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Productions, Characterizations, and Applications of Monoclonal and Recombinant Antibodies against Daidzin and Paclitaxel

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Introduction

Daidzin (DIN) is a useful secondary metabolite from *Glycine max*, *Pueraria lobata*, and *Pueraria mirifica*, which these medicinal plants exhibited beneficial effects against cardiovascular, cerebrovascular and hyperlipidemia diseases, and estrogen deficiency related disorders in clinical trials. Thus, DIN-quantified products are important to ensure their safety and efficacy. By the way, paclitaxel (PTX) had been approved for ovarian and breast cancer, and it showed efficacy for various cancer treatments. However, some patients were suffered due to their toxicities. Therefore, convenient analytical methods might be useful to monitor drug concentration in serum for avoiding of life threatening side effects. Antibodies were extensively developed for analytical applications. Design of antibody is determinant of analytical performance of immunoassays. In response to high demand of antibody fragments as an analytical tool, effect of antibody structure on analytical characteristics should be investigated. Moreover, simple and efficient methods of antibody fragment production are crucial to facilitate their structural and functional investigations. It this thesis, it was aimed to evaluate (1) effect of DIN-carrier protein conjugate formats on binding characteristics of responding antibodies against DIN, (2) effect of peptide linker lengths on binding characteristics of anti-DIN scFvs and anti-PTX scFvs, and (3) effect of heavy chain constant region 1(C_H1) sequence on refolding and reactivity of anti-PTX Fabs

Methods and Results

Firstly, monoclonal antibody (mAb) against DIN were produced using two formats of immunogenic DIN conjugate including the native bovine serum albumin (nBSA)-DIN and cationized BSA (cBSA)-DIN conjugates. Therefore, two different mAbs were obtained, in which anti-DIN mAbI and anti-DIN mAbII were produced using nBSA-DIN and cBSA-DIN, respectively. The results (Table 1) revealed that design of carrier protein-DIN conjugate affected largely on binding specificity of anti-DIN mAb, which anti-DIN mAbI showed group-specific

binding against isoflavonoids, and anti-DIN mAbII binds specifically against DIN. Both anti-DIN mAbs exhibited high sensitivity, precision, and accuracy for DIN determination. However, only anti-DIN mAbII (linearity of determination: 1.56–100 ng/ml, coefficient of variation: 1.28 – 4.57%, and DIN recovery: 98.99 – 104.9%) was applied for specific determination of DIN in plant samples and involved products. DIN content of these samples were ranged from 1.837 to 6123 μg/100 mg dry weight, which were in agreement with DIN content analyzed by the HPLC-UV method. With advantages of immunoassays, this is an attractively alternative method for DIN analysis in various field.

Table 1 Cross-reactivity of anti-DIN mAbs against isoflavonoids.

Compound	Cross-reactivity (%)	
	Anti-DIN mAbI	Anti-DIN mAbII
Isoflavonoid glycosides		
DIN	100	100
Genistin	82.3	0.044
Glycitin	11.9	0.481
Tectoridin	539	< 0.040
Kakkalide	15.4	< 0.040
Puerarin	3.37	< 0.040
Isoflavonoids		
Daidzein	16.2	1.57
Genistein	24.4	< 0.040
Glycitein	1.02	< 0.040
Tectorigenin	142	< 0.040
Irisolidone	11.4	< 0.040
Biochanin A	11.0	< 0.040
Formononetin	2.35	< 0.040
Prunetin	15.7	< 0.040

Until now the influence of a peptide linker on binding characteristics of scFv against low molecular weight compounds have not been evaluated yet. Therefore, variable region of anti-DIN mAbI was used as a model to investigate the effect of peptide linker. The BmNPV bacmid system was applied to express multiform of anti-DIN scFvs with a different length of linker between V_H and V_L. All forms of anti-DIN scFvs were produced and secreted into body fluid of *B. mori* larvae. Then, the reactivity of purified scFvs were evaluated by indirect ELISA. Lengthening of a peptide linker not only increased a reactivity of anti-DIN scFvs against DIN, but also slightly decreased their binding specificity. The length of a linker between V_H and V_L affect heavily sensitivity of anti-DIN scFvs-based indirect competitive ELISA (Figure 1). By using indirect competitive ELISA, the DIN concentration given 50% inhibitory effect (IC_{50}) of control was 1272, 125.1, 84.95, and 79.34 ng/ml for anti-DIN scFv5, 15, 25 and 35, respectively

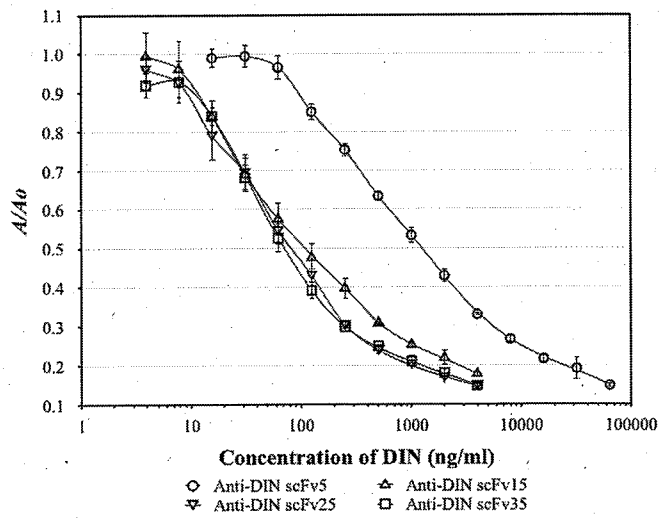

Figure 1 Reactivity of anti-DIN scFvs against DIN evaluated by indirect competitive ELISA

Two clones (3A3 and 1C2) of anti-PTX scFvs confirmed that length of a peptide linker between V_H and V_L affects reactivity of scFv. Both clones of anti-PTX scFvs were constructed, expressed using *E. coli*, and then refolded *in vitro*. Lengthening of a linker from 15 amino acids (scFv15) to 25 amino acids (scFv25) improved refolding and reactivity of anti-PTX scFvs (Fig. 2). Since the reactivity was not sufficiently recovered using a refolding procedure, BmNPV bacmid expression system was applied for expression of active anti-PTX scFvs. The results also confirmed that lengthening of the linker improved their reactivity (Fig. 3), which their reactivity were comparable to their parental mAb.

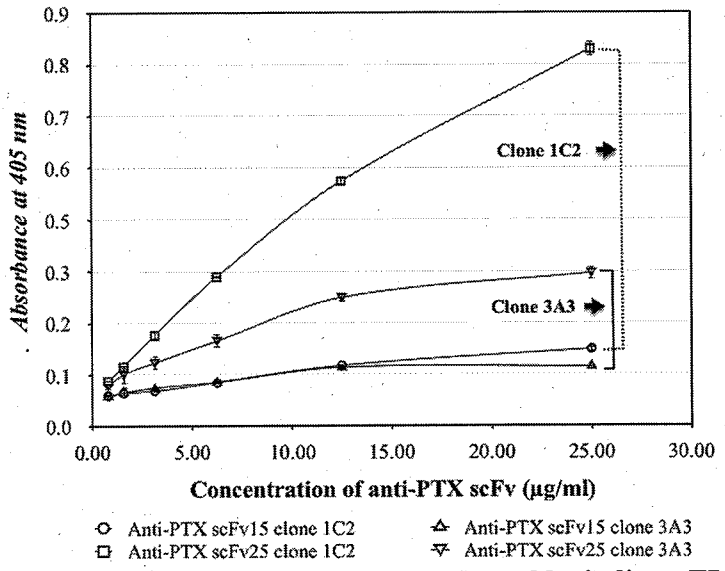

Figure 2 Reactivity of anti-PTX scFvs against immobilized PTX evaluated by indirect ELISA

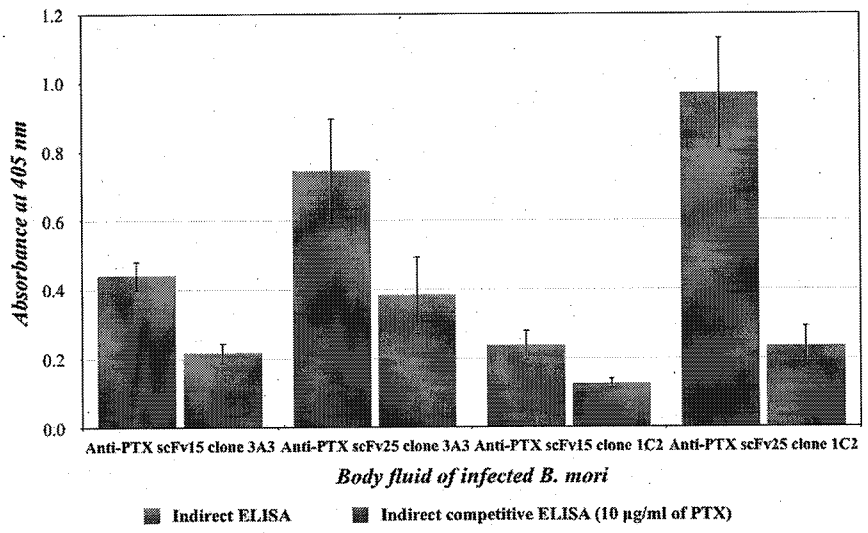

Figure 3 Reactivity of anti-PTX scFvs against PTX evaluated by indirect competitive ELISA. The reactivity against immobilized and free PTX was improved when a peptide linker was lengthened to 25 amino acids.

The reactivity of anti-PTX scFv clone 1C2 was not fully recovered (binding specificity) by *in vitro* refolding, and its low expression was observed in the *B. mori* expression system. Therefore, anti-PTX Fab was constructed from anti-PTX mAb clone 1C2 (IgG2a). V_H-C_H1 and V_L-C_L were constructed and expressed using *E. coli*, and then both domains were refolded to form anti-PTX Fab. Because of low efficacy of assembly between light chain (V_L-C_L) and V_H-

C_H1 (IgG2a), amino acid sequence of C_H1 was modified to be IgG1, IgG2b, and IgG3 subtypes to evaluate the difference of assembly between each V_H-C_H1 and V_L-C_L. The results showed that modification of C_H1 affect anti-PTX Fab refolding (Fig. 4) and their binding reactivity (Fig. 5). Judging from the SDS-PAGE analysis, assembly between V_H-C_H1 and V_L-C_L was estimated as follows; anti-PTX Fab IgG1 > anti-PTX Fab IgG2b > anti-PTX Fab IgG2a > anti-PTX Fab IgG3.

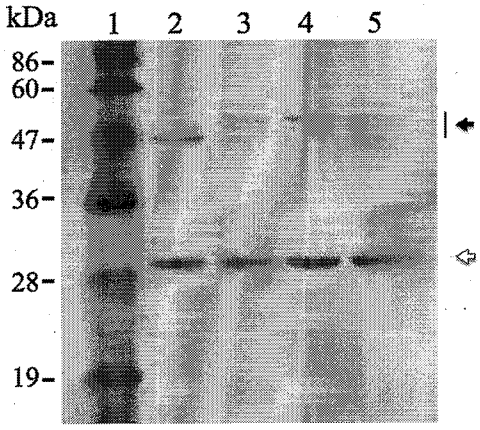

Figure 4 Analysis of refolded anti-PTX Fabs using SDS-PAGE (non-reducing condition). Lane 1 represents protein markers, whereas lane 2, 3, 4, and 5 are mixture of refolded anti-PTX Fab IgG1 (1 μg), anti-PTX Fab IgG2a (1 μg), anti-PTX Fab IgG2b (1 μg), and anti-PTX Fab IgG3 (1 μg), respectively. The solid arrow indicates anti-PTX Fabs, whereas the transparent arrow indicated unassembled V_L-C_L.

Their reactivity against PTX was investigated using indirect competitive ELISA. Refolded anti-PTX Fabs reacted in a concentration-dependent manner of free PTX (Fig. 5). According to IC_{50}, the reactivity of anti-PTX Fabs against PTX was in the order of anti-PTX Fab IgG1 (IC_{50} = 82.05 ng/ml) > anti-PTX Fab IgG2a (IC_{50} = 126.2 ng/ml) > anti-PTX Fab IgG2b (IC_{50} = 190.8 ng/ml) > anti-PTX Fab IgG3 (IC_{50} > 1000 ng/ml), where their parental mAb showed IC_{50} of 59.87 ng/ml. Therefore, anti-PTX Fab IgG1 exhibited the highest sensitivity for PTX determination using anti-PTX Fab-based indirect competitive ELISA. Modification of C_H1 improved refolding efficiency and reactivity of anti-PTX Fab.

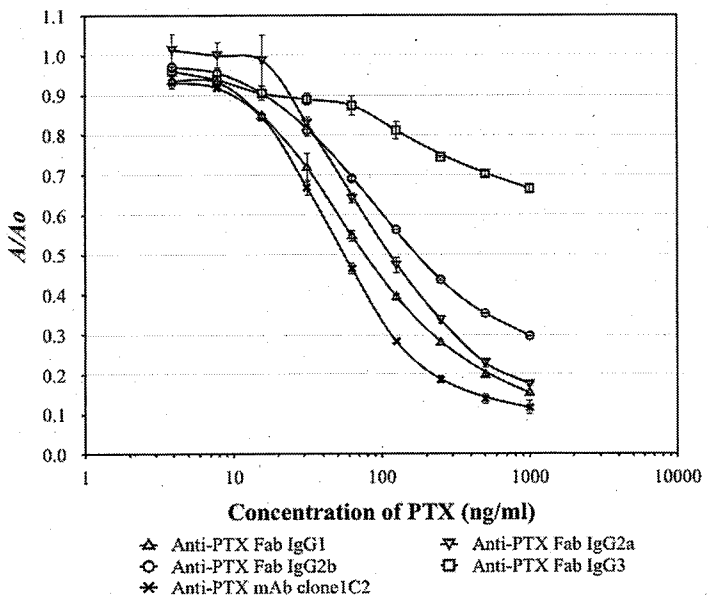

Figure 5 Reactivity of anti-PTX Fabs against PTX evaluated by using indirect competitive ELISA.