenone, which is not correlated to any fatal toxic effects in animals or humans. Some of the other major types of Fusarium toxins include beauvercin and enniatins, butenolide, equisetin and fusarins (Desjardins et al., 2007).

1.2. Aflatoxins

Aflatoxins occur in nuts, cereals and rice under conditions of high humidity and temperature and present a risk to human health that is insufficiently recognized. The two major *Aspergillus* species that produce aflatoxins are *A. flavus*, which produces only B aflatoxins, and *A. parasiticus*, which produces both B and G aflatoxins. Aflatoxins M_1 and M_2 are oxidative metabolic products of aflatoxins B_1 and B_2 produced by animals following ingestion, and so appear in milk (both animal and human), urine and faeces. Aflatoxicol is a reductive metabolite of aflatoxin B_1.

Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. The main target organ for toxicity and carcinogenicity is the liver. Among 18 different types of

aflatoxins identified, major members are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and aflatoxin M_2 (AFM_2) (Fig. 1-2).

The evaluation of epidemiological and laboratory results carried out in 1987 by the International Agency for Research on Cancer (IARC) found that there is sufficient evidence in humans for the carcinogenicity of naturally occurring mixtures of aflatoxins, which are therefore classified as Group 1 carcinogens, except for aflatoxin M_1, which is possibly carcinogenic to humans (Group 2B) (Report of an IARC Expert Committee, 1987).

In 1974, a major outbreak of hepatitis due to aflatoxin was reported in the states of Gujrat and Rajasthan in India, resulting in an estimated 106 deaths (Krishnamachari et al., 1975). The outbreak lasted for 2 months and was confined to tribal people whose main staple food, maize, was later confirmed to contain aflatoxin. The preliminary analysis confirmed that consumption of *A. flavus* had occurred (Krishnamachari et al., 1975; Bhat et al., 1978).

Another outbreak of aflatoxin affecting both humans and dogs was reported in northwest India in 1974 (Tandon et al., 1977; Bhat et al.,

1978; Reddy et al., 2007). A major aflatoxin exposure outbreak was subsequently documented in Kenya in 1981 (Ngindu et al., 1982). Since 2004, multiple aflatoxicosis outbreaks have been reported worldwide, resulting in 500 acute illness and 200 deaths (Centers for Disease Control and Prevention [CDCP], 2004; Azziz-Baumgartner et al., 2005). Most outbreaks have been reported from rural areas of the East Province of Kenya in 2004 and occurred because of consumption of home grown maize contaminated with molds. Preliminary testing of food from affected areas revealed the presence of aflatoxin as reported in 1981 (Ngindu et al., 1982). In 2013, countries in Europe including Romania, Serbia, and Croatia reported the nationwide contamination of milk with aflatoxin (https://en.wikipedia.org/wiki/2013_European_aflatoxin_contamination).

The major sources of aflatoxins are fungi such as *A. flavus*, *A. parasiticus*, and *A. nomius* (Kurtzman et al., 1987), although they are also produced by other species of *Aspergillus* as well as by *Emericella* spp. (Reiter et al., 2009). There are more than 20 known aflatoxins, but the four main ones are AFB_1, AFB_2, AFG_1, and AFG_2 (Inan et al., 2007), while AFM_1 and AFM_2 are the hydroxylated metabolites of AFB_1 and AFB_2 (Giray et al., 2007; Hussain et al., 2008).

Aspergillus species are an industrially important group of microorganisms distributed worldwide. *A. niger* has been listed as Generally Recognized as Safe (GRAS) status by the USFDA (Schuster et al., 2002). However, some species have negative impacts and cause diseases in grape, onion, garlic, peanut, maize, coffee, and other fruits and vegetables (Lorbeer et al., 2000; Magnoli et al., 2006; Waller et al., 2007; Rooney-Latham et al., 2008). Moreover, *Aspergillus* section *nigri* produces mycotoxins such as ochratoxins and fumonisins in peanut, maize, and grape (Astoreca et al,. 2007a, b; Frisvad et al., 2007; Mogensen et al., 2009).

Plant-pathogen interactions have been studied using molecular markers such as green fluorescent protein (GFP) isolated from *Aequorea victoria* (Prasher et al., 1992). The GFP gene has been successfully inserted into *Undifilum oxytropis* (Mukherjee et al., 2010), *Fusarium equiseti* (Macia-Vicente et al., 2009), and *Muscodor albus* (Ezra et al., 2010) and utilized to study the expression of different proteins and production of mycotoxins. *A. flavus* and *A. parasiticus* infect many crops in the field, during harvest, in storage, and during processing. *A. flavus* is dominant in corn, cottonseed, and tree nuts, whereas *A. parasiticus* is dominant in peanuts. *A. flavus* consists of mycelium,

conidia, or sclerotia and can grow at temperatures between 12 and 48℃ (Hedayati et al., 2007). *A. flavus* produces AFB_1 and AFB_2, whereas *A. parasiticus* produces AFG_1, AFG_2, AFM_1, AFB_1, and AFB_2. *A. flavus* produces a number of airborne conidia and propagules that infect plants such as cotton (Lee et al., 1986). A high number of propagules was reported in soil, air, and on cotton leaves during mid- to late August, while soilborne inoculum increased drastically between April and December in cotton fields in Arizona (Ashworth et al., 1969). This fungus can even colonize moribund rye cover crop and peanut fruit debris (Griffin et al., 1976).

Among the mycotoxins affecting foods and feed, aflatoxin is the major one in foods that ultimately harms human and animal health (Boutrif, 1998). The level of toxicity associated with aflatoxin varies with the types present, with the order of toxicity being $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Jaimez et al., 2000).

Aflatoxins specifically target the liver organ (Abdel-Wahhab et al., 2007). Early symptoms of hepatotoxicity of liver caused by aflatoxins comprise fever, malaise and anorexia followed with abdominal pain, vomiting, and hepatitis; however, cases of acute poisoning are exceptional and rare (Etzel, 2002). Chronic toxicity by aflatoxins

comprises immunosuppressive and carcinogenic effects. Evaluation of the effects of AFB_1 on splenic lymphocyte phenotypes and inflammatory cytokine expression in male F344 rats have been studied (Qian et al., 2014). AFB_1 reduced anti-inflammatory cytokine IL-4 expression, but increased the pro-inflammatory cytokine IFN-γ and TNF-α expression by NK cells. These findings indicate that frequent AFB_1 exposure accelerates inflammatory responses via regulation of cytokine gene expression. Furthermore, Mehrzad et al. (2014) observed that AFB_1 interrupts the process of antigen-presenting capacity of porcine dendritic cells, suggested this perhaps one of mechanisms of immunotoxicity by AFB_1.

Aflatoxins cause reduced efficiency of immunization in children that lead to enhanced risk of infections (Hendrickse, 1997). The hepatocarcinogenicity of aflatoxins is mainly due to the lipid peroxidation and oxidative damage to DNA (Verma, 2004). AFB_1 in the liver is activated by cytochrome p450 enzymes, which are converted to AFB_1-8, 9-epoxide, which is responsible for carcinogenic effects in the kidney (Massey et al., 1995). Among all major mycotoxins, aflatoxins create a high risk in dairy because of the presence of their derivative, AFM_1, in milk, posing a potential health hazard for human

consumption (Van Egmond, 1991; Wood, 1991). AFB_1 is rapidly absorbed in the digestive tract and metabolized by the liver, which converts it to AFM_1 for subsequent secretion in milk and urine (Veldman et al., 1992). Although AFM_1 is less mutagenic and carcinogenic than AFB_1, it exhibits high genotoxic activity. The other effects of AFM_1 include liver damage, decreased milk production, immunity suppression and reduced oxygen supply to tissues due to anemia (Aydin et al., 2008), which reduces appetite and growth in dairy cattle (Akande et al., 2006). Several studies have shown the detrimental effects of aflatoxins exposure on the liver (Sharmila Banu et al., 2009), epididymis (Agnes et al., 2001), testis (Faisal et al., 2008), kidney and heart (Mohammed et al., 2009; Gupta et al., 2011). It has been found that aflatoxin presences in post-mortem brain tissue (Oyelami et al., 1995) suggested that its ability to cross the blood brain barrier (Qureshi et al., 2015). AFTs also cause abnormalities in the structure and functioning of mitochondrial DNA and brain cells (Verma, 2004). The effects of aflatoxin on brain chemistry have been reviewed in details by Bbosa et al. (2013). Furthermore, few reports have described the effects of AFB_1 administration on the structure of the rodent central nervous system (Laag et al., 2013).

The liver toxicology of aflatoxin is also a critical issue (IARC, 2002; Iqbal et al., 2014). Limited doses are not harmful to humans or animals; however, the doses that do cause-effects diverse among aflatoxin groups. The expression of aflatoxin toxicity is regulated by factors such as age, sex, species, and status of nutrition of infected animals (Williams et al., 2004). The symptoms of acute aflatoxicosis include oedema, haemorrhagic necrosis of the liver and profound lethargy, while the chronic effects are immune suppression, growth retardation, and cancer (Gong et al., 2004; Williams et al., 2004; Cotty et al., 2007).

1.3. Detection methods of aflatoxins

Aflatoxin contamination in grain poses a great threat to human and livestock health as well as international trade. Confirmation of the extent of contamination is the essential first step in reducing exposure to aflatoxins. Detection of aflatoxins is necessary in a variety of situations ranging from in the field to in controlled laboratory settings. Accordingly, the technologies being developed range from those which can be conducted rapidly with minimal technical expertise, such as immunoassays and biosensors, to those which can be conducted by

technical personnel.

Aflatoxins are usually detected and identified according to their absorption and emission spectra, with peak absorbance occurring at 360 nm. AFB toxins exhibit blue fluorescence at 425 nm, while AFG toxins show green fluorescence at 540 nm under UV irradiation. This florescence phenomenon is widely accepted for aflatoxins.

Thin layer chromatography (TLC) is one of the oldest techniques used for aflatoxin detection (Fallah et al., 2011), while high performance liquid chromatography (HPLC), liquid chromatography mass spectroscopy (LCMS), and enzyme linked immune-sorbent assay (ELISA) are the methods most frequently used for its detection (Choi et al., 2004; Cho et al., 2005; Min et al., 2010; Tabari et al., 2011; Andrade et al., 2013; Sulyok et al., 2015).

Aflatoxin determination in foods and feed is currently performed by high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS) (CAST, 2003; Kolosova et al., 2006). Although most of these methods are sensitive and accurate, they are laborious, expensive, time-consuming and unsuitable for analysis of large number of samples and also require costly equipment and

extensive clean-up procedures (Kolosova et al., 2006).

Fast, reliable and sensitive analytical methods are needed, due to the strict guidelines on mycotoxin contamination that have been imposed by importing countries. This demand has led to the development of quantitative or semi-quantitative methods for mycotoxin screening, based on immunochemical techniques, such as enzyme-linked immunosorbent assays (ELISAs), since they do not require costly instrumentation, are able to analyse a large number of samples simultaneously and require no sample clean-up (Krska et al., 2008; Li et al., 2009; Zhang et al., 2011). In general, ELISAs are rapid, simple, specific and sensitive, they can be used in the field and have become the most common rapid methods for mycotoxin detection in foods and feed (Zheng et al., 2005). Additionally, the detection limits of ELISA can be comparable with or even lower than those obtained by instrumental methods (Kolosova et al., 2006).

However, commercial ELISA kits are expensive which makes their inclusion in routine analysis in developing countries difficult (Devi et al., 1999); therefore investments in immuno-reagent production are an alternative to reduce costs.

Production of high quality antibodies is required for the assay development of aflatoxin estimation. High-throughput phage display technology is currently an attractive and effective method of choice to obtain antibodies, wherein affinity maturation can be modified (Hoogenboom et al., 1991; Smith, 1985) and the molecule can be further engineered. In addition, several serological techniques require an enzyme as a label to detect measurable signal, for example alkaline phosphatase (AP), which is one of the widely used enzymes. It has a wide range of applications in diagnostics, immunology and molecular biology, serving as a biochemical marker in the quantitative measurement of analytes.

1.4. Antibodies for detection of mycotoxins

Among the sensitive analytical methods to identify and quantify mycotoxins, immunoassays based on specific interactions between antibodies and antigens are the most useful tools for rapidly monitoring mycotoxins (Zheng et al., 2006). These assays have a number of advantages, including high selectivity toward the target analytes, greater than or comparable sensitivity to the instrumental methods and

ease of operation and high-throughput screening ability (Vasylieva et al., 2017) for mycotoxins in agricultural foodstuffs. In the case of the ELISA method (immunoassay method), antibodies used as a recognition element are highly specific to their antigens (analyte). The affinity of antibodies to specific mycotoxins is especially more important when the mycotoxin concentrations in foods or feed matrices are very low (~nM). Since mycotoxins are low molecular weight haptens, mycotoxin-specific antibody production is not easy, being costly and highly laborious. Despite these difficulties, antibodies to most mycotoxins are commercially available and widely used in immunoassays. A large number of antibodies against mycotoxins have been developed, including polyclonal antibodies (pAbs) (Shiu et al., 2010; Zhuang et al., 2014), monoclonal antibodies (mAbs) (Zhang et al., 2009; KalayuYirga et al., 2017) and recombinant antibodies (rAbs) (Li et al., 2013; Arola et al., 2017).

Polyclonal antibodies are obtained by immunization of animals followed by extraction from blood. Being inexpensive and easy produced in a mass scale, they can show insufficient selectivity toward structurally relative mycotoxins and proteins used as conjugates on the immunization step. Monoclonal antibodies are produced from

hybridomas by fusing myeloma or spleen cells from immunized mice. They are more uniform in affinity toward analytes against polyclonal antibodies. Despite high sensitivity and specificity of analyte recognition, antibodies show some limitations, i.e., need of animals for their production, at least at first stages, susceptibility to denaturation and hydrolytic degradation and incompatibility with many organic solvents required for mycotoxin extraction. Besides whole IgG molecules, their fragments obtained by specific cleavage with proteolytic enzymes or produced by recombinant bacterial cells are used in the assembly of immunosensors. Reduced parts of immunoglobulins can contain the tips of Y-shape molecule (Fab) or single-chain variable fragment (scFv) of both heavy and light chains. Reduced IgG parts are much smaller than intact antibodies and can be easily implemented in the immunosensor assembly. For the same reason, their application minimizes steric hindrance in heterogeneous systems including immunosensors.

1.5. Single-chain variable fragments (scFvs)

A single-chain variable fragment (scFv) is an artificial minimal antibody in which the variable regions from the heavy and light chains (V_H and V_L) of immunoglobulin are joined by a flexible peptide linker (Fig. 1-3). The resulting fragment is the smallest antibody which still retains affinity and specificity of its parent antibody. ScFv has many advantages in therapeutic and diagnostic applications for its small size and easy mass production. There is a lot of interest in the possibility of using scFvs as therapeutic agents (Mohr L. et al., 2004). Compared to the much larger Fab', $F(ab')_2$, and IgG forms of monoclonal antibodies *in vivo*, scFv proteins may be cleared more quickly from the blood, and penetrate tissues with a rapid and even distribution. The lack of the Fc domain makes its immunogenicity lower than that of the native antibody *in vivo*. In addition, recombinant antibody genes can be manipulated genetically to increase target sensitivity and specificity, and can be engineered to meet requirements of detection methods and therapy (Chowdhury P. S. et al., 1999; Ahmad Z. A. et al., 2012; Roopali R. et al., 2015). It is, moreover, produced by simple techniques at low cost. Among the recombinant antibodies, the fidelity of scFv, that is, whether the combining site of these proteins is a bonafide

replica of that of native antibodies, is often taken for granted because scFv is known to have similar structural properties as the native molecule and, moreover, useful and high-affinity scFv is continually found by many previous studies. Because of these merits, scFv can be applied to antibody microarray, that is called a protein chip.

A protein chip with scFv has many advantages because scFv enable to measure relative abundance of hundreds of native proteins in one experiment and to identify variations in protein levels among a variety of biological samples.

1.6. Improving the properties of antibody fragments

In some cases fine-tuning of properties is required for better performance of the antibody fragments. Various libraries have been constructed and knowledge-based approaches have been applied to improving the properties of an antibody obtained from hybridoma cell lines or from the above-mentioned primary libraries.

The antibody-antigen interaction is a combination of steric and electrostatic complementarity between the paratope of an antibody and the epitope of an antigen. Hydrophobic interactions, hydrogen bonds

and van der Waals interactions may all contribute to the binding. By increasing the number of specific interactions and the size of the buried surface area it is possible to improve the affinity. The binding properties of the antibodies have been improved by single mutations, random mutations to selected residues, random mutagenesis for the whole or some part of the fragment, CDR-walking, insertions in selected CDRs and chain shuffling. In general, the knowledge-based site-specific approaches to improve the affinity have not been widely used due to the lack of detailed high resolution structural data of antibodies and to the difficulty in predicting the possible conformational changes during antigen binding (Dougan et al., 1998). Homology modeling has been used successfully to target single residues or regions for mutagenesis (Roberts et al., 1987; Ruff-Jamison et al., 1993; Iba et al., 1998; Hemminki et al., 1998). At present mutagenesis, random or targeted, followed by selection for improved affinity continues to be a convenient method.

Sometimes the association or the dissociation behavior of the antibody has been the special target for affinity-tuning (Duenas et al., 1996; Söderlind et al., 2001; Jirholt et al., 2001; Hugo et al., 2003; João C. E. et al., 2017). In many antibody applications the kinetic parameters of

the binding process are important. In diagnostic and immune-affinity applications as well as in therapeutic applications and antibody chip technologies a high association rate is crucial. Katakura et al. (2004) developed a kinetic model to improve the isolation of such antibodies. On the other hand the dissociation rate constants are very important in applications such as continuous monitoring and affinity chromatography applications, which prefer a rapid dissociation (Hugo et al., 2003).

Specificity requirements for the antibody fragment depend on the applications. In general, high specificity is preferred and some examples of antibody engineering to decrease cross-reactivity are listed in Table 1-1. Random mutagenesis to defined areas has been a successful approach for specificity optimization. High specificity is important for diagnostic antibodies and especially in cases in which the cross-reacting compound may be present in samples at high concentrations.

Determination of different steroid hormone levels in human sera samples is a good example of a high requirement of specificity of anti-hapten antibodies (Pope et al., 1996; Hemminki et al., 1998; Lamminmaki et al., 1999). On the other hand in applications such as

affinity chromatography and antibody-based solid-phase extraction the binding of related substances can sometimes be considered advantageous. Cross-reactivity for structurally similar compounds makes it possible to capture all of them simultaneously and to subject the enriched mixture to further identification and quantitation (Houben et al., 1999; Delaunay-Bertoncini et al., 2004).

Stability is an important requirement for all antibodies, independent of specificity. *In vitro* applications may require antibodies with a long half-life, high stability in organic solvents and resistance to surface denaturation and dry atmosphere. Natural antibody frameworks have very different stability properties (Ewert et al., 2003). Single amino acids may have a great impact on stability (Honegger et al., 2001; Shuang W. et al., 2014). Two aspects of stability engineering, structure-based framework engineering and CDR-grafting to more stable frameworks, were studied by Ewert et al. 2004. CDR-grafting is a way to introduce the valuable binding properties of CDRs to a superior framework. Targeted 35 mutations can be used to refine the stability of an antibody. Mutations affecting stability may influence e.g. the packing of the hydrophobic core, hydrogen bonding or charge interactions. The importance of the disulfide bridges for stability was

indicated by several authors (Glockshuber et al., 1990; Proba et al., 1997, 1998), although scFv with sufficient stability can tolerate the loss of both disulfide bridges (Wörn et al., 1998). High temperatures or denaturants have been used to introduce selection pressure for increased stability of antibody fragments (Proba et al., 1998; Jung et al., 1999). Examples of successful stability engineering are presented in Table 1-2. Different aspects of stability engineering of scFvs were reviewed (Wörn et al., 2001; Brian R. M. et al., 2010; Andreas L. et al., 2015). In addition to the protein engineering approach, chemical cross-linking by e.g. glutaraldehyde has been used to stabilize the antibody fragments (Glockshuber et al., 1990; Vuolanto et al., 2004).

The stability of an antibody sometimes correlates with the expression yield (Jung and Plückthun, 1997; Jung et al., 1999; Ewert et al., 2003; Lombana T. N. et al., 2015; Kathrin Z. et al., 2009) and even a single amino acid replacement can improve both stability and expression level (Hugo et al., 2003). The balance between the correct folding and aggregation may also depend on a single amino acid (Knappik et al., 1995; Shuang W. et al., 2014). The best correlations between stability and production level have been obtained with cytoplasmic expression, whereas the correlation of stability with periplasmic expression is less

straightforward, e.g. mutations increasing the periplasmic expression of anti-fluorescein scFv had no influence on stability (Nieba et al., 1997).

Targeted and knowledge-based engineering has been applied to improving the crystallization properties of antibodies. Surface mutagenesis was applied to introduce a packing motif to the β-strand of variable domain in order to promote their crystallization propensity (Wingren et al., 2003). Immunoreactivity, expression and overall structures of the antibodies were maintained. Rational design of packing motifs promoting crystallization also opens new possibilities for anti-hapten antibody applications.

1.7. Heterologous expression of antibody fragments

Most often the antibody fragments have been produced in *E. coli* and different expression strategies have been applied. Correctly folded antibody fragments can be produced in the periplasmic space between the inner and outer membranes of *E. coli* (Better et al., 1988; Skerra et al., 1988; Carter et al., 1992; Pack et al., 1993). The periplasmic space has reducing conditions and chaperones, leading to the formation of disulfide bridges and correct folding of the polypeptides. Production of

antibody fragments in the periplasm may lead to leakage or partial lysis of the outer membrane and antibody fragments can then be collected from the culture supernatant. Single mutations to V_H (Forsberg et al., 1997) or optimized vector and growth conditions have shown to prevent leakage and lysis of the bacterial cells (Pack et al., 1993; Horn et al., 1996). If the signal sequence, which guides the synthesized polypeptide to the periplasm, is not included then the antibody fragments tend to accumulate in the cytoplasm and may form insoluble aggregates, inclusion bodies (Bird et al., 1988; Proba et al., 1995; Sinacola et al., 2002). Refolding of the 26 antibody fragments *in vitro* is then necessary and the success of this procedure is antibody-dependent (Sinacola et al., 2002).

Using optimized expression vectors and high cell density-fermentation product concentrations up to 1-3 g/L of the functional antibody fragment have been obtained. (Carter et al., 1992; Pack et al., 1993; Horn et al., 1996). The expression concentration of soluble proteins can be improved by increasing the total expression level and also the folding yield, which is connected to the susceptibility of the antibody fragment to proteolytic degradation (deHaard et al., 1998). Co-expression of periplasmic chaperones has in some studies improved the

functional expression of the antibody fragment (Bothmann et al., 1998, 2000; Mavrangelos et al., 2001). The choice of the expression vector, bacterial strain and conditions for cultivation, e.g. media composition and temperature, affect the final yield of soluble active antibody fragment. In addition to these extrinsic factors especially the amino acid sequences of the antibody but also the origin of the antibody and fragment type can exert an effect on the final yield. Despite identical production vectors and cultivation conditions up to 10-fold differences in product concentration of different antibodies have been observed (Knappik et al., 1995). Even a single mutation can have a dramatic effect on the expression (Duenas et al., 1995; Knappik et al., 1995). Critical residues on the surface or at the domain interface can have a great impact on expression (Knappik et al., 1995). The importance of the framework regions was demonstrated in one study in which humanization of an antibody improved the production by 10-100 folds compared to the parental murine antibody (Carter et al., 1992). The exposed hydrophobic patches in scFv antibodies may promote aggregation, which necessitates the substitution of key hydrophobic residues by hydrophilic ones for improving yields (Nieba et al., 1997).

Studies to identify the critical positions in antibody frameworks for improved expression have been performed for various antibodies. A systematic study with the human variable domains revealed differences in production levels of different germline family consensus domains and different combinations of the domains (Ewert et al., 2003). Careful consideration is needed if mutations are applied to a different framework, as the studies of deHaard et al. (1998) and Kipriyanov et al. (1997) have indicated. The change of glutamine to glutamate at the position V_H6 caused a decrease in productivity of the class IIB antibody without affecting the affinity, whereas the same mutation for several class IIA antibodies decreased their production levels and also induced loss in binding activity.

In addition to bacteria other hosts such as yeast (Horwitz et al., 1988; Shusta et al., 1998; Lange et al., 2001), insect cells (Laroche et al., 1991; Reavy et al., 2000), mammalian cells (Dorai et al., 1994), filamentous fungi (Nyyssönen et al., 1993) and various species of plants (Churchill et al., 2002) have also been used to produce antibody fragments. A comparison of bacteria, yeast, insect and mammalian expression systems in the production of antibody fragments was presented (Verma et al., 1998; Lee et al., 2015).

1.8. Production of heterologous proteins using *E. coli*

E. coli is one of the organisms of choice for the production of heterologous proteins. Its use as a cell factory is well-established and it has become the most popular expression platform. For this reason, there are many molecular tools and protocols at hand for the high-level production of heterologous proteins, such as a vast catalog of expression plasmids, a great number of engineered strains and many cultivation strategies.

The advantages of using *E. coli* as the host organism are well known. (i) It has unparalleled fast growth properties. In glucose-salts media and given the optimal environmental conditions, its doubling time is about 20 min (Sezonov et al., 2007). This means that a culture inoculated with a 1/100 dilution of a saturated starter culture may reach a stationary phase in a few hours. However, it should be noted that the expression of a recombinant protein may impart a metabolic burden on the microorganism, causing a considerable decrease in generation time (Seo et al., 1985). (ii) High cell density cultures are easily achieved. The theoretical density limit of an *E. coli* culture is estimated to be about 200 g dry cell weight/l or roughly 1×10^{13} viable bacteria/ml

(Lee, 1996; Shiloach et al., 2005). However, exponential growth in complex media leads to densities near that number. In the simplest laboratory setup (i.e., batch cultivation of *E. coli* at 37 ℃, using LB media), $<1 \times 10^{10}$ cells/ml may be the upper limit (Sezonov et al., 2007), which is less than 0.1% of the theoretical limit. For this reason, high cell-density culture methods were designed to boost *E. coli* growth, even when producing a recombinant protein (Kim et al., 2006; Kim et al., 2011). Being a workhorse organism, these strategies arose thanks to the wealth of knowledge about its physiology. (iii) Rich complex media can be made from readily available and inexpensive components. (iv) Transformation with exogenous DNA is fast and easy. Plasmid transformation of *E. coli* can be performed in as little as 5 min (Pope et al., 1996).

1.9. Research objectives

This study is concerned with (1) construction of a single-chain variable fragment with improved affinity to aflatoxin B_1 by back mutation in the framework region of the heavy chain and (2) effective production of the functional single-chain variable fragment against AFB_1 using recombinant *E. coli* by extracellular secretion. To carry out these objectives successfully, the detailed strategies are listed as follows;

1. Construction of a single-chain variable fragment with improved affinity to aflatoxin B_1
   1) Construction of individual back mutant scFvs for the 6 mutated residues to characterize the contribution of each mutation to affinity improvement
   2) Extracellular production of scFvs in recombinant *E. coli*
   3) Analyses of the affinity of scFvs through surface plasmon resonance (SPR) and specificity to other mycotoxins by competitive indirect enzyme-linked immunosorbent assay
   4) Biophysical comparison of scFvs using CD spectrometry and analytical gel filtration chromatography to assess the effect of the back mutation

2. Effective production of the functional scFv against aflatoxin B_1 using recombinant *E. coli*

1) Production of functional form of scFv via the extracellular secretion using recombinant *E. coli*

2) Purification of the secreted scFv from the culture medium

3) Various biochemical analyses by MALDI-TOF MS, ELISA and CD spectrometry

Table 1-1. Examples of antibody engineering to decrease cross-reactivity and alter specificities.

Target area	Hapten	Change in specificity	Reference
Heavy chain	Estradiol	Reduced cross-reactivity	Saviranta et al., 1998
CDRs	Testosterone	Reduced cross-reactivity	Hemminki et al., 1998
3 CDR	17-OHP	Broadened specificity	Iba et al., 1998
SDRs	Digoxin	Altered specificities	Chen et al., 1999
CDR-H2	Estradiol	Reduced cross-reactivity	Lamminmaki et al., 1999
3 CDR + random V_H	11-deoxycortisol	Altered specificities	Miyazaki et al., 1999
5 or 10 residues	Digoxin	Altered specificities	Short et al., 2001
Whole fragment	Sulphonamides	Broadened specificity	Korpimake et al., 2003

Table 1-2. Examples of stability engineered recombinant antibody fragments.

Target area	Origin	Fragment	Note	Reference
Loop grafting	Murine	scFv	Also improved expression	Jung and Plückthun, 1997
Single replacements	Murine	scFv		Wörn and Plückthun, 1998
Single replacements	Humanised	scFv	Also improved expression and affinity	Jung et al., 1999
Whole fragment	Human	scFv		Jermutus et al., 2001
Six replacement	Human	scFv	Also improved expression	Ewert et al., 2003
Single replacement	Murine	scFv/Fab	L34, also improved expression	Hugo et al., 2003

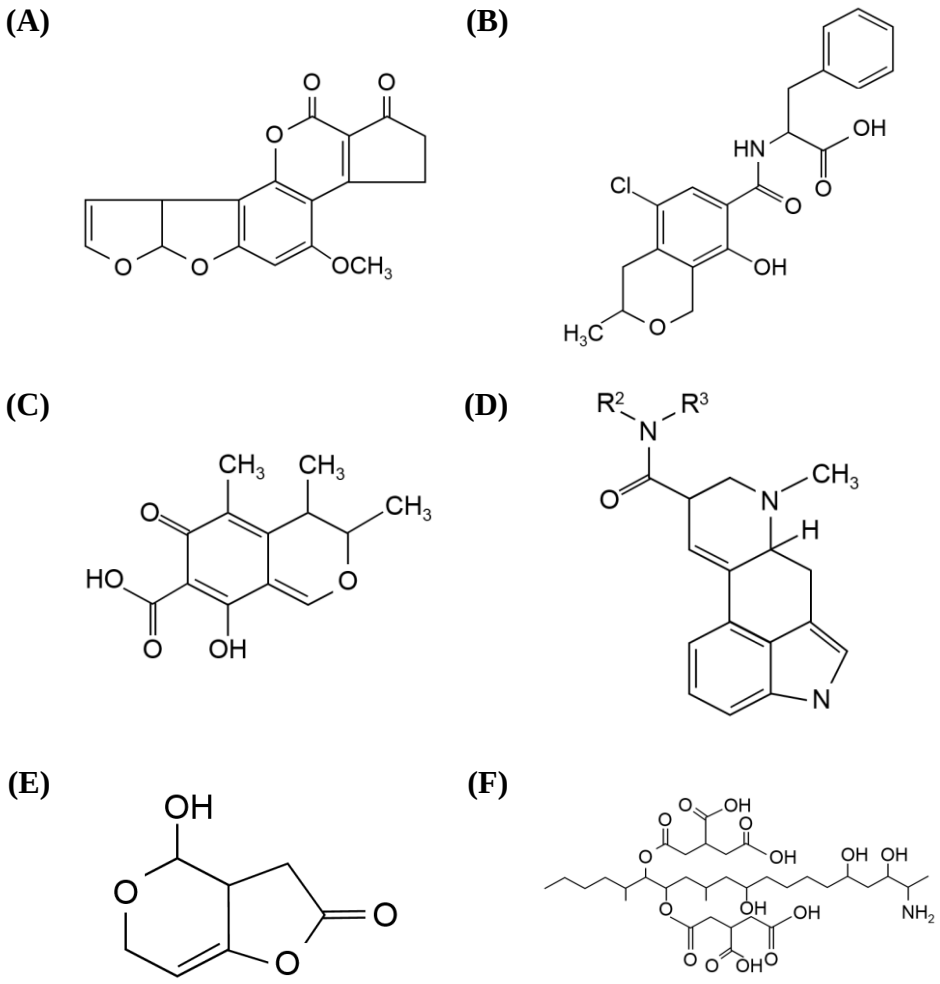


Figure 1-1. Chemical structure of representative mycotoxins. (A) Aflatoxin B_1, (B) Ocharatoxin A, (C) Citrinin, (D) Ergot alkaloid, (E) Patulin and (F) Fumonisin.

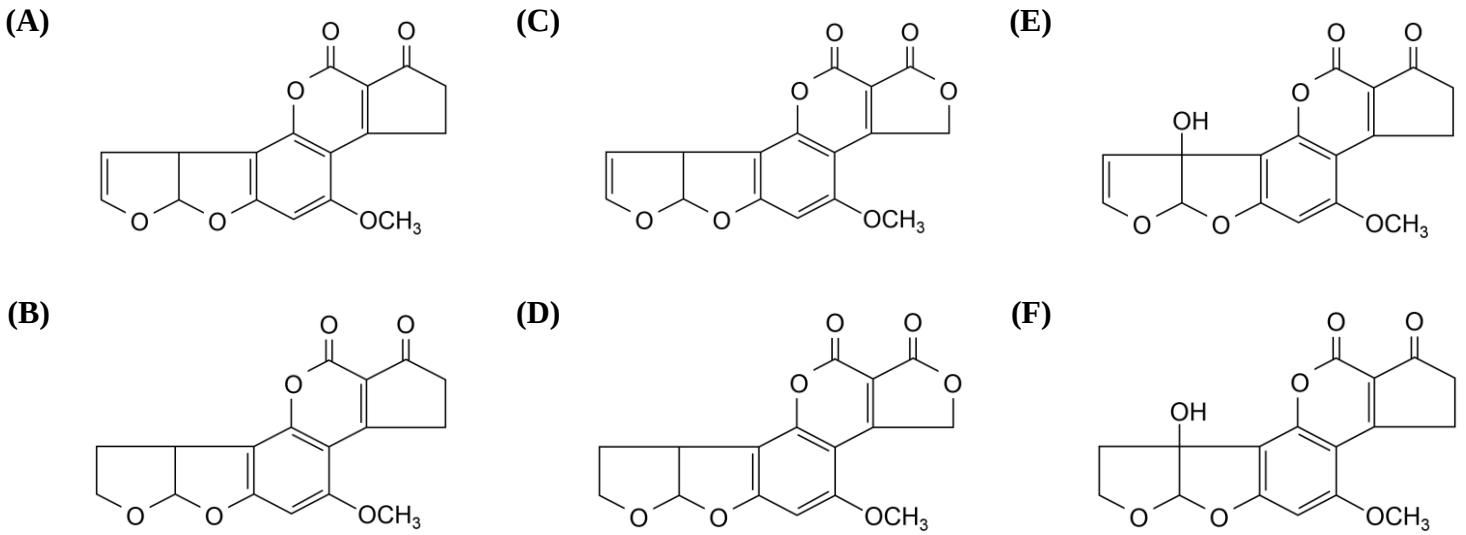


Figure 1-2. Chemical structure of aflatoxins. (A) Aflatoxin B_1, (B) Aflatoxin B_2, (C) Aflatoxin G_1, (D) Aflatoxin G_2, (E) Aflatoxin M_1 and (F) Aflatoxin M_2.

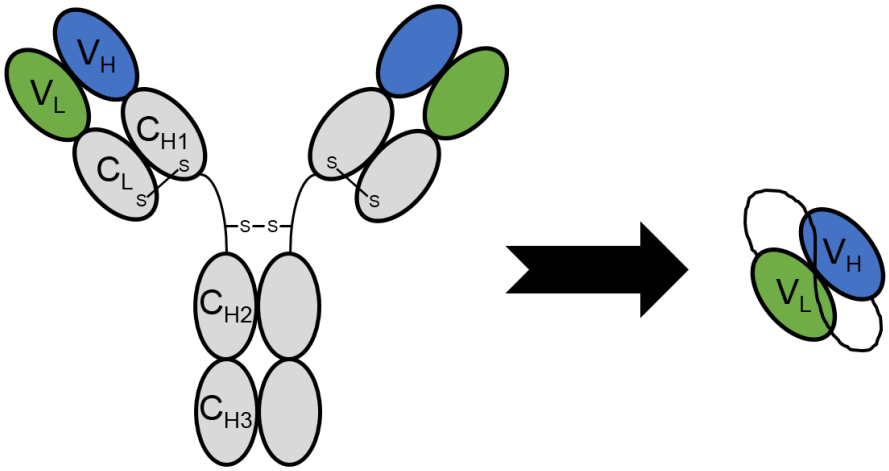


Figure 1-3. Structure of single-chain variable fragments. Single-chain variable fragments are a minimal form of functional antibodies, consisting of the variable regions of only the heavy and light chain of a full IgG antibody, connected by a small linker peptide(V_L; variable region on light chain, C_L; constant region on light chain, V_H; variable region on heavy chain, C_H; constant regions on heavy chains, S-S; disulfide bridge).

Chapter 2

Construction of a single-chain variable fragment with improved affinity to aflatoxin B_1

2.1. SUMMARY

Aflatoxin B_1 (AFB_1) produced in *A. flavus* is a major hepatocarcinogen found in foods and feed. For effective immunological detection of AFB_1 at low concentrations, the development of high affinity antibody for AFB_1 is required. Previously, an affinity improved single-chain variable fragment containing 6 mutations (scFv-M37) was isolated from an artificial mutagenic library using yeast surface display combined fluorescence-activated cell sorting, which showed a 9-fold higher affinity than its wild type scFv (Min et al., 2015). When compared with the wild type scFv, the scFv-M37 showed an increased association rate constant (k_a) and an decreased dissociation rate constant (k_d), which was ascribed to 6 mutations that are located in a complementarity determining region (CDR: 1 residue), framework regions (FRs: 4 residues) and a linker (Lk: 1 residue) of the scFv-M37. Even though 6 mutated residues contributed overall to the affinity improvement, it was not identified which mutation brought positive or negative effects on an affinity improvement since the 6 mutations in the scFv-M37 were generated by random mutagenesis using error-prone PCR.

In this study, the effect of the 6 mutated residues on the affinity improvement was characterized using surface plasmon resonance analysis, which identified a deleterious mutation (V_H-A110T) located on a framework region of the scFv-M37. The back mutation of V_H-A110T resulted in a 3.2-fold affinity improvement (Fig 2-1), which was attributed to a decrease of dissociation rate constant (k_d) in the interaction between AFB_1 and the back mutant scFv. The biophysical analyses using circular dichroism and gel filtration revealed that the back mutation of V_H-A110T caused a subtle conformational change of the scFv toward tighter binding to AFB_1.

2.2. MATERIALS AND METHODS

2.2.1. Strains, plasmids and site-directed mutagenesis

Bacterial strains and plasmids used in this study are listed in Table 2-1. *E. coli* DH5α and *E. coli* BL21(DE3) were used for genetic manipulation and protein expression, respectively. The site-directed mutagenesis to construct expression plasmids for the back mutants of scFv-M37 was carried out according to the instruction of the QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) with the oligonucleotides (Table 2-2) and pET26-scFv-M37 as a template.

2.2.2. Expression and purification of scFv proteins

The recombinant *E. coli* BL21(DE3) cells harboring each expression plasmid were cultured at 37 ℃ in a 500-mL baffled flask (Duran Schott, Mainz, Germany) containing M9-glucose medium supplemented with 50 mg/L kanamycin. Expression of each gene was induced at the logarithmic growth phase (OD_{600} = 0.5 ~ 1.0) by adding final 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the cells were grown

for an additional 12 h at 25 ℃. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (Optizen POP; Mecasys, Daejon, Korea). The soluble scFv protein secreted into culture medium was purified by nickel affinity chromatography. The culture broth collected by centrifugation at 6,000 rpm for 10 min was injected to a HisTrap FF column (GE healthcare, Piscataway, NJ) equilibrated with binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole). After washing unbound proteins with 10 column volumes of the biding buffer, the scFv bound to the resin was eluted with stepwise gradient of an elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 500 mM imidazole). The elution fractions were pooled and dialyzed against a binding buffer (20 mM sodium phosphate, pH 6.5) and then subjected to cation exchange chromatography using the HiTrap SP FF column (GE healthcare, Piscataway, NJ). The scFv proteins bound to the cation exchange resin (SP Sepharose FF) were eluted by linear gradient of NaCl (0-1.0 M). The purity of the scFv protein in the eluents was determined by SDS-PAGE and densitometric analyses.

2.2.3. Surface plasmon resonance analysis

The affinity of the scFvs to AFB_1 (K_D) was measured by the surface plasmon resonance (SPR) method using a BIAcore 2000 (GE heathcare, Uppsala, Sweden). The pH scouting and immobilization of AFB_1-BSA conjugate were carried out in the Fc-2 of a CM5 sensor chip (GE heathcare, Uppsala, Sweden) activated by EDC/NHS, according to the manufacturer's instruction. The AFB_1-BSA conjugate (100 g/mL in 50 mM sodium acetate buffer (pH 4.5)) was fed into the activated Fc-2 at a flow rate of 5 L/min and the Fc-2 was washed with 50 mM NaOH for removing the non-specifically cross-linked AFB_1-BSA conjugate.

Immobilization of BSA onto the activated Fc-1 followed the same procedure as above. The scFvs in the reaction buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20) were injected at 20 L/min flow rate in the periods of association and dissociation. The sensorgrams were analyzed using the BIAevaluation software 3.1 (GE heathcare, Uppsala, Sweden). The equilibrium dissociation constant (K_D) was defined as the ratio of the dissociation rate constant (k_d) to the association rate constant (k_a).

2.2.4. Structure modeling of the scFv-M37

The homology model of the scFv-M37 was generated by MODELER in Discovery Studio 4.3 (Biovia, San Diego, CA). The anti-cytochrome C oxidase Fv fragment crystal (PDB 1MQK), the Fab fragment crystal of a murine IgG1 (PDB 1GIG) and the anti-carbohydrate Fab crystal (PDB 1F4W) were used as templates for modeling the heavy chain (V_H), light chain (V_L) and the entire scFv-M37, respectively. The sequence similarity between each template and the scFv-M37 were of 97.8% for V_H, 96.6% for V_L and 94.4% for the entire scFv-M37.

2.2.5. Gel filtration chromatography analysis

Analytical gel filtration chromatography was performed on an ÄKTA Prime Plus (GE Healthcare, Uppsala, Sweden) using a Superdex 200 HR 10/30 column (GE Healthcare, Piscataway, NJ). The purified scFv was injected to the column that was equilibrated with 20 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl and separated at a flow rate of 0.5 mL/min at ambient temperature.

2.2.6. Competitive indirect ELISA assay

The 96-well ELISA plates were coated overnight at 4 ℃ with 0.2 g of AFB_1-BSA conjugate. The coated plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with a blocking buffer (5% skim milk in PBS). After washing the plates with PBST, one hundred μL of purified scFvs (2 μg/mL) and 100 μL of aflatoxin derivatives (AFB_1, AFG_1, AFG_2, and AFM_1) and mycotoxins (OTA, FMB_1, FMB_2, DON and T-2 toxin) in a dilution series (0 to 10 μg/mL) were added consecutively to each well. After addition of anti-His IgG conjugated horse radish peroxidase (HRP), the plates were incubated for 1 h at room temperature, followed by washing with PBST. The plates were developed by adding 100 μL of HRP substrate (Sigma), according to the manufacturer's instructions. The optical density at 450 nm was read on a microplate reader (Molecular Devices). The specificities for other aflatoxin derivatives and mycotoxins were calculated according to the following equation: [Cross-reactivity = (IC_{50} AFB_1/IC_{50} aflatoxins or IC_{50} mycotoxins) × 100].

2.2.7. Circular dichroism (CD) analysis

Far-UV Circular dichroism (CD) measurements were carried out with an automated Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). The spectrum was measured over a wavelength range of 260 to 190 nm using a 2-mm pathlength. The temperature was maintained at 25 ℃. The background CD spectrum of the buffer was subtracted from that of the protein sample to obtain the spectrum of scFv protein. The thermostability was monitored through the change in ellipticity at 200 nm as a function of temperature over the range from 25 to 75 ℃. The ellipticity was measured at 1 ℃ intervals with an equilibration time of 1 min at each temperature. Data points were collected at each interval for 5 sec.

2.3. RESULTS AND DISCUSSION

2.3.1. Extracellular production of scFv-M37 mutants

The 6 mutated residues of the scFv-M37 are distributed on framework regions (FRs) and a linker, as well as a complementarity-determining region (CDR) (Fig. 2-2 and 2-3). To examine the contribution of individual mutations of the scFv-M37 to the affinity improvement, total 6 back mutants were constructed via site-directed mutagenesis for pET26-scFv-M37, where the scFv gene is intervened with the genes of the N-terminal pelB signal peptide and the C-terminal 6-histidine tag for the periplasmic expression and the Ni-affinity purification, respectively. When expressed in *E. coli*, all the scFv proteins accumulated mainly as inclusion bodies in periplasm (data not shown). The soluble scFv proteins were found in culture media. This extracellular secretion of soluble scFv into culture medium was frequently observed for many other proteins expressed in the periplasm of *E. coli* (Choi et al., 2004). Therefore, scFvs were purified from the culture media using Ni-affinity chromatography. Because of the co-purification of *E. coli* cellular proteins binding to Ni-Sepharose resin (Fig. 2-4 (B), lane 'N'), the elution fractions of the Ni-affinity

chromatography were pooled and then subjected to cation exchange chromatography to increase their purities. Most scFv proteins was electrostatically bound to the cation exchange resin and then eluted by increasing NaCl concentrations (Fig. 2-4). All the scFv proteins were purified with at least 90% purity based on the SDS-PAGE and the gel filtration chromatography analyses (Fig. 2-8).

2.3.2. Affinity analyses for scFv-M37 back mutants

The purified scFv proteins were subjected to SPR analysis to compare their affinities to AFB_1. The SPR analyses using a BIAcore system have been used to determine binding kinetics and a K_D value of the purified scFv proteins, which is widely used to describe the binding affinity of antibodies. Generally, a K_D value obtained in SPR assay is inversely correlated with the antigen-binding affinity of an antibody. While the K_D value of the scFv-M37 was determined to be 9.2×10^{-8} M, the K_D values of the 6 back mutants were in a range of 2.7 to 27.7×10^{-8} M, representing 0.3 to 3.2-fold change in the affinity to AFB_1 compared with the scFv-M37 (Fig. 2-5 and Table 2-3). As expected, the back-mutation of L_k-S5P in the flexible $(Gly_4Ser)_3$ linker between the heavy

and light chain variable regions did not change the affinity of the scFv to AFB_1. However, the back mutation of V_L-A57V located in CDR-II of the light chain resulted in the most significant reduction of the affinity among the 6 back-mutations, suggesting that the V_L-A57V mutation was pivotal in the affinity improvement of the scFv-M37.

The back mutations in the FRs also caused the affinity decreases as observed in the scFv-BM1, BM2 and BM4 containing the back mutations of V_H-T28P, V_H-A94V and V_L-E40V, respectively. However, the scFv-BM3 carrying the back mutation of V_H-A110T in FR-IV of the heavy chain showed a 3.2-fold higher affinity than the scFv-M37, for which k_a was not changed but k_d was decreased from 1.9×10^{-3} s^{-1} to 0.6×10^{-3} s^{-1}. When compared with the scFv-WT, 31-fold affinity improvement was measured (Table 2-3). The K_D value of the scFv-BM3 was determined to be 2.7×10^{-8} M, which means that the scFv-BM3 is capable of detecting aflatoxin B_1 at a concentration of 27 nM.

These results suggest that the negative effect of V_H-A110T was compensated by the positive contributions of V_H-T28P, V_H-A94V, V_L-E40V and V_L-A57V mutations in the scFv-M37. The V_H-A110T mutation likely arose at an early stage during error-prone PCR and might be preserved in a large fraction of the scFv mutant clones,

because it is one of the 4 common mutated residues present in high affinity scFv clones isolated from the random mutagenic library of scFv-WT (Min et al., 2015).

Despite the additional affinity improvement by the V_H-A110T back mutation, it affected little the high specificity for AFB_1. As shown in the competitive indirect ELISA assay of the scFv-BM3 (Fig. 2-6), it exhibited low levels of cross-reactivities to other aflatoxin derivatives (AFG_1, AFG_2, AFM_1 and AFM_2) and other mycotoxins (OTA, FMB_1, FMB_2, DON and T-2) at a concentration range of 0.1 to 1,000 ng/ml, which was comparable to the results of the scFv-WT and M37 (Min et al., 2011; Min et al., 2015).

The detection limits for the aflatoxin B_1 contamination were 7 nM of mAb, 52 nM of other scFvs and 27 nM of scFv-BM3 respectively. When comparing with the mAb, the scFv-BM3 showed relatively low affinity to aflatoxin B_1, but exhibited similar affinity to aflatoxin B_1 with other scFvs.

2.3.3. Comparison of the scFv-M37 and the scFv-BM3

In the homology model of scFv-M37 (Fig. 2-2), the mutated residues responsible for the affinity improvement were located in CDR-III of the light chain or Vernier zones close to CDRs (Min et al., 2015). However, the V_H-A110 residue is not close to CDRs and even located at the opposite site of CDRs in the scFv-M37 homology model (Fig. 2-2). It has been reported that mutations in FRs indirectly affect the conformation of CDRs, which in turn contributed to the affinity enhancement (Baek et al., 2015; Leyton et al., 2009). In order to verify whether the V_H-A110T back mutation affected the conformation of the scFv, the secondary structure of the scFv-M37 and BM3 were analyzed using circular dichroism spectrometry. Both the scFvs showed very similar CD spectra exhibiting positive and negative peaks of molar ellipticity at around 200 and 216 nm, respectively, suggesting that the conformational change caused by the back mutation of V_H-A110T was subtle (Fig. 2-7 (A)). These peaks in the CD spectra are indicative of the proteins rich in the antiparallel β-sheet as previously reported for many scFvs and antigen-binding fragments (Pini et al., 1997; Pledger et al., 1999; Vendel et al., 2012).

To ascertain whether the thermostability of the scFvs was affected by the back mutation of V_H-A110T, the temperature-induced denaturation properties of the scFvs were studied using CD spectrometry. Since a significant decrease of CD signal at 200 nm was observed in the CD spectrum analysis of the scFv-BM3 at 75 ℃ (Fig. 2-7 (A)), thermal denaturation profiles of the scFvs were monitored at 200 nm to determine the temperature of unfolding transition (T_m). Overall thermal denaturation profiles of the two scFvs were similar each other in a range from 25 to 75 ℃, exhibiting a gradual decrease of CD signal near at 45 ℃, and then a sharp sigmoidal decrease due to the protein aggregation (Fig. 2-7 (B)). A slight decrease of thermostability was observed in the scFv-BM3, of which the T_m value was determined to be about 50 ℃, which is 2 ℃ lower than the scFv-M37, representing a partial destabilization of the scFv-BM3 by the back mutation.

Analytical gel filtration chromatography was used to assess the effect of the back mutation on the folded state of the scFvs. Both scFvs eluted as single peaks close to that expected for a monomer (Fig. 2-8). A small difference between the two scFvs was that the scFv-BM3 exhibited a slight shoulder close to the peak. There was no detectable peak for oligomers that are frequently observed in gel filtration analysis of many

recombinant antibodies including scFv (Dolezal et al., 2003; Vendel, et al., 2012). These results also indicate that the scFvs produced in this study are homogeneously folded proteins as a monomeric form. ScFv-BM3 is 3.2-fold more sensitive to AFB_1 than scFv-M37 through back mutation of Ala by Thr at the position of V_H:110 in scFv-M37. In the structural aspect, its position of V_H:110 is known to be the FR-IV region, which connects the variable region with the constant region and Thr at the position of V_H:110 is more close to the aflatoxin B_1 binding pocket than Ala in superposition of the scFv-M37 and scFv-BM3 (Fig. 2-9), so mutation from Thr to Ala in 110 amino acid residue influences an affinity improvement of scFv-BM3 via conformational structure of CDRⅢ-V_H.

Table 2-1. Bacterial strains and plasmids used in this study.

Strain and plasmid	Relevant characteristic or construction	Reference
Strains		
E. coli DH5α	F^- (Φ80Δ*lac*ZΔM15) *rel*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17(r_K^-,m_K^+) *sup*E44 Δ(lacZYA-argF) U169	Invitrogen
E. coli BL21(DE3)	F^- *ompT hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmids		
pET26-scFv-M37	anti-AFB_1-scFv-M37, 6 mutations (V_H-T28P, V_H-A94V, V_H-T110A, L_k-S5P, V_L-E40V, V_L-A57V)	Min et al., 2015
pET26-scFv-BM1	anti-AFB_1-scFv-BM1, back mutation of V_H-Pro28 in scFv-M37 to Thr	This study
pET26-scFv-BM2	anti-AFB_1-scFv-BM2, back mutation of V_H-Val94 in scFv-M37 to Ala	This study
pET26-scFv-BM3	anti-AFB_1-scFv-BM3, back mutation of V_H-Ala110 in scFv-M37 to Thr	This study
pET26-scFv-BM4	anti-AFB_1-scFv-BM4, back mutation of L_k-Pro5 in scFv-M37 to Ser	This study
pET26-scFv-BM5	anti-AFB_1-scFv-BM5, back mutation of V_L-Val40 in scFv-M37 to Glu	This study
pET26-scFv-BM6	anti-AFB_1-scFv-BM6, back mutation of V_L-Val57 in scFv-M37 to Ala	This study

Table 2-2. Oligonucleotides for site-directed mutagenesis used in this study.

Clone	Residue for back mutation	Oligonucleotide	Nucleotide sequence *
scFv-BM1	V_H-T28P	BM1-F	GCAGCCTCTGGATTCACTTTCAGTACCTATGCC
		BM1-R	GGCATAGGTACTGAAAGTGAATCCAGAGGCTGC
scFv-BM2	V_H-A94V	BM2-F	GCCATGTATTACTGTGCAAGCCATGGCCTACTA
		BM2-R	TAGTAGGCCATGGCTTGCACAGTAATACATGGC
scFv-BM3	V_H-T110A	BM3-F	TGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
		BM3-R	TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCA
scFv-BM4	L_k-S5P	BM4-F	TCCTCAGGTGGCGGTGGCTCCGGCGGTGGTGGCAGCGGT
		BM4-R	ACCGCTGCCACCACCGCCGGAGCCACCGCCACCTGAGGA
scFv-BM5	V_L-E40V	BM5-F	GCCAACTGGGTCCAAGAAAAACCAGATCATTTA
		BM5-R	TAAATGATCTGGTTTTTCTTGGACCCAGTTGGC
scFv-BM6	V_L-A57V	BM6-F	GGTACCAACAACCGAGCTCCAGGTGTTCCTGCC
		BM6-R	GGCAGGAACACCTGGAGCTCGGTTGTTGGTACC

The underlined sequences correspond to the amino acid for each back mutation.

Table 2-3. Affinity and binding kinetics of purified scFv antibodies.

scFv clone	Residue for back mutation	K_D ($M^{-1} \times 10^{-8}$)	k_a ($M^{-1}\ s^{-1} \times 10^{-4}$)	k_d ($s^{-1} \times 10^{-3}$)	Fold improvement	Chi^2 value*	Reference
Wild-type	-	82.7	0.7	6.0	1	3.01	Min et al., 2015
M37	-	9.2	2.1	1.9	9	2.98	Min et al., 2015
BM1	V_H-P28T	14.2	4.0	5.6	5	1.57	This study
BM2	V_H-V94A	16.0	2.8	4.4	8	3.54	This study
BM3	V_H-A110T	2.7	2.1	0.6	31	2.10	This study
BM4	L_k-P5S	8.5	2.1	1.8	10	0.49	This study
BM5	V_L-V37E	22.6	0.6	1.4	3	3.99	This study
BM6	V_L-V54A	27.7	0.8	2.3	3	0.29	This study

Chi^2 values represent information about the goodness of fitting in generating theoretical sensorgram data.

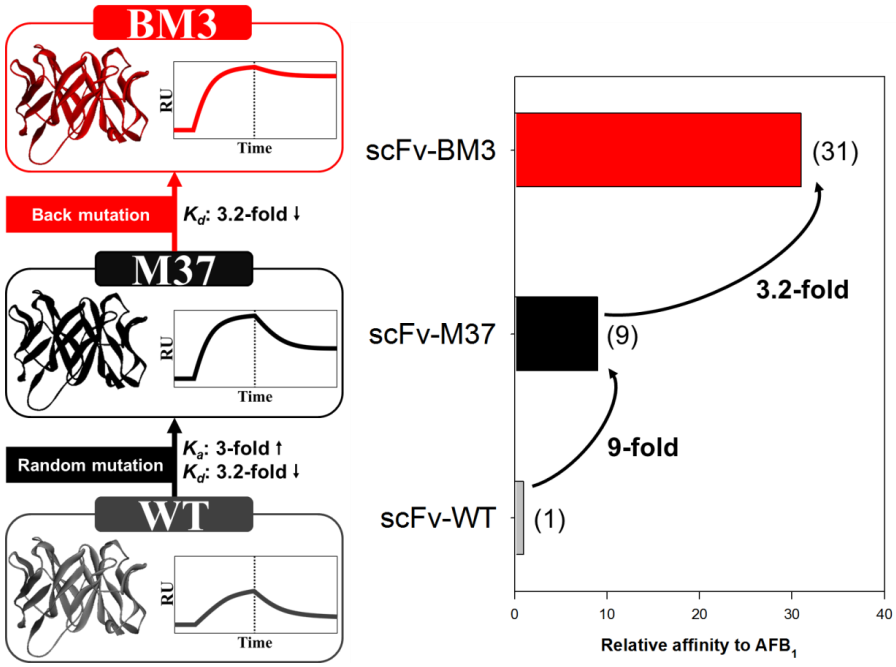


Figure 2-1. Schematic representation depicting affinity improvements of scFv-M37 and BM3. The affinity improvement of the scFv-M37 was due to both 3-fold increase of k_a and 3.2-fold decrease of k_d (Min et al., 2015). The additional 3.2-fold decrease of k_d in the scFv-BM3 resulted in a 31-folds affinity improvement of the scFv-BM3 compared with its wild type. The numbers in parentheses of the graph refer relative affinity to AFB_1.

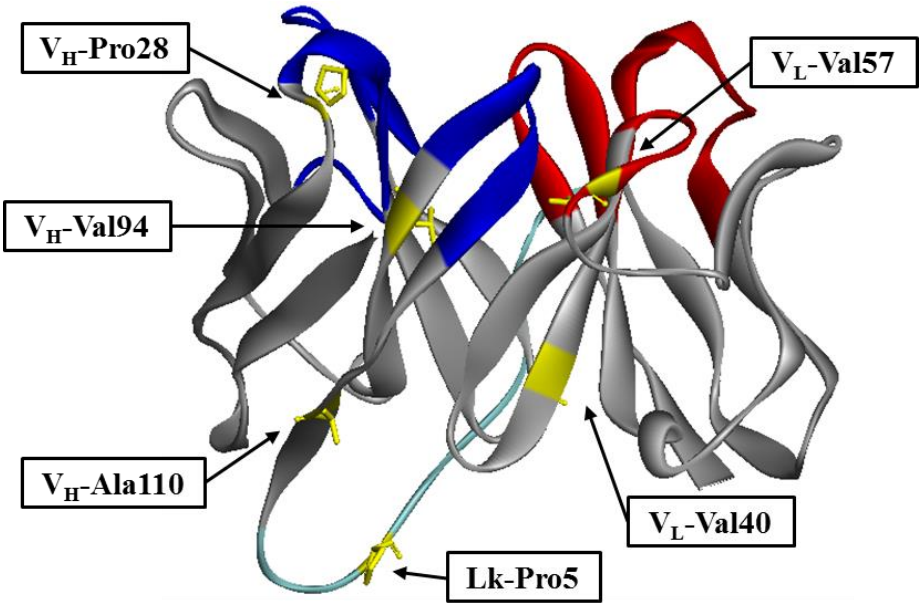


Figure 2-2. Homology model structure of scFv-M37. FRs are colored grey, heavy chain CDRs blue, light chain CDRs red and linker blue green. The residues for back mutation are represented as stick model in yellow color.

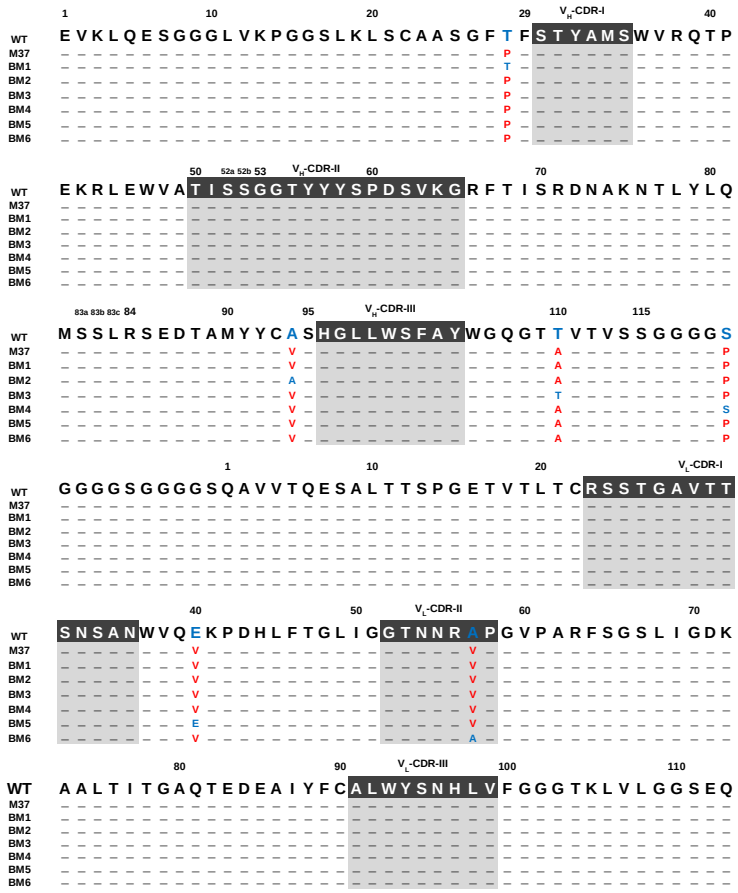
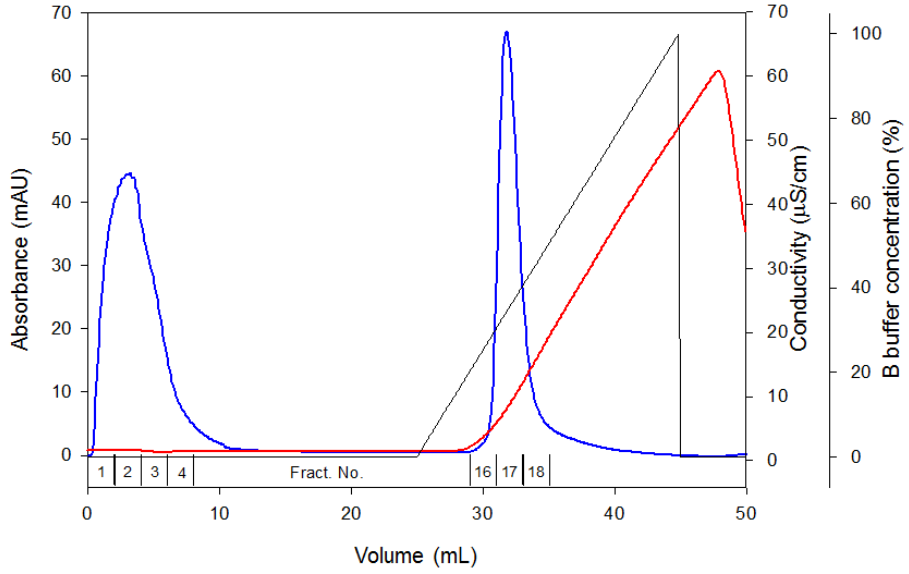


Figure 2-3. Protein sequence alignment of scFv-WT, M37, and 6 back mutants (scFv-BM1 to BM6). Antibody numbering, and all CDRs and FRs of the V_H and V_L domains were determined according to Chothia antibody sequence database's rule (Al-Lazikani et al., 1997). The different residues were presented as one letter code of amino acid and the same amino acids were presented as hyphen (-).The underlined sequences correspond to glycine and serine residues in synthetic $(Gly_4Ser)_3$ linker between V_H and V_L.

(A)


Absorbance (mAU)
Conductivity (µS/cm)
B buffer concentration (%)
Fract. No.
1
2
3
4
16
17
18
Volume (mL)

(B)

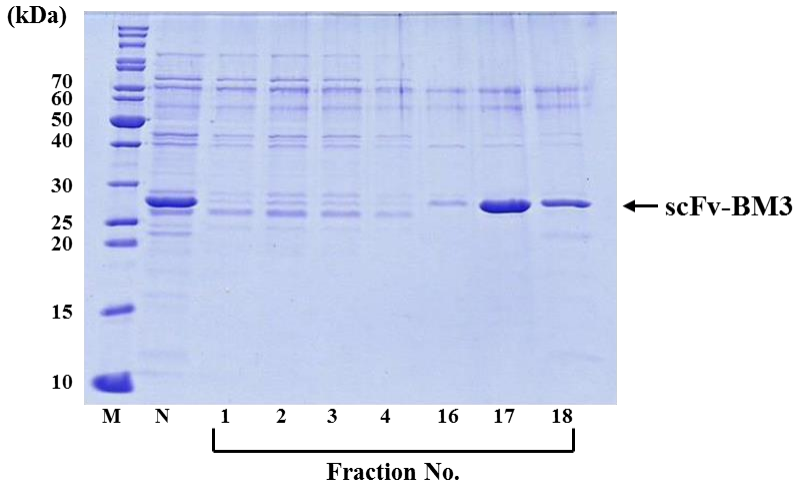

(kDa)
70
60
50
40
30
25
20
15
10
M
N
1
2
3
4
16
17
18
Fraction No.
scFv-BM3

Figure 2-4. Purification of scFv-BM3. (A) Cation exchange chromatography with SP Sepharose FF. The blue, black, red lines indicate the absorbance at 280 nm, the elution buffer concentration (1 M NaCl) and conductivity, respectively. (B) SDS-PAGE analysis for the elution fractions. Lane M and N denote marker proteins and the sample injected to the column which is the pooled elution fraction in Ni-affinity chromatography.

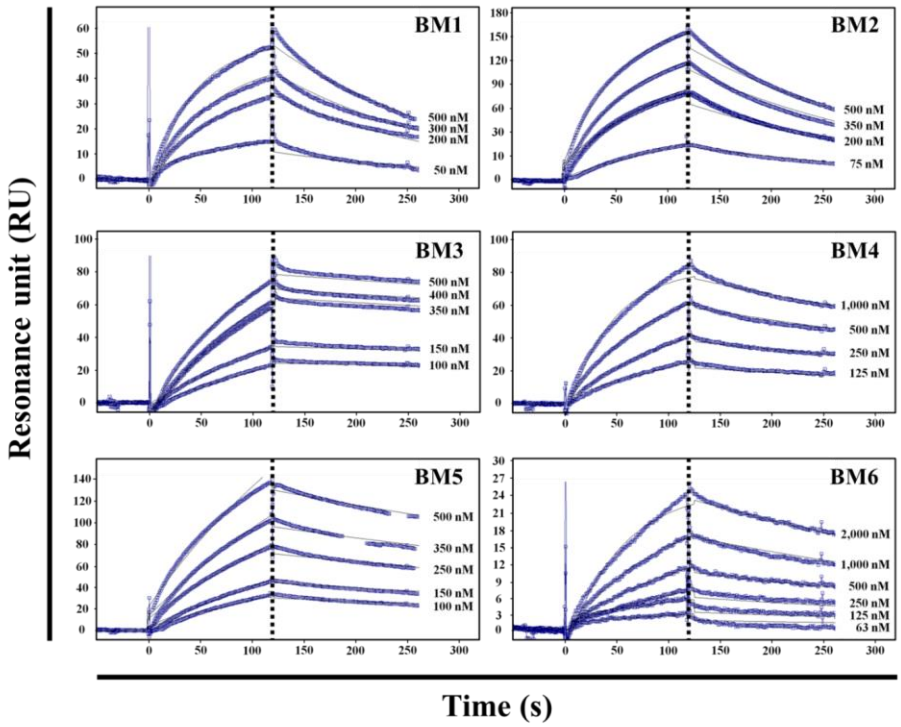


Figure 2-5. Sensorgrams for binding of scFv-M37 back mutants to AFB_1. Various concentrations of purified scFv-M37 back mutants (scFv-BM1 to BM6) as analyte were injected into the AFB_1-BSA conjugate immobilized on CM5 chip in BIAcore. Each data was monitored by BIAevaluation software 3.1.

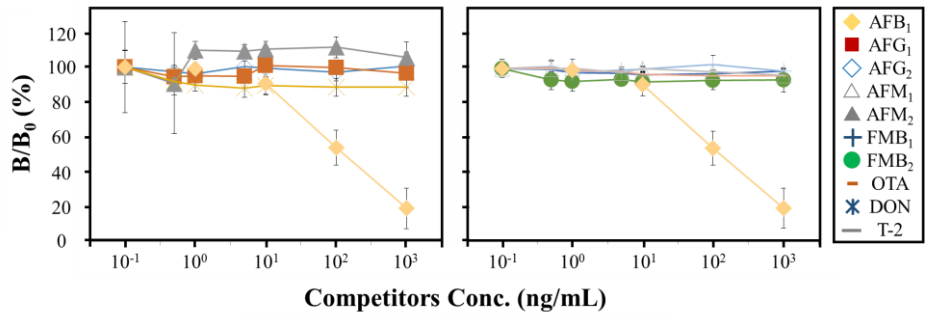


Figure 2-6. Specificity assay of scFv-BM3 using competitive indirect ELISA. For AFB derivatives (left panel), a range of 0.1-1,000 ng/mL of AFB_1, aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2) were used as competitors. For other mycotoxins (Right pannel), 0.1-1,000 ng/mL of fumonisin B_1 (FMB_1), fumonisin B_2 (FMB_2), deoxynivalenol (DON), ochratoxin (OTA) and T-2 toxin were used. B_0 is an absorbance at 450 nm without competitors and B is an absorbance at 450 nm with various concentrations of each competitor.

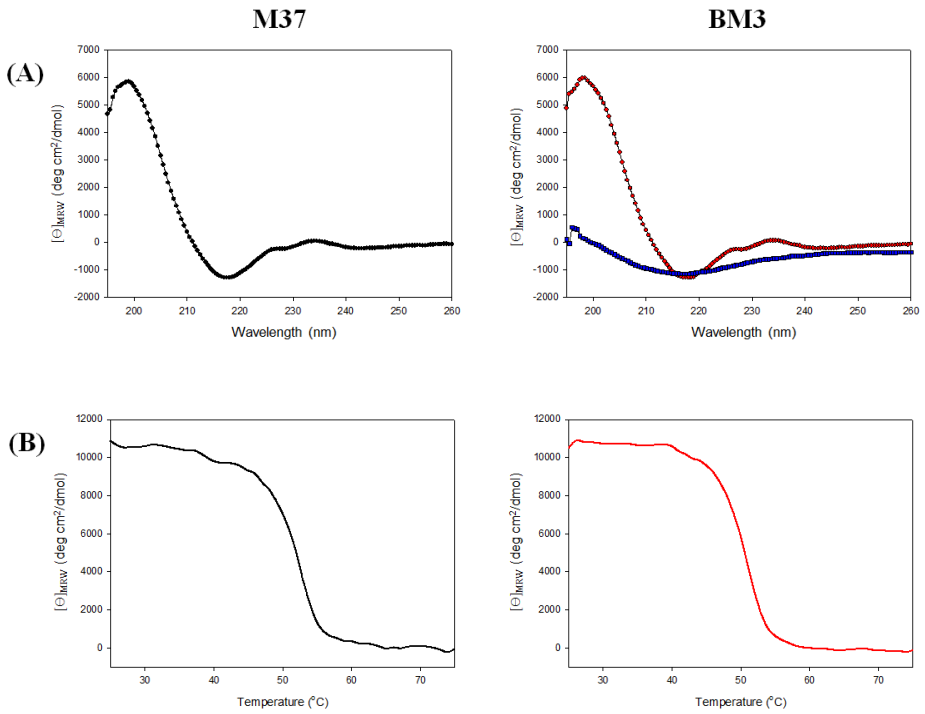


Figure 2-7. Far-UV circular dichroism spectra (A) and thermal denaturation profiles of scFv-M37 and BM3 (B). The CD signal was converted to the mean residue ellipticity and plotted versus the wavelength or the temperature. Thermal denaturation was monitored at 200 nm in a range from 25 ℃ to 75 ℃.

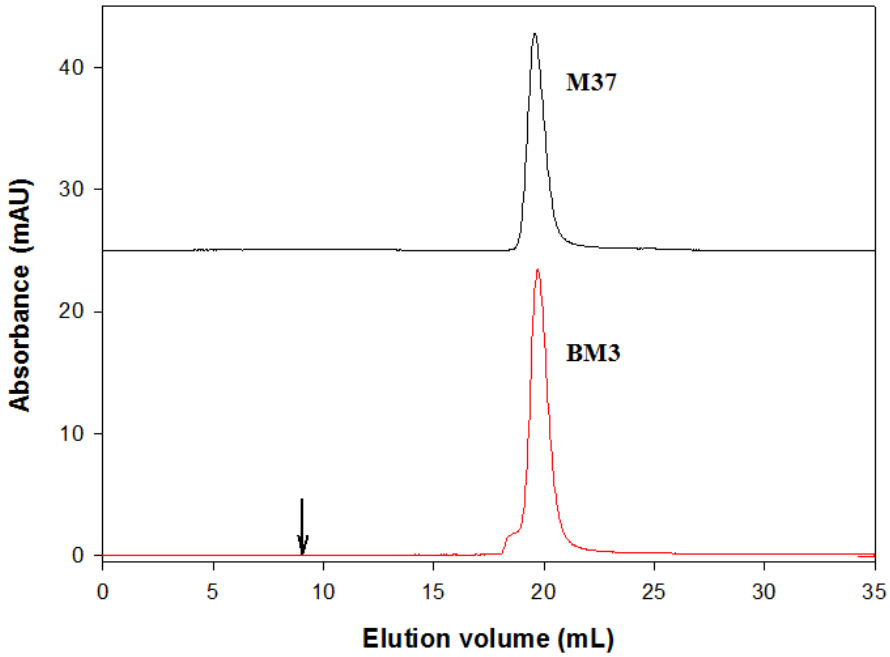


Figure 2-8. Analytical gel filtration chromatography for scFv-M37 and BM3. The column volume of the gel filtration column (Superdex 200 10/300 GL) was 24 mL. The void volume (V_0) determined with blue dextran was 9.05 mL as indicated by the arrow.

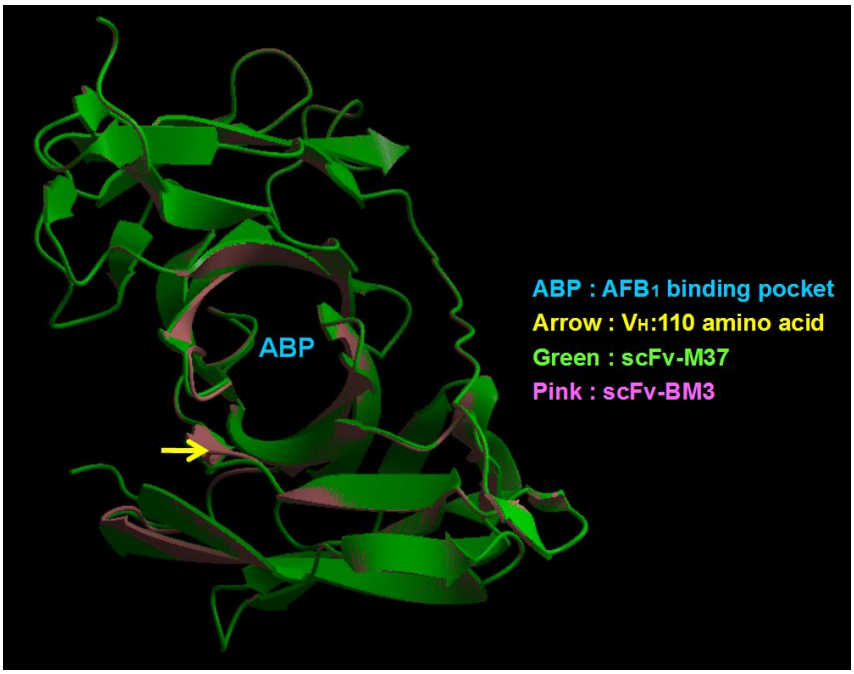


Figure 2-9. Superposition of scFv-M37 and scFv-BM3. The green colored structure is scFv-M37 and the pink colored structure is scFv-BM3.

Chapter 3

Effective production of the functional scFv against aflatoxin B_1 using recombinant *Escherichia coli*

3.1. SUMMARY

ScFv-BM3 is a single-chain variable fragment (scFv) against aflatoxin B_1 (AFB_1) engineered by affinity maturation and site-directed mutagenesis, and thus has a 31-fold higher affinity than its wild-type. In order to apply scFv-BM3 to immunological detection of AFB_1, periplasmic expression in *E. coli* was attempted to produce a functional form of scFv-BM3. ScFv-BM3 accumulated as inactive aggregates in the cells. However, it was found that the scFv-BM3 secreted into the culture medium had binding activity to AFB_1. Expression conditions for scFv-BM3 were further manipulated to enhance secretion into the culture medium. This extracellular secretion of functional scFv-BM3 was significantly improved by supplementation with Triton X-100 and optimization of expression conditions. The scFv-BM3 purified from the culture medium exhibited a typical anti-parallel β-sheet structure and adopted a proper conformation to bind AFB_1 with high affinity and specificity in various biophysical and biochemical analyses.

3.2. MATERIALS AND METHODS

3.2.1. Strains and plasmids

E. coli BL21(DE3) was used for protein expression. Since the scFv-BM3 gene was cloned into pET26b at the *Nco*I and *Xho*I sites, the PelB signal peptide and the 6-histidine tag were fused to scFv-BM3 at the N-terminus and the C-terminus, respectively (Min et al., 2016). The plasmid pET26-scFv-BM3 was transformed into *E. coli* BL21(DE3) for expression of scFv-BM3. ScFv-BM3 was expressed under the control of the T7 promoter, which can be induced by adding isopropyl- β-D-thiogalactopyranoside (IPTG).

3.2.2. Expression and purification of the scFv-BM3

The recombinant *E. coli* BL21(DE3) cells harbouring each expression plasmid were cultured at 37 ℃ in a 500-mL baffled flask (Duran Schott, Mainz, Germany) containing M9-glucose medium supplemented with 50 mg/L kanamycin. The expression of each gene was induced at the logarithmic growth phase (OD_{600} = 0.5 ~ 1.0) by adding IPTG at a final concentration of 0.1 mmol/L, and the cells were grown for an

additional 12 h at 25 ℃. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (Optizen POP; Mecasys, Daejon, Republic of Korea). The soluble scFv protein secreted into culture medium was purified by Ni-affinity chromatography. The culture broth collected by centrifugation at 4,300 *g* for 10 min was injected to the HisTrap FF column (GE healthcare, Piscataway, NJ, USA) equilibrated with binding buffer (20 mmol/L sodium phosphate, pH 7.4, 500 mmol/L NaCl, 20 mmol/L imidazole). After washing unbound proteins with 10 column volumes of the binding buffer, the scFv-BM3 bound to the resin was eluted with stepwise gradient of elution buffer (20 mmol/L sodium phosphate, pH 7.4, 500 mmol/L NaCl, 500 mmol/L imidazole). The elution fractions were pooled and dialyzed against binding buffer (20 mmol/L sodium phosphate, pH 7.0) and then subjected to cation exchange chromatography using a HiTrap SP FF column (GE healthcare, Piscataway, NJ, USA). The scFv-BM3 bound to the cation exchange resin (SP Sepharose FF) was eluted by linear gradient of NaCl (0 ~ 500 mmol/L).

3.2.3. Western blotting analysis

The extracellular secretion of scFv-BM3 into the culture medium was confirmed by Western blot analysis with a mouse anti-6-histidine tag IgG (Abcam, Cambridge, UK) and a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad, Hercules, CA, USA). The bands of scFv-BM3, which specifically reacted with the antibodies, were visualized by the Opti-4CN substrate and a detection kit (Bio-Rad, Hercules, CA, USA).

3.2.4. Enzyme-linked immunosorbent assay (ELISA)

The principle of ELISA analysis for scFv-BM3 is schematically described in Fig. 3-1. A 96-well ELISA microplate was coated with 100 μL of 2 μg/mL AFB_1-BSA conjugate in 25 mmol/L carbonate buffer (pH 9.6) at 4 ℃ overnight, washed 3 times using phosphate buffered saline with 0.05% Tween-20 (PBST), and blocked using 2% BSA in PBST. Subsequently, 100 μL of samples containing scFv-BM3 were added to the wells and incubated for 1 h at 37 ℃. After washing the microplate 3 times, 100 μL of HRP-conjugated anti-6-histidine tag antibody (Abcam, Cambridge, UK) of 1 μg/mL was added and the

plates were incubated for 1 h at 37 ℃. The microplate was developed by adding 100 μL of 3,3',5,5'-Tetramethylbenzidine (TMB) supplemented with H_2O_2 (Sigma Aldrich, St. Louis, MO, USA). After H_2SO_4 of 1 mol/L solution was added to each well, the absorbance at 450 nm was read on a microplate reader (PowerWave, BioTek, Winooski, VT, USA).

3.3. RESULTS AND DISCUSSION

3.3.1. Insoluble expression of the scFv-BM3 in *E. coli*

Since most scFvs contain two intramolecular disulfide bonds, which are required for their correct folding, the PelB signal peptide was fused to the N-terminus of scFv-BM3 and then expressed in *E. coli* BL21(DE3) for secretion into the periplasm of oxidative conditions that are favorable for disulfide bond formation. However, most of the scFv-BM3 was expressed as insoluble inclusion bodies (Fig. 3-2 (A)). The apparent molecular weight of the scFv-BM3 inclusion bodies was approximately 26 kDa, which is nearly identical to its theoretical molecular weight of the signal peptide-less scFv-BM3 (26.2 kDa), indicating that the scFv-BM3 was exported into the periplasm and then aggregated as insoluble inclusion bodies. When the nickel (Ni) affinity chromatography was attempted to purify the scFv-BM3 containing the C-terminal 6-histidine tag in the soluble fraction at a low proportion (Fig. 3-2, lane S), the scFv-BM3 was not purified (Fig. 3-3). In addition, there was no detectable increase in absorbance at 450 nm for the soluble fraction in the ELISA analysis using the AFB_1-bovine serum albumin (BSA) conjugate and the horseradish peroxidase (HRP)-

conjugated anti-6-histidine tag IgG (Fig. 3-6), indicating that there was no functional scFv-BM3 in *E. coli*.

3.3.2. Extracellular secretion of the functional scFv-BM3

The expression of scFv-BM3 in the *E. coli* periplasm was not suitable for the production of functional scFv-BM3. Since many functional recombinant proteins expressed in *E. coli* have been purified from culture media (Gupta et al., 2016), the culture medium of *E. coli* BL21(DE3)/pET26-scFv-BM3 was subjected to Western blotting analysis using the HRP-conjugated anti-6-histidine tag IgG to verify its extracellular secretion. The 26 kDa band of scFv-BM3 was detected by developing with the HRP substrate, indicating the extracellular secretion of scFv-BM3 in the culture medium (Fig. 3-2 (B)). In addition, an obvious increase in absorbance at 450 nm was observed for the culture medium in the ELISA analysis, which is contrary to the negligible increase in absorbance for the soluble fraction (Fig. 3-6). These results clearly demonstrated that the scFv-BM3 secreted into culture medium was a functional form able to bind to AFB_1.

It has been reported that the supplementation of Triton X-100 or glycine to culture medium facilitates the extracellular secretion of target proteins from *E. coli* cells (Yang et al. 1998; Holm et al. 2007). Therefore, various concentrations of Triton X-100, Tween 80, Tween 20 and glycine were added to the culture medium upon isopropyl-β-$_{D}$-thiogalactopyranoside (IPTG) induction of scFv-BM3. The addition of Triton X-100 improved the extracellular secretion of functional scFv-BM3 compared to other additives (Fig. 3-4). In addition, when comparing culture media (M9 or LB medium) for extracellular production of scFv-BM3, the expression level of scFv-BM3 to the culture medium was improved when the M9 medium was used (Fig. 3-5). This result might be due to the properties of ingredients in complex LB medium, since trypton and yeast extract maintained the integrity of the outer membrane of *E. coli*.

The optimal concentration of Triton X-100 for the extracellular production of scFv-BM3 was determined to be 0.25% in the ELISA analysis (Fig. 3-6). However, the addition of glycine decreased the extracellular production of scFv-BM3 compared with the control (Fig. 3-7). While glycine induces swelling *E. coli* cells and enlargement of the periplasmic space by interfering with peptidoglycan synthesis,

Triton X-100 disrupts the integrity of the outer membrane (Yang et al., 1998; Bao et al., 2016). Triton X-100 did not significantly improve the refolding yield of scFv-BM3 (Fig. 3-8), suggesting that the enhanced extracellular production of scFv-BM3 was not due to the artificial chaperone effect of Triton X-100 (Yazdanparast et al., 2007; Wang et al., 2010). The addition of 0.25% Triton X-100 to the culture medium upon IPTG induction resulted in a slight decrease (7%) of colony forming units (CFU), indicating that cell lysis by Triton X-100 was not significant at this concentration (Fig. 3-9). Therefore, the supplementation with 0.25% Triton X-100 might weaken the integrity of the outer membrane, resulting in the improved leakage of scFv-BM3 from the periplasmic space into the culture medium. For further improvement of the extracellular production of scFv-BM3, temperatures and IPTG concentrations were optimized. The optimal temperature and IPTG concentration for the extracellular production of scFv-BM3 were 25 ℃ and 0.1 mmol/L, respectively (Fig. 3-10).

3.3.3. Purification of the scFv-BM3 secreted into culture medium

The culture medium of the recombinant *E. coli* cells expressed with the addition of 0.1 mmol/L IPTG and 0.25% Triton X-100 at 25 ℃ was subjected to Ni-affinity chromatography to purify the scFv-BM3 secreted into the culture medium. Although the band of scFv-BM3 in the culture medium was indistinguishable because of its low secretion level, the distinct band of scFv-BM3 in the elution fraction was clearly observed in the SDS-PAGE gel, indicating that scFv-BM3 of ca. 26 kDa was bound to the Ni-sepharose FF resin and eluted by increasing imidazole concentration (Fig. 3-11 (A)). However, some contaminant proteins co-eluted with the scFv-BM3 were observed in the SDS-PAGE analysis. The contaminant proteins were likely *E. coli* cellular proteins that were secreted into the culture medium and co-purified with the scFv-BM3. Approximately 95% of *E. coli* cellular proteins had isoelectric points (pI) at weak acidic pH in wild-type *E. coli* proteome analysis (Kweon et al., 2002). However, the scFv-BM3 has a net positive charge at pH 7.0 because the pI value of scFv-BM3 is 7.93. Therefore, the elution fractions of the Ni-affinity chromatography were dialyzed against 20 mmol/L sodium phosphate buffer at pH 7.0 and

then subjected to cation exchange chromatography with the same buffer to improve the purity of scFv-BM3 (Fig. 3-11 (B)). As expected, most of the contaminant *E. coli* proteins did not bind to the cation exchanger (SP sepharose FF) and were found in the flow-through fraction, whereas the scFv-BM3 was bound to the cation exchanger and then eluted by increasing NaCl concentration with a linear gradient, which resulted in the high purity of scFv-BM3 without the contamination of *E. coli* proteins. Elution fractions 1 to 3 were pooled and used for biophysical analyses because a contaminant band of ca. 23 kDa was observed in elution fraction 4. The scFv-BM3 purified via the two chromatography steps was 2.86 mg from a 1-liter culture medium of *E. coli* BL21(DE3)/pET26-scFv-BM3.

3.3.4. Characterization of the scFv-BM3 purified from culture medium

Various physicochemical analyses were carried out to verify whether the purified scFv-BM3 had a correctly folded conformation to recognize AFB_1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis revealed that the

scFv-BM3 purified from the culture medium has 26,250.5 Da of molecular mass that is nearly identical to the theoretical molecular weight (26,188.2 Da) of the signal peptide-less scFv-BM3 (Fig. 3-12 (A)). The correct processing of the PelB signal peptide was verified by a peptide mass fingerprint (PMF) method that showed a distinct peptide mass peak corresponding to the N-terminal peptide (MEVKLVESGGGLVK) of scFv-BM3 ((Fig. 3-12 (B)), and any mass peak derived from the PelB signal peptide was not detected in the mass spectrum. These results suggest that the scFv-BM3 was exported into the periplasm via the PelB signal peptide-mediated Sec-dependent pathway (Thie et al., 2008) and then secreted into the culture medium. To assess whether the secreted and purified scFv-BM3 had an affinity for its antigen, the specific binding of the purified scFv-BM3 to AFB_1 was measured by ELISA using a microplate coated with the AFB_1-BSA conjugate or BSA as the control. As shown in Fig. 3-12 (C), the purified scFv-BM3 showed a high binding affinity to AFB_1.

The far-UV circular dichroism (CD) spectrum of scFv-BM3 exhibited positive and negative peaks of molar ellipticity at approximately 200 and 218 nm, respectively (Fig. 3-12 (D)). These peaks in the CD spectrum are indicative of the proteins rich in the antiparallel β-sheet

that are a typical structure of scFvs (Pini et al., 1997; Pledger et al., 1999; Vendel et al., 2012). In thermal unfolding analysis using CD spectrometry at 200 nm, the scFv-BM3 showed a gradual decrease in the CD signal near 45 ℃ and then a sharp sigmoidal decrease due to protein denaturation (Fig. 3-12 (E)). The melting temperature (T_m) value of scFv-BM3 was determined to be approximately 47.5 ℃. The CD spectrum and the thermal unfolding profile of scFv-BM3 were in accordance with previous results (Min et al., 2016). When the thermal unfolding analysis was performed in the presence of 4 μmol/L of AFB_1, the T_m value was increased to 53.5 ℃, which is 6 ℃ higher than that in the absence of AFB_1. This significant improvement of thermal stability is clear because the overall structure of the scFv-BM3 was stabilized by binding to AFB_1 at its complementarity determining regions (CDRs), accounting for 24.8% of the scFv-BM3 polypeptide. Taken together with the above results, it is obvious that the scFv-BM3 exported from the periplasm of *E. coli* to the culture medium adopted a properly folded structure suitable for the immunological detection of AFB_1.

This study demonstrated that the functional scFv-BM3 could be produced by *E. coli*. While the scFv-BM3 was expressed as inactive aggregates in *E. coli*, its functional form was secreted into the culture

medium that was facilitated by the addition of Triton X-100. Various biophysical and biochemical analyses revealed that the scFv-BM3 purified from the culture medium had a functional structure to bind AFB_1 specifically. This extracellular production method can be applied to the production of other recombinant antibodies such as scFv and Fab using *E. coli*.

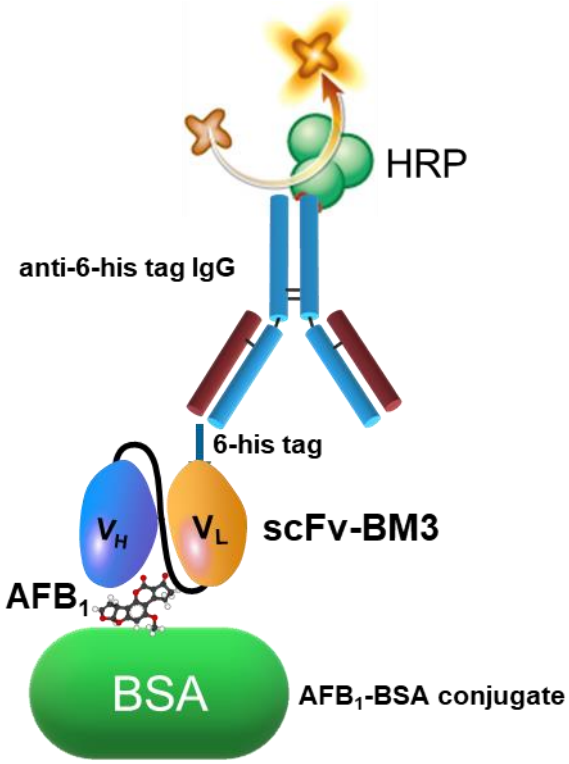
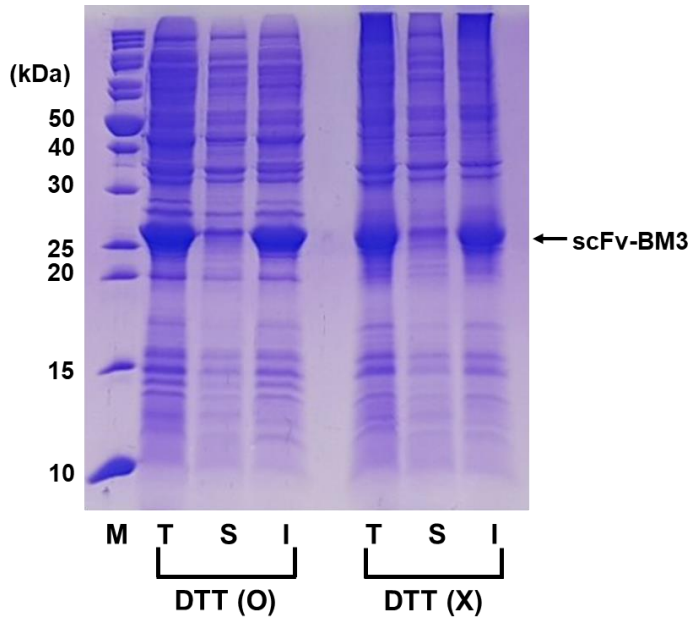


Figure 3-1. A schematic representation of ELISA analysis for the scFv-BM3. The scFv-BM3 binds to AFB_1 of the AFB_1-BSA conjugate coated on the wells of an ELISA plate. The HRP conjugated anti-6-his IgG recognizes and binds to the C-terminal 6-histidine tag of scFv-BM3 bound to the AFB_1-BSA conjugate. Therefore, the intensity of absorbance at 450 nm developed by HRP reaction with TMB substrate is positively correlated with the amount of scFv-BM3 bound to AFB_1-BSA conjugate.

(A)


(kDa)
50
40
30
25
20
15
10
scFv-BM3
M
T
S
I
T
S
I
DTT (O)
DTT (X)

(B)

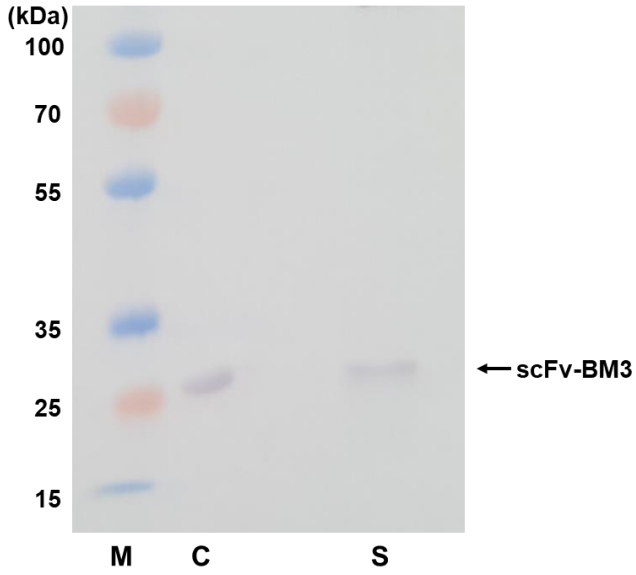

(kDa)
100
70
55
35
25
15
scFv-BM3
M
C
S

Figure 3-2. Intracellular and extracellular scFv-BM3 expressed in *E. coli*. (A) SDS-PAGE analysis for total, soluble and insoluble fractions of *E. coli* cell lysate. The recombinant *E. coli* cells induced by IPTG for 4 h were harvested and disrupted, and their lysates were fractionated into total cell lysate (T), soluble fraction (S) and insoluble fraction (I). Lane M denotes a protein size marker. The presence and the absence of dithiothreitol (DTT) represent reducing and non-reducing SDS-PAGE, respectively. (B) Western blotting analysis for scFv-BM3 secreted into culture medium. The purified scFv-BM3 as a control (C) and the culture medium (S) of the recombinant *E. coli* cells induced by IPTG for 4 h were subjected to SDS-PAGE, followed by Western blotting analysis using a mouse anti-6-histidine tag IgG. Lane M denotes a protein size marker.

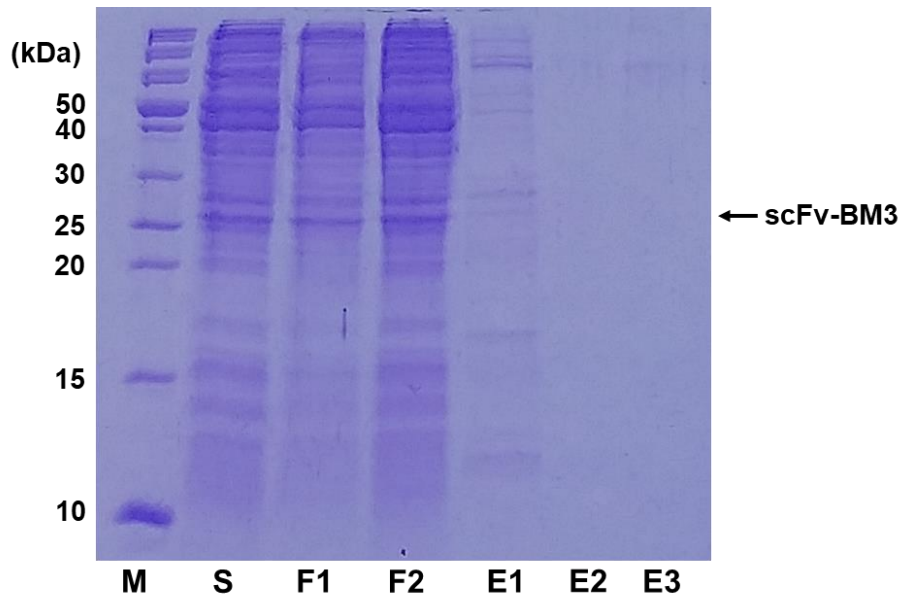


Figure 3-3. SDS-PAGE analysis for purification of scFv-BM3 in soluble fraction via Ni-affinity chromatography. A 25 mL aliquot of the soluble fraction (S) was injected into a HisTrap FF 1 mL column equilibrated with a binding buffer. The proteins bound to Ni-sepharose FF resin were eluted by increasing the imidazole concentration to 500 mmol/L. The fractions of flow-through (FT) and elution (E) were subjected to SDS-PAGE analysis. Lane M denotes marker proteins.

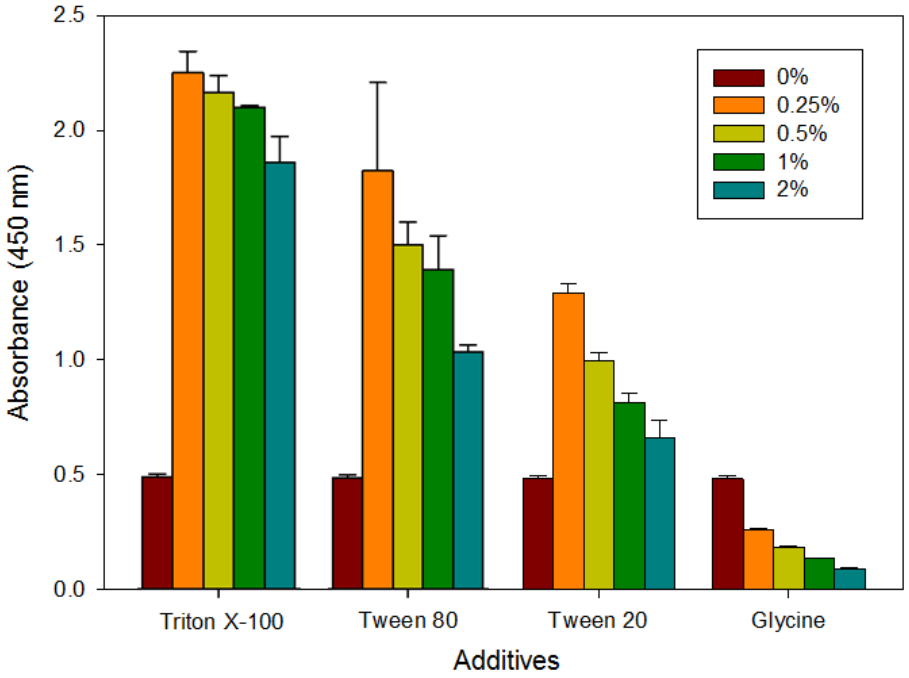


Figure 3-4. Comparison of various additives on extracellular production of scFv-BM3. The relative concentrations of scFv-BM3 secreted into culture medium at various concentrations of Triton X-100, Tween 80, Tween 20 and Glycine were compared by ELISA using a 96-well plate coated with the AFB_1-BSA conjugate.

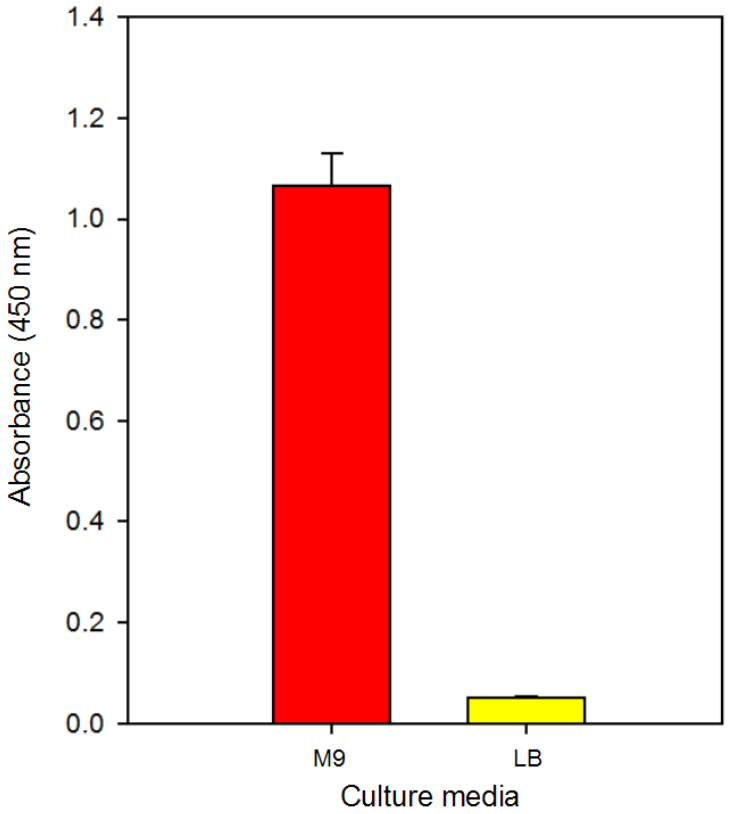


Figure 3-5. Comparison of culture media on extracellular production of scFv-BM3. The relative concentrations of scFv-BM3 secreted into culture medium were compared by ELISA using a 96-well plate coated with the AFB_1-BSA conjugate.

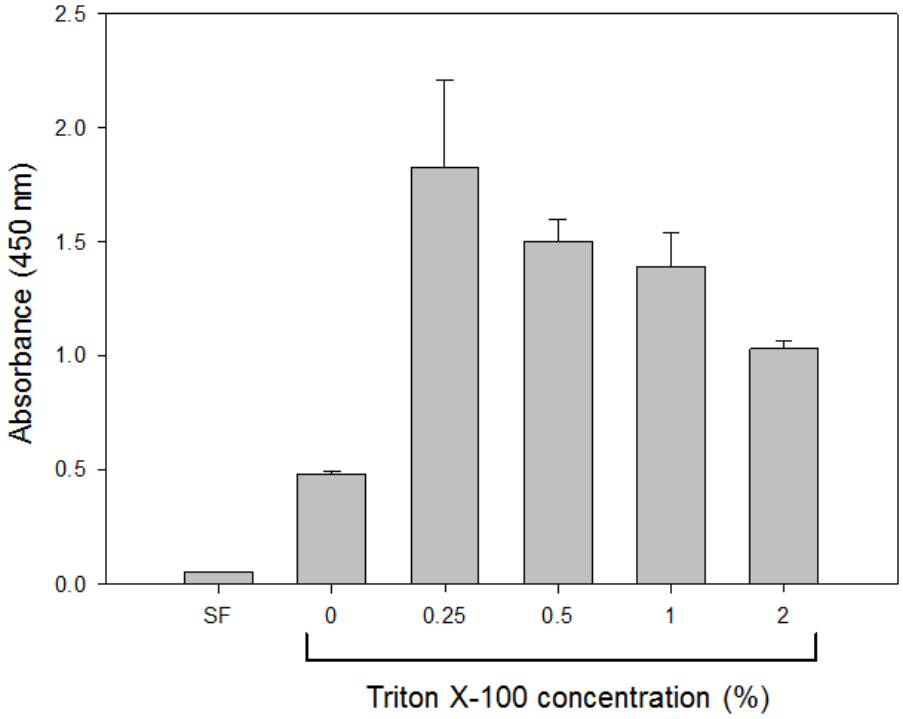


Figure 3-6. ELISA analysis for functional scFv-BM3. The relative concentrations of the functional scFv-BM3 in the soluble fraction (SF) of the cell lysate and in the culture medium at various concentrations of Triton X-100 were compared by ELISA using a 96-well plate coated with the AFB_1-BSA conjugate.

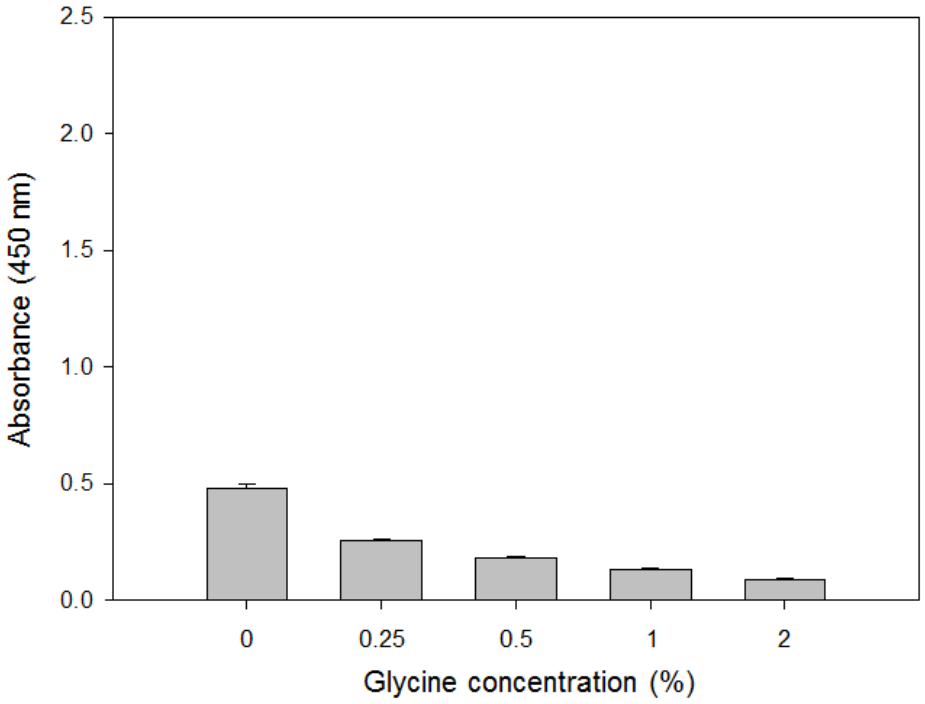


Figure 3-7. Effect of glycine on extracellular production of scFv-BM3. The relative concentrations of scFv-BM3 secreted into culture medium at various concentrations of glycine were compared by ELISA using a 96-well plate coated with AFB_1-BSA conjugate.

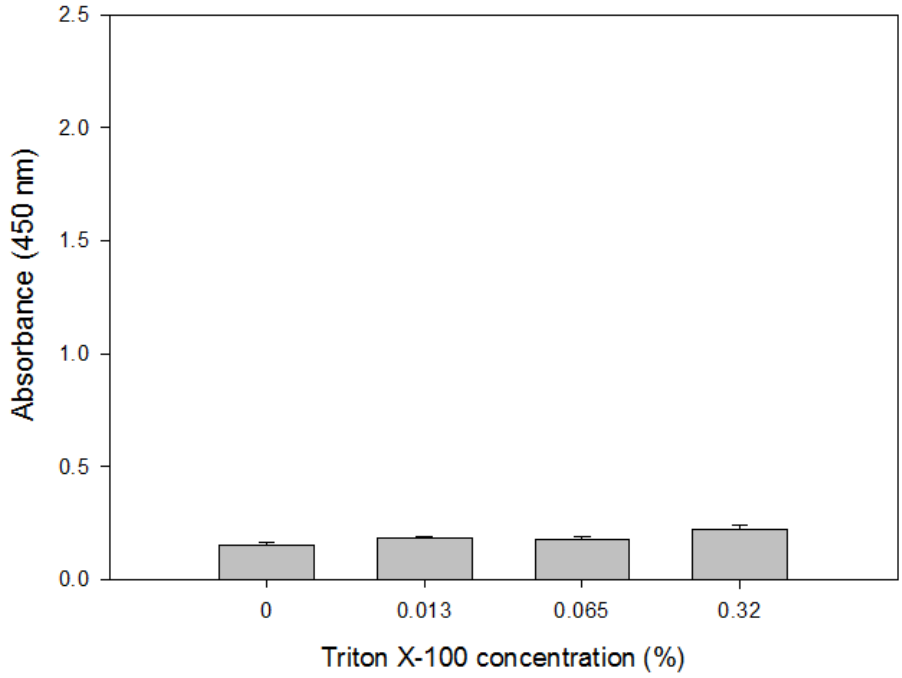


Figure 3-8. Effect of Triton X-100 on refolding of scFv-BM3. The inclusion bodies of the scFv-BM3 were solubilized in 20 mM Tris buffer (pH 8.5) containing 6 M guanidine HCl and then refolded by a 20-fold dilution with a refolding buffer (20 mmol/L Tris, pH 8.5, 1 mmol/L GSH and 0.1 mmol/L GSSH) containing various concentrations of Triton X-100. The relative concentrations of scFv-BM3 refolded at various concentrations of Triton X-100 were compared by ELISA using a 96-well plate coated with AFB_1-BSA conjugate.

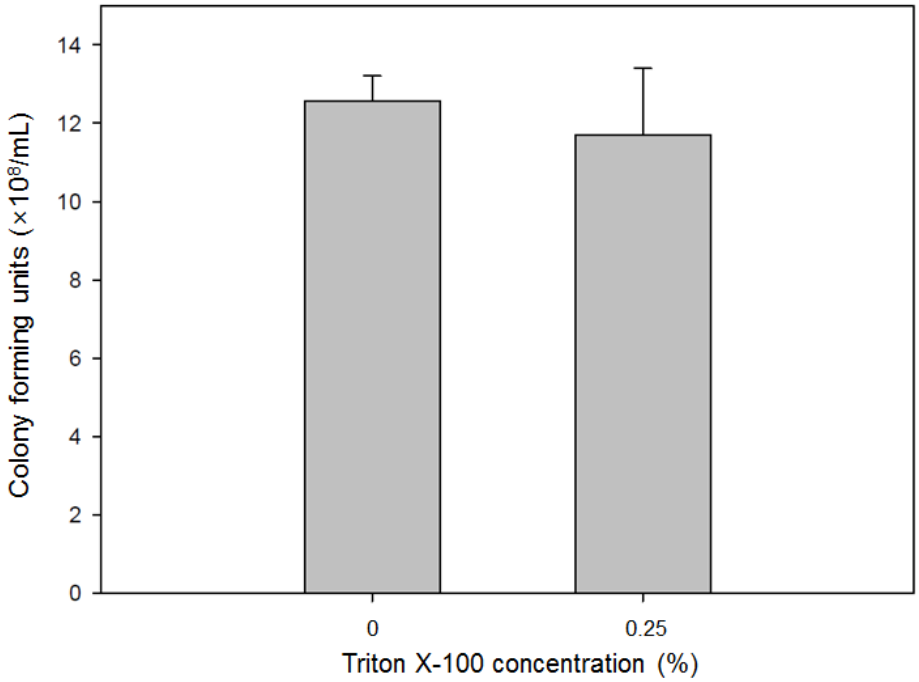
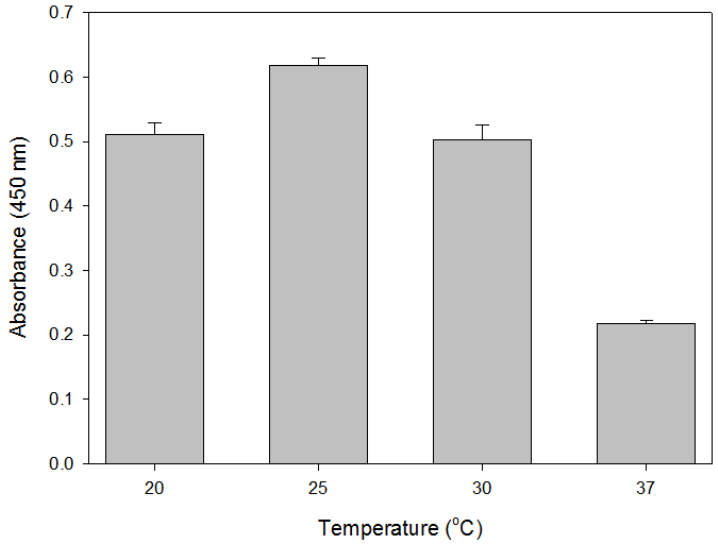


Figure 3-9. Effect of 0.25% Triton X-100 on cell growth. Colony forming units (CFU) of *E. coli* BL21(DE3) harboring pET26-scFv-BM3 with and without 0.25% Triton X-100 were compared. The *E. coli* cells of 4 h after addition of 0.1 mmol/L IPTG or 0.1 mmol/L IPTG and 0.25% Triton X-100 were serially diluted to 10^{-7} (10-fold steps) and plated on LB agar medium containing 50 mg/L kanamycin. CFU were counted after 24 h incubation at 37℃.

(A)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance (450 nm)
20
25
30
37
Temperature (°C)

(B)

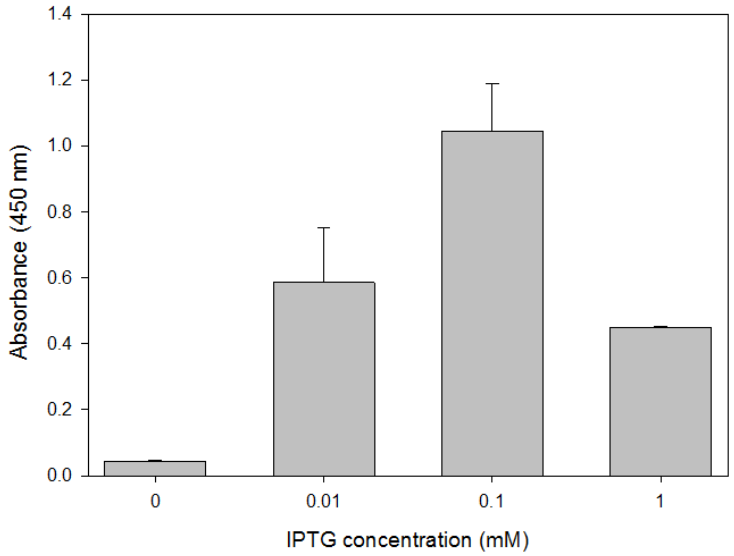
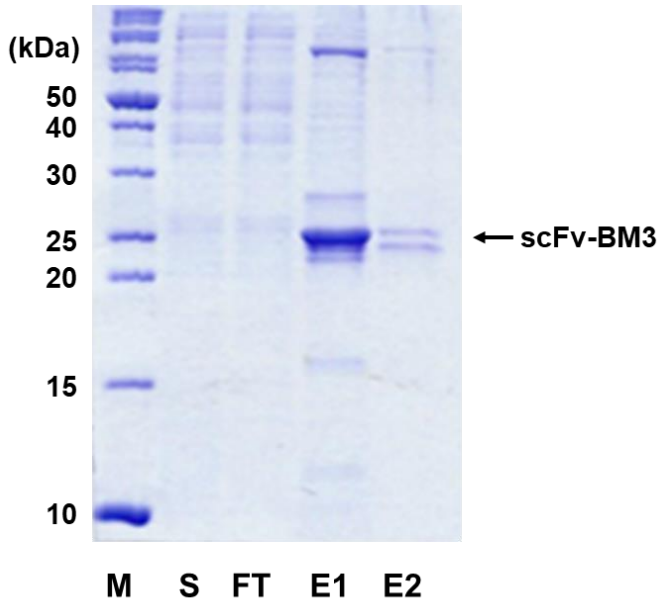

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (450 nm)
0
0.01
0.1
1
IPTG concentration (mM)

Figure 3-10. Effect of expression temperature and IPTG concentration on extracellular production of scFv-BM3. The relative concentrations of scFv-BM3 secreted into culture medium at various expression temperatures (A) and IPTG concentrations (B) were compared by ELISA using a 96-well plate coated with AFB_1-BSA conjugate.

(A)


(kDa)
50
40
30
25
20
15
10
scFv-BM3
M
S
FT
E1
E2

(B)

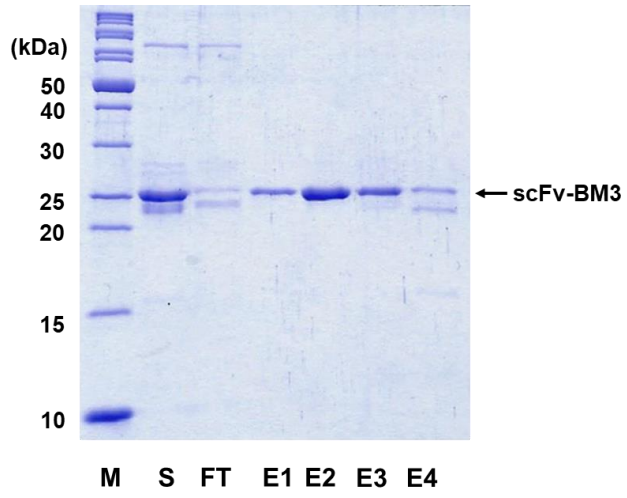
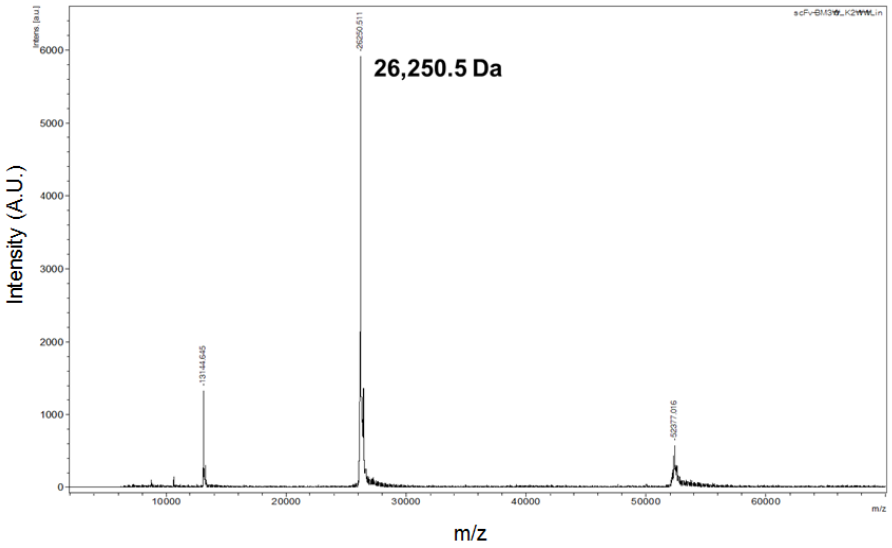
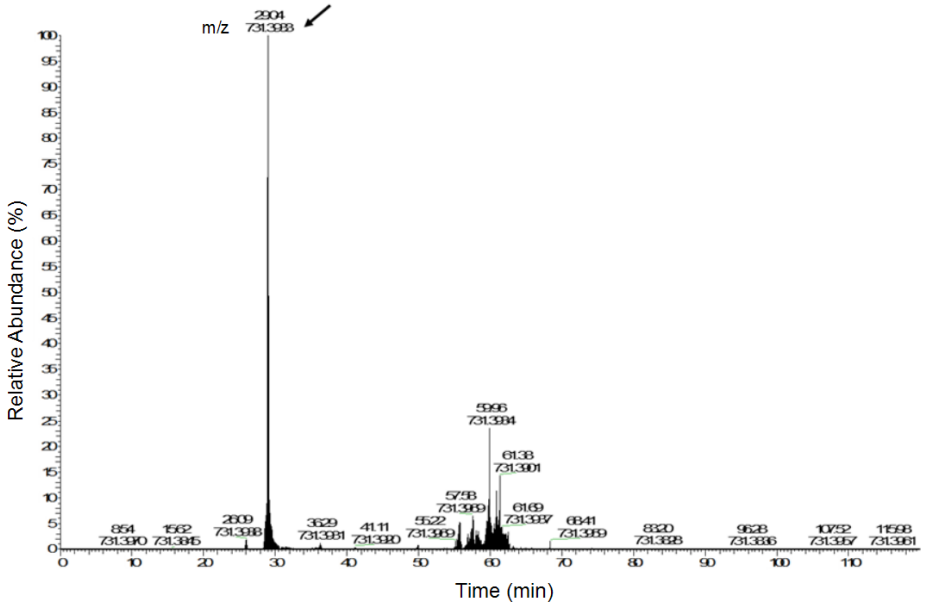

(kDa)
50
40
30
25
20
15
10
scFv-BM3
M
S
FT
E1
E2
E3
E4

Figure 3-11. Purification of scFv-BM3 secreted into culture medium via Ni-affinity and cation exchange chromatography. (A) Ni-affinity chromatography. SDS-PAGE analysis for the sample injected (S), the flow-through (FT) and the elution (E) fractions of the Ni-affinity purification to purify scFv-BM3 secreted into the culture medium. (B) Cation exchange chromatography. SDS-PAGE analysis for the sample injected (S), the flow-through and the elution fractions of cation exchange chromatography to purify the scFv-BM3 in the elution fractions of the N-affinity chromatography. Lane M denotes a protein size marker.

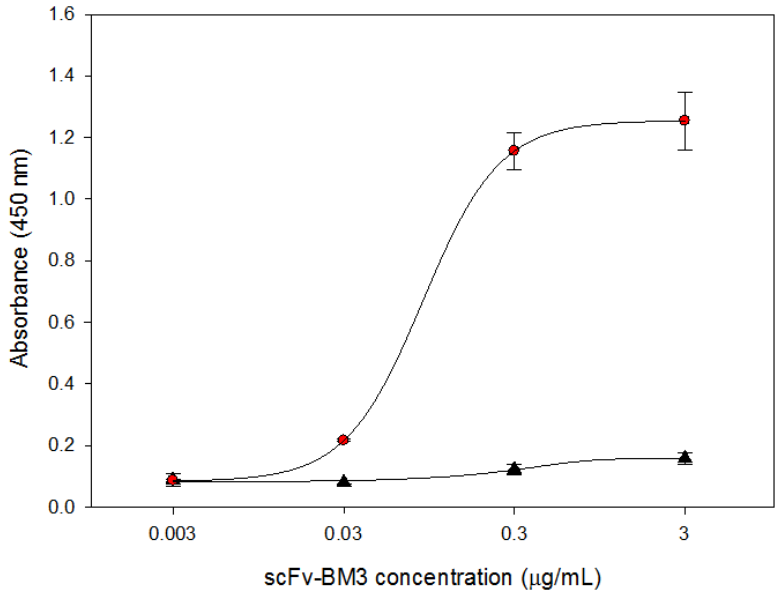
(A)


26,250.5 Da
Intensity (A.U.)
m/z

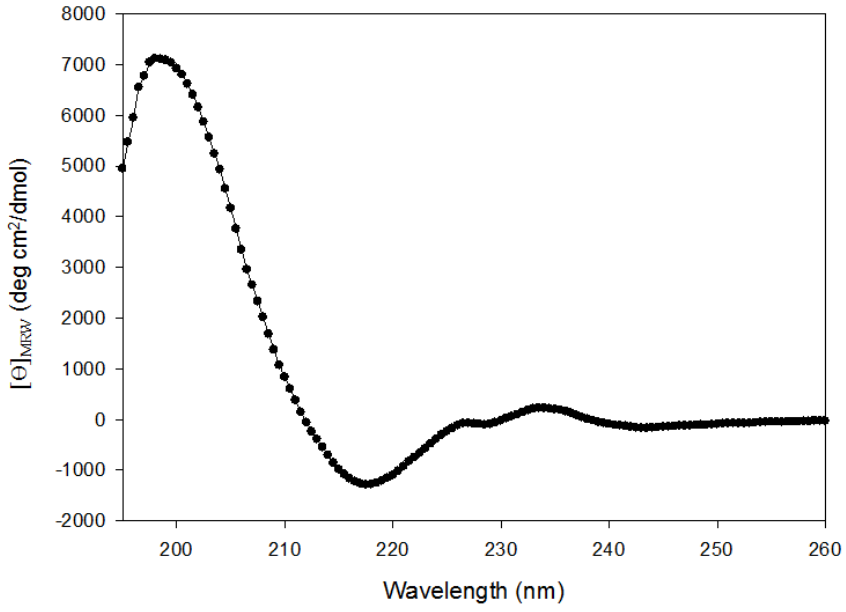
(B)


m/z
2904
731.3383
Relative Abundance (%)
Time (min)

(C)


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (450 nm)
0.003
0.03
0.3
3
scFv-BM3 concentration (μg/mL)

(D)


8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
[Θ]MRW (deg cm2/dmol)
200
210
220
230
240
250
260
Wavelength (nm)

(E)

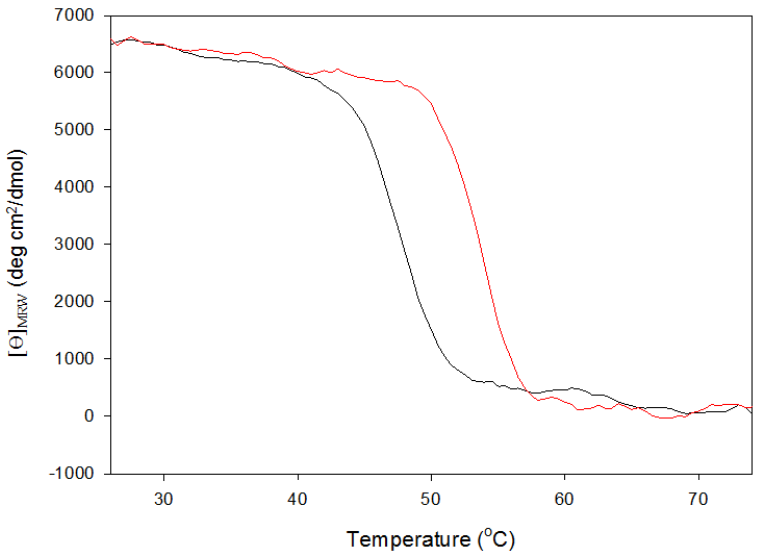


Figure 3-12. Biochemical analyses of purified scFv-BM3. (A) MALDI-TOF MS. The molecular mass analysis of the purified scFv-BM3 was conducted on an Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The mass range was measured in the range of 2,000 to 100,000 Da with a linear mode, and the mass spectrum was accumulated with individual 500 laser shots. (B) Identification of N-terminal peptide fragment of purified scFv-BM3. The band of the purified scFv-BM3 in the SDS-PAGE gel was excised, digested with trypsin, and subjected to peptide mass analysis using LC-ESI-MS. The arrow indicates the mass peak of the N-terminal peptide (MEVKLVESGGGLVK) of scFv-BM3. The mass peak of 731.4 Da

represents the N-terminal peptide with double charges and a methionine oxidation. (C) ELISA. The purified scFv-BM3 was diluted to a range of 0.003 to 3 μg/L for the ELISA analysis. The absorbance at 450 nm was obtained from wells coated with the AFB_1-BSA conjugate (red circles) or BSA as a negative control (black triangles). (D) Far-UV CD spectrum. The spectrum was measured over a wavelength range of 260 to 190 nm at 25 ℃. The CD signal was converted to the mean residue ellipticity and plotted versus the wavelength. (E) Thermal denaturation profile. The CD signal at 200 nm was monitored from 25 ℃ to 75 ℃. The red and black lines represent the change in the mean residue ellipticity for scFv-BM3 in the presence and absence of 4 μmol/L AFB_1, respectively. The CD signal was converted to the mean residue ellipticity and plotted versus the temperature.

CONCLUSIONS

For effective immunological detection of AFB_1 at low concentrations, the development of high affinity antibodies for AFB_1 is required. Immunological detection of low molecular weight toxins such as AFB_1 using a single-chain variable fragment (scFv) is a potentially novel and safe method of diagnosing fungal infection and food contamination.

The effect of the 6 mutated residues on the affinity improvement was characterized using surface plasmon resonance analysis, which identified a deleterious mutation (V_H-A110T) located on a framework region of the scFv-M37. The back mutation of V_H-A110T (scFv-BM3) resulted in a 3.2-fold affinity improvement, which was attributed to a decrease in dissociation rate constant (k_d) in the interaction between AFB_1 and the back mutant scFv, and thus has a 31-fold higher affinity than its wild-type. The biophysical analyses using circular dichroism and gel filtration revealed that the back mutation of V_H-A110T caused a subtle conformational change of the scFv toward tighter binding to AFB_1.

This fine-tuning approach via back mutation of randomly mutated residues in the affinity-maturated scFv can be applied to affinity-maturated antibodies for additional affinity improvement.

To apply scFv-BM3 to immunological detection of AFB_1, periplasmic expression in *E. coli* was attempted to produce a functional form of scFv-BM3. While scFv-BM3 was expressed as inactive aggregates in the periplasm of *E. coli*, its active form was secreted into the culture medium that was facilitated by the addition of Triton X-100. This extracellular secretion of functional scFv-BM3 was significantly improved by supplementation with Triton X-100 and optimization of expression conditions. The scFv-BM3 purified from the culture medium exhibited a typical anti-parallel β-sheet structure and adopted a proper conformation to bind AFB_1 with high affinity and specificity in various biophysical and biochemical analyses. This extracellular production method can be applied to the production of other functional antibody fragments such as scFv and Fab using *E. coli*.

In conclusion, an affinity improvement of the single-chain variable fragment against aflatoxin B_1 was achieved via the back-mutation of an scFv and a simple and efficient method to produce active form of functional antibody fragment was demonstrated using recombinant *E. coli* by extracellular secretion.

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국 문 초 록

아플라톡신 B_1(AFB$_1$)은 식품과 사료에 존재하는 곰팡이 독소 중 가장 강한 독성을 가진 독소이다. 아플라톡신 B_1은 아스퍼질러스속 곰팡이에 의해 생산되고, 인체 간독성, 기형발생, 돌연변이를 유발하는 특성에 따라 심할 경우 간염, 출혈, 면역저하, 간암종을 일으킬 수 있다. 이렇게 유해한 아플라톡신 B_1의 검출을 위해서는 면역학적 방법을 통하여 가능한 낮은 농도에서 효과적으로 검출할 수 있는 기술 개발이 필요하다. 이에 대한 구체적 방법으로 항체 단쇄가변분절(scFv)을 이용하여 안전하고 효과적으로 식품 등에 곰팡이 감염 또는 오염 여부를 진단할 수 있다. 이전의 연구에서 항체 단쇄가변분절의 항원(아플라톡신 B_1)에 대한 친화력 증대를 위하여 인위적으로 만든 돌연변이 라이브러리로부터 6개의 돌연변이체를 분리하였고, 이 중 scFv-M37은 야생형의 scFv에 비해 9배나 높은 친화력을 나타내었다. 본 연구에서는 6개의 돌연변이 부위에 대해 역돌연변이 방법을 사용하여 각 부위의 돌연변이체에 대한 항원(아플라톡신 B_1) 친화력을 분석하였고, 결과적으로 V_H의 110번 아미노산인 알라닌(A)을 본래 야생형의 아미노산인 트레오닌(T)으로 변이시킬 경우 3.2배 친화력이 증가되는 것을 알 수 있었고, 이는 본래 야생형의 scFv에 비해 31배의 친화력이 증대되는 결과를 보였다. 원편광 이색성 측정 및 젤 여과법 등 다양한 생물물리적 분석을 통해 이러한 돌연변이는 항원과 결합시 미묘한 입체구조 변화에 따라 친화력이 변하는 것을 알 수 있었고, 이러한 미세하고 정교한 조정을 통하여 항원과 친화력을 증대시킬 수 있음을 보여주고 있다.

보통의 경우 대장균을 통해 항체 단쇄가변분절을 발현하였을 때, 대부분 비활성형의 응집체 형태로 쌓임에 따라, 이를 활성형 단백질의 형태로 효과적으로 발현하기 위하여 대장균 배양시 Triton X-100을 첨가하여 발현되는 단백질의 세포외 배출을 늘리고, 발현조건을 최적화하는 연구를 수행하였다. 추가적으로 최종 정제된 항체 단쇄가변분절은 역 평행 β-sheet 구조를 나타내었고, 생물물리적 및 생화학적 분석법을 통하여 항원과의 높은 친화력과 아플라톡신 B_1에 특이성을 가지고 있음을 알 수 있었다. 이러한 대장균의 세포외 발현 방법은 다른 scFv나 Fab와 같은 항체 단편에 대한 효율적 생산에 적용될 수 있다. 결과적으로, 본 연구에서는 역돌연변이의 방법을 통해 아플라톡신 B_1에 특이적이고 친화력이 증대된 항체 단쇄가변분절을 선별하였고, 이렇게 선별된 단쇄가변분절을 재조합 대장균을 통해 활성형 단백질의 형태로 효과적으로 생산할 수 있는 방법을 찾아냄으로써 향후 다양한 형태의 단백질 생산에 응용될 수 있을 것으로 기대한다.

주요어 : 단쇄가변분절, 아플라톡신 B_1, 재조합 대장균, 역돌연변이, 친화력 증대, 세포외 배출

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