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Production and Characterization of Single Chain Fv Directed against β_2-Agonist Clenbuterol

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The single chain Fv (scFv) directed against β_2-agonist clenbuterol (CBL) was produced by using phage display technology. The heavy chain and light chain variable region genes (V_H, V_L) were amplified by the polymerase chain reaction (PCR) from CBL specific hybridoma cell lines 5D1 and assembled as a single chain Fv (scFv) fragment with linker peptide $(Gly_4Ser)_3$. Then the scFv DNA fragment was cloned into M13 phagemid vector pCANTAB5E and the anti-CBL antibody libraries were constructed. Phages displaying scFv were enriched by panning with CBL−ovalbumin (CBL−OVA) conjugate. After only one round of panning, antigen-positive recombinant phage clones were successfully selected by ELISA. The positive phage was used to infect *Escherichia coli* HB2151, and the expression of soluble scFv was then induced by IPTG. The scFv showed an improved sensitivity (with IC_{50} of 0.78 ± 0.005 ng/mL ($n = 4$)) when compared with the parent monoclonal antibody (MAb) (with IC_{50} of 1.34 ± 0.006 ng/mL ($n = 4$)) in competitive indirect ELISA (CI-ELISA). Cross-reactivity studies showed that the specificity of scFv was similar to that of MAb. The recombinant scFv prepared in this study could be potentially used instead of conventional antisera or MAb for development of a rapid and affordable immunoassay for the detection of residual CBL in biological matrices.

KEYWORDS: Single chain Fv; phage display; clenbuterol

INTRODUCTION

Clenbuterol (CBL) is a β_2-agonist drug that has been therapeutically used in human and veterinary medicine for treatment of asthma. At supratherapeutic dose, CBL is a repartitioning agent, increasing muscle growth and decreasing carcass fat. Because of these effects, CBL was also used illegally in meat-producing animals for promoting lean body mass. As has been demonstrated, residues of CBL that is accumulated in animal edible tissue (lung, liver, kidney, and muscle) could present a potential risk for the health of the consumer. Accordingly, the use of CBL as growth promoters in livestock has been banned in many countries. However, the abuse of CBL is still encouraged in the black market due to the substantial economic profit. Thus, it is necessary to continuously monitor CBL misuse in order to meet public demands for residue-free animal products.

For official control purposes of CBL, a two-stage program with immunochemical screening followed by gas chromatography−mass spectrometry (GC−MS) or liquid chromatography−mass spectrometry (LC−MS) confirmation is commonly applied. The limits of detection (LODs) using GC−MS for the identification and quantification of clenbuterol are 0.2−0.5 ng/mL with the linear range being 5−200 ng/mL, while the LODs using LC−MS for detecting clenbuterol are 0.1−1 ng/mL (*1*, *2*). Instrumental methods can unequivocally identify the analyte in the sample, but are too expensive and time-consuming for routine work. Immunoassay (IA), as a convenient screening tool, showed several advantages, such as being simple, rapid, sensitive, specific, and generally cost-effective for large sample loads. Since Yamamoto and Iwata reported the first competitive enzyme immunoassay for CBL (*3*), several methods have been developed (*4*−*8*), and various EIA kits are now commercially available. It has been reported that the LOD of ELISA for detecting of clenbuterol is 0.15 ng/g while the LOD of immunobiosensor assay is 0.27 ng/mL (*4*, *8*). These methods can meet the requirement of 1 ng/mL of the official residue control plans of the EU member states. However, to our knowledge, CBL immunochemical assays were mainly based on conventional polyclonal antibodies (PAb) and monoclonal

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Table 1. PCR Primer Sets for Amplication of V_h, V_L, and Assembly ScFv Fragment

primer	sequence[a]
V_{HBACK}	5′ CAG GTS MAR CTG CAG SAG TCW GG 3′
V_{HFOR}	5′ TGA GGA GAC GGT GAC CGT GGT GCC 3′
V_{LBACK}	5′ GAC ATC GAG CTC ACT CAG TCT CCA 3′
V_{LFOR}	5′ CCG TTT TAT TTC CAG CCT GGT CCC 3′
LINKER1	5′ GGC ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GG 3′
LINKER2	5′ TGG AGA CTG AGT GAG CTC GAT GTC CGA TCC GCC ACC GCC AGA GCC ACC TCC GCC 3′
pSfiI	5′ GTC CTC GCA ACT GC*G GCC CAG CCG GCC* ATG GCC CAG GTC AAA CTG CAG GAG TCA GG 3′
pNotI	5′ GAG TCA TTC T*GC GGC CGC* CCG TTT TAT TTC CAG CCT GGT CCC 3′

[a] Restriction site sequences on the primers are italicized and underlined. Degeneracy of nucleotides was represented by M:A or C; R:A or G; S:C or G; W:A or T.

antibodies (MAb). PAb are easiest and quickest to produce, but they are not single molecular entities and sometimes cause nonspecific reactivity. MAb are single molecular entities, and multiple clones are available for selection in the development process, but the preparation of MAb is more complex, and expensive cell culturing facilities are required for large scale production.

Recently, advances in the field of recombinant antibody (RAb) technology have provided an alternative means to engineer low-cost antibodies with desirable affinity and specificity. Especially taking advantage of phage display technology, the active antibody fragments could be selected rapidly from a large number of phage antibody libraries bypassing hybridoma technology and even animal immunization (*9*). Once the gene was cloned, the RAb could be expressed rapidly in a large amount in *Escherichia coli* at considerably lower cost, and mutagenesis of immunoglobulin genes could create antibody with different affinities and specificities. Because of these advantages, beyond the medical and biochemical diagnostics, several research groups have examined the potential of RAb technology for developing more useful antibodies for food and environment diagnostics in recent years (*10*, *11*). A growing number of RAb against low molecular weight antigens (hapten) such as pesticides, toxins, antibiotics, and some environment pollutants have been successfully produced, and these RAb showed desired sensitivity and specificity compared with parent MAb (*12*−*15*).

In this study, functional single chain Fv (scFv) directed against CBL was produced using phage display technology, and the sensitivity and specificity of scFv were characterized. This is the first report in the literature describing the production of RAb directed against CBL. This research established a foundation for development of a novel immunoassay for CBL screening using RAb instead of conventional PAb or MAb.

MATERIALS AND METHODS

Total RNA Extraction and cDNA synthesis. Total RNA was extracted from 10^7 Hybridoma cells 5D1 that secreted monoclonal antibody against CBL using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. About 2 μg of RNA was reverse transcribed using random hexamer primers and the first-strand cDNA synthesis kit (Promega).

ScFv DNA Construction. The first-strand cDNA product (2 μL) was used as a template for the amplification of the variable heavy (V_H) and light (V_L) domains of the immunoglobulin genes. The primers used in the PCR amplification are shown in **Table 1**. The first round of PCR was carried out for 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with an initial denaturation for 2 min at 94 °C. Then the scFv genes were generated by assembly of V_H, the $(Gly_4\text{-}Ser)_3$ linker (**Table 1**), and V_L fragments using overlap extension PCR. Finally, the full-length scFv fragments containing the *Sfi*I and *Not*I sites were amplified by using external primers (pSfiI and pNotI).

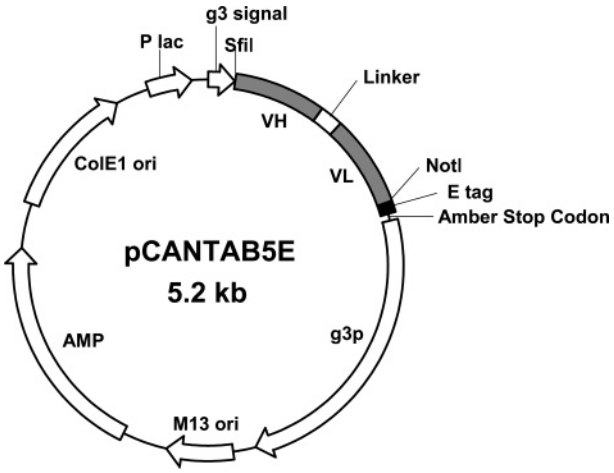


Figure 1. pCANTAB5E phagemid vector for cloning, phage displaying, and expression of soluble scFv. *Sfi*I and *Not*I restriction sites are for cloning of scFv fragment. E tag fusing expression with scFv is for detection. Amber stop codon between the E tag and g3p is for scFv phage dislplaying (in TG1 strain) or expression of soluble scFv (in HB2151 strain).

Phage Display ScFv Library Construction. The scFv DNA fragment was digested by restriction enzymes *Sfi*I and *Not*I (NEB) and ligated into the phagemid pCANTAB5E (Amersham Biosciences) which was precut with the same enzymes (**Figure 1**). The ligated products were transformed into competent cells of *E. coli* TG1 (suppressor strain), and the transformed cells were infected with M13KO7 helper phage for rescuing of scFv-displaying phage. Then, the phage particles were purified with PEG-NaCl for next biopanning.

Biopanning. The microtitration plate (Corning) was coated with 100 μg/mL clenbuterol conjugated to ovalbumin (CBL−OVA) and blocked with 5% skim milk. After washing with PBST, about 10^{11} pfu/mL recombinant phages were added to wells (100 μL/well) and incubated for 2 h at 37 °C. The wells were washed 20 times with PBST and 20 times with PBS to discard the unbound phage particles. One hundred microliters of log phase *E. coli* TG1 was added to wells and reinfected at 37 °C for 1 h. The infected cells were plated on an LB agar plate containing 100 μg/mL ampicillin and 2% glucose (LB-AG) and incubated overnight at 30 °C. The panning procedure was handled for only one round.

Screening of Recombinant Phage Antibody Clones. Single clones were first checked for full-length inserts by colony PCR. The individual clones containing full-length inserts were then used for producing scFv-displaying phages as describing as above. The antigen-positive clones were identified by phage-ELISA. Briefly, microtitration plates were coated with 1 μg/mL CBL−OVA or OVA (negative control) and blocked with 2% skim milk. Phage solution diluted with equivalent blocking buffer was added to the plate and incubated for 2 h at 37 °C. After washing 4 times with PBST, 100 μL of HRP-conjugated anti-M13 antibody (Amersham Biociences) diluted 1:5000 in PBST to detect bonded recombinant phage scFv was added and incubated for 1 h at 37 °C. After washing as above, 100 μL of substrate H_2O_2/3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well for color develop-

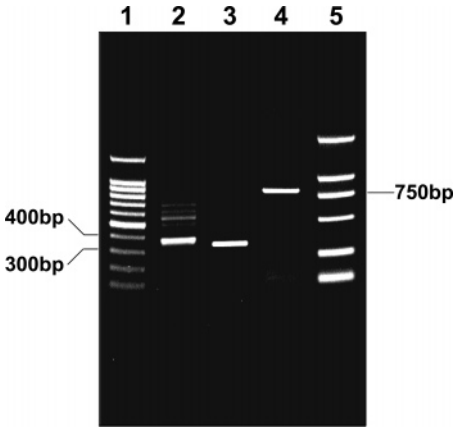


Figure 2. V_H and V_L PCR amplification and assembly scFv. Lanes: 1 and 5, DNA ladder; 2, V_H PCR product (about 350 bp); 3, V_L PCR product (about 320 bp); 4, scFv PCR product (about 760 bp).

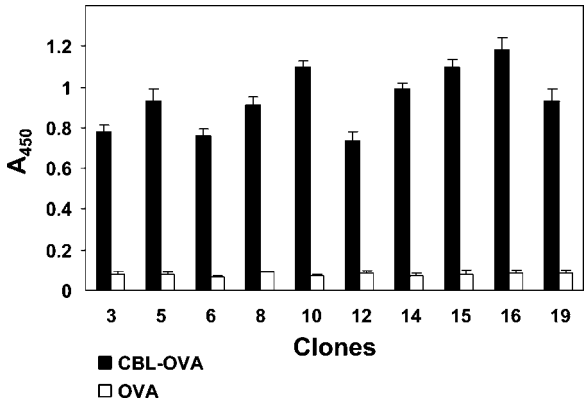


Figure 3. Phage ELISA to determine the specificity of 10 individual clones recovered after the one round of panning. Ten random selected clones were isolated, and recombinant phages from each clone were produced separately. Each of them was reacted to two coating antigens in parallel, namely, CBL–OVA and OVA (negative control). All phages from different clones bound to CBL–OVA only but not to OVA.

ment and the reaction was stopped with 10% H_2SO_4. The absorbance was determined at 450 nm with a microtiter plate reader (LT2, Biocell).

Expression of Soluble ScFv Fragments. Recombinant phages from antigen-positive clones were used to infect *E. coli* HB2151 for production of soluble scFv antibodies. The infected *E. coli* HB2151 cells were cultured in 2×YT medium supplemented with 100 μg/mL ampicillin at 30 °C with 250 rpm shaking until they reached an OD_{600} of 0.5. Isopropyl β-D-thiogalacoside (IPTG) was then added to 1 mM final concentration, and the cells were incubated overnight on a shaker at 30 °C. The culture solution was centrifuged at 4000*g* for 10 min at 4 °C. Both supernatants and pellets were collected. Periplasmic scFv was extracted by osmotic shock (*16*). The scFv from both supernatant and periplasm was detected by using SDS−PAGE and Western blotting, as well as indirect ELISA.

SDS−PAGE and Western Blotting. SDS−PAGE and Western blotting were performed as previously reported (*17*). The expressed proteins were probed with HRP-anti-E tag (Amersham Biociences) diluted 1:8000 in PBST. The visualizing bands were then developed with the electrogenerated chemiluminescence (ECL) method.

Indirect ELISA. The indirect ELISA was used to detect the concentration of soluble scFv in supernatant or periplasm. Microtitration plates were coated and blocked as described above. One hundred microliter volumes of gradient diluted supernatants or periplasmic extracts were added to wells, and the mixtures were incubated for 1 h at 37 °C. After washing 4 times with PBST, 100 μL of HRP-anti-E tag diluted 1:8000 in PBST was added, and the mixture was incubated for 1 h at 37 °C. After washing, the color was developed and the absorbance value was determined as above.

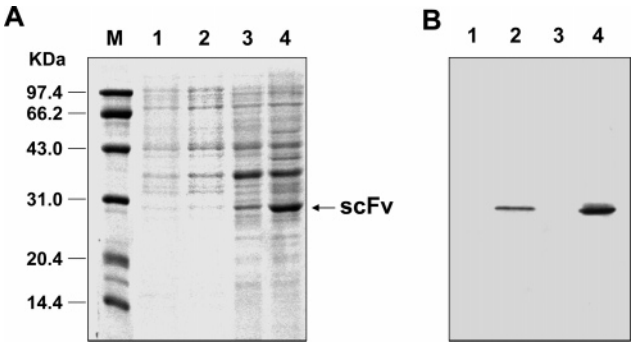


Figure 4. SDS–PAGE and Western-blotting analysis of soluble scFv expression. (A) SDS–PAGE analysis: M, low molecular weight protein standards; lane 1, supernatant of negative control (host cells alone); lane 2, supernatant of host cells containing recombinant plasmid and induced by IPTG; lane 3, periplasmic extract of negative control; lane 4, periplasmic extract of host cells containing recombinant plasmid and induced by IPTG. (B) Western blotting of duplicate gel of A.

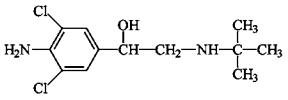
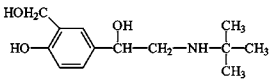
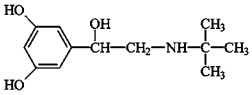
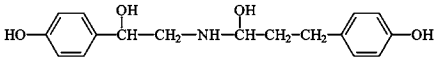
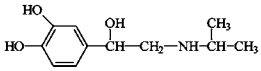
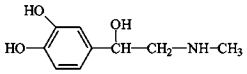
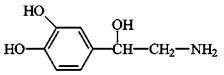
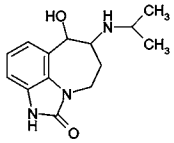
Characterization of the Soluble ScFv. The sensitivity and specificity of soluble scFv were determined by competitive indirect ELISA (CI-ELISA). Supernatant sample and MAb were determined in parallel. Microtitration plates were coated and blocked as described above. Next, serial diluted CBL standards or its structure analogues (50 μL) were added to each coated well and simultaneously incubated with 50 μL of supernatant or MAb properly diluted in PBST. The plates were incubated for 1 h at 37 °C and then washed 4 times with PBST. The amount of soluble scFv or MAb bound was determined by incubation with 100 μL of HRP-anti-E tag conjugate (for scFv) or HRP-goat-anti-mouse IgG conjugate (for MAb) (Amersham Biociences) diluted 1:8000 in PBST, and the color was developed as above. The absorbance value was measured and the data were fitted with a four-parameter logistic equation to determine the value of IC_{50}. The IC_{50} was defined as the concentration of inhibitor required inhibiting color development by 50% compared to control wells containing no competitors. The cross reactivity was calculated as the ratio of $IC_{50(CBL)}$ to $IC_{50(analogue)}$.

RESULTS AND DISCUSSION

PCR Amplification. V_H and V_L genes from hybridoma cell lines secreting CBL MAb were amplified by PCR and assembled into a scFv-encoding DNA fragment. As shown in **Figure 2**, amplification of V_H generated a major DNA fragment with approximate 350-bp length, while V_L generated an expected 320-bp fragment. PCR assembling and amplification of scFv produced an expected fragment with 760-bp length. This fragment was used for next ligating.

Phage Displaying of ScFv Antibody, Biopanning, and Screening. The gel-purified scFv gene fragments were digested and ligated into phagemid pCANTAB5E and then transformed *E. coli* TG1 cells, which is an amber suppressor strain. In TG1 cells, the amber stop coden (TAG) between scFv E tag DNA sequence and g3p can be read through. In the presence of helper phage M13KO7, the scFv E tag-g3p fusion protein was displayed on the surfaces of the recombinant phage, allowing for biopanning and affinity screening. In this study, few clones showed positive to CBL in phage-ELISA before panning (data not shown). However, after one round of panning, 10 of 20 clones contained inserts and were shown to be antigen-positive. Meanwhile, the positive recombinant scFvs were only specific to CBL but not to carrier protein OVA (**Figure 3**). Hybridomas are good sources of mRNA for facile cloning of variable region gene of immunoglobulin; the V_H and V_L genes could be directly cloned from hybridomas' mRNA without using further panning (*18*−*22*). However, in many hybridomas, some nonfunctional

Table 2. Cross Reactivity of the ScFv with CBL Analogues[a]

substance	chemical structure	% cross reactivity	
		MAb	ScFv
clenbuterol		100	100
salbutamol		<0.01	<0.01
terbutaline		<0.01	<0.01
ractopamine		<0.01	<0.01
isoproterenol		<0.01	<0.01
adrenalin		<0.01	<0.01
noradrenalin		<0.01	<0.01
zilpaterol		<0.01	<0.01

[a] % cross reactivity = $IC_{50\,(\mathrm{CBL})} / IC_{50\,(\mathrm{analogues})}$

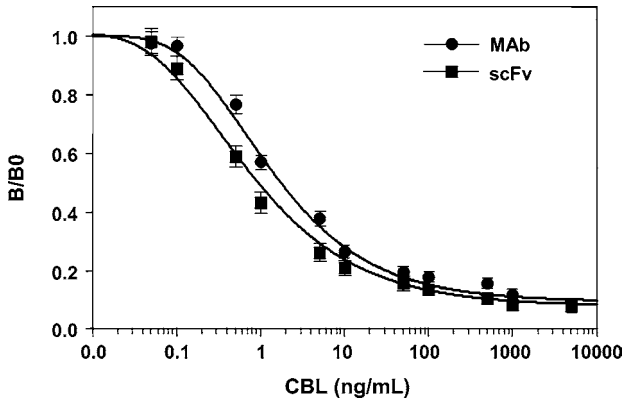


Figure 5. Competitive indirect ELISA (CI-ELISA) to determine sensitivity of soluble scFv. Parent MAb and scFv were compared. The data were fitted with a four-parameter-logistic equation to calculate the IC_{50}. (–●–) MAb. The IC_{50} was 1.34 ± 0.006 ng/mL. (–■–) ScFv. The IC_{50} was 0.78 ± 0.005 ng/mL.

immunoglobulin variable regions that do not recognize the target antigen might be expressed and could also be amplified during PCR. Moreover, mutant could be generated in the PCR or assembly reaction, and this condition would also result in abortive gene products (*17*, *23*). In spite of using hybridomas that produce high-affinity antibodies as sources of RNA, panning was still necessary for improving the chances of screening the antibody of interest with desired specificity.

Soluble ScFv Expression and Analysis. The antigen-positive recombinant phages were used to infect the nonsuppressor strain *E. coli* HB2151 for producing soluble scFv. In HB2151 cells, the amber stop codon between E tag and g3p is read as stop codon and a soluble scFv-E tag fusion protein is produced as a consequence. The expression of scFv-E tag fusion protein was induced by IPTG. The soluble scFv that secreted into supernatant and periplasm was determined simultaneously. Approximately 29 kDa scFv protein could be detected only in a periplasmic extract sample compared to negative control (host cell alone) in SDS−PAGE analysis (**Figure 4A**). Nevertheless, the expected specific band both in the supernatant and in the periplasmic sample was detected in Western-blotting analysis (**Figure 4B**), demonstrating soluble scFv expressed both in supernatant and in periplasm. Indirect ELISA also showed a similar result. Supernatant sample produced a measurable signal even in 1:160 dilution, and the periplasmic extract sample was in 1:1600 dilution. Since the volume (about 50 mL) of supernatant was larger than the volume of periplasmic extract (about 2 mL), the absolute amount of scFv in supernatant may be higher than in periplasm. This phenomenon might be due to the leakiness of the outer membrane or a partial cell lysis of the host cell, resulting in the secretion of soluble scFv into culture medium

(*24*, *25*). Additionally, the primary sequence of scFv might also play an important role (*26*).

The Characterization of Soluble ScFv. The sensitivity and specificity of scFv were determined by CI-ELISA. As shown in **Figure 5**, the IC_{50} value of scFv to CBL was 0.78 ± 0.005 ng/mL ($n = 4$), which was less than that of MAb (1.34 ± 0.006 ng/mL ($n = 4$), **Figure 5**). These results suggested that the sensitivity of scFv was better than that of parent MAb. Several previous studies also demonstrated similar results (*19*, *27*−*29*). The increased sensitivity of the scFv fragment may be due to the difference of the number of antigen binding sites between the scFv fragment and MAb. In heterogeneous competition ELISA, incubation of bivalent MAb with hapten at low concentration would result in only one binding site occupied while another binding site was still available for capture of coating antigen. In contrast, antigen-bound monovalent scFv fragment would not be captured.

A limited number of reactive analogues were selected for cross-reactivity detection. As shown in **Table 2**, similar to parent MAb, all compounds showed little cross reactivity to CBL-scFv. This result indicated that the CBL-scFv shared a similar structure of the antigen binding site with the parent MAb. Both MAb and scFv against CBL showed no high cross reactivity to salbutamol and terbutaline, which shared a very similar chemical structure to CBL except for substituents in the benzene ring. This could be due to the fact that the substituents present on the aromatic ring differ widely in terms of both polarity and dimension (*30*).

Conclusions. In this study, an immunoglobulin variable fragment gene from hybridoma cell lines producing anti-CBL MAb was successfully cloned, and phage display scFv antibody libraries were constructed. After one round of biopanning, phage-displaying scFvs against CBL were selected. The positive recombinant phage was used for infecting *E. coli* HB2151, and the functional soluble scFv were expressed. The scFv showed greater sensitivity when compared with parent MAb (IC_{50} value of 0.78 ± 0.005 ng/mL for the scFv vs 1.34 ± 0.006 ng/mL for the MAb, $p < 0.05$). As an alternative to antiserum or MAb, the recombinant scFv produced in this study could be used to develop a rapid and sensitive immunoassay, especially competitive ELISA for detection of CBL.

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