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Construction, Bacterial Expression, and Characterization of Hapten-Specific Single-Chain Fv and Alkaline Phosphatase Fusion Protein[1]

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We have designed and constructed a bacterial expression vector to produce a fusion protein of hapten-specific single-chain Fv (ScFv) and alkaline phosphatase (PhoA) in *Escherichia coli*. The ScFv gene was assembled using genes encoding the heavy and light chain variable domains of anti-NP (4-hydroxy-3-nitrophenyl acetyl) mouse monoclonal antibody. The ScFv gene was then fused to the 5′ terminus of the *E. coli* PhoA coding region. The expressed fusion protein ScFv(NP)-PhoA was purified using an NP affinity column, and gel-filtration. Characterization of the fusion protein was then performed. The estimated molecular weight by gel filtration was approximately 151 kDa, suggesting the dimerization of the protein. Kinetic constants of ScFv(NP)-PhoA were calculated and compared with those of wild-type PhoA. The k_{cat} values of ScFv(NP)-PhoA and wild-type PhoA were 103 (s^{-1}) and 96.1 (s^{-1}), respectively, showing that PhoA activity was somewhat increased by tethering the molecules. The equilibrium binding constant of ScFv(NP)-PhoA was determined using two different haptens, NP-capronate and NIP(3-iodo-4-hydroxy-5-nitrophenyl acetyl) by means of fluorescence quenching measurements. The obtained binding constants were 2.2×10^5 (M^{-1}) for NP-capronate and 1.0×10^6 (M^{-1}) for NIP, respectively. No apparent difference in binding constants was seen between ScFv(NP) and ScFv(NP)-PhoA, showing that sufficient specificity and binding affinity were retained when ScFv(NP) was tethered to alkaline phosphatase. ScFv(NP)-PhoA can be used to detect nanogram concentrations of NP-BSA in ELISA without the use of chemically conjugated secondary antibodies.

Key words: alkaline phosphatase, antibody engineering, fusion protein, NP, single-chain Fv.

Immunoassays are commonly used to detect minute amounts of analytes in various diagnostic tests. They are based on the specific association of antigens and antibodies, and analytes are detected with specific antibody labeled with sensitive reporter molecules. Horseradish peroxidase (HRP) and alkaline phosphatase (PhoA) are most commonly used as labeling enzymes, and they are mostly conjugated chemically to antibodies by means of chemical crosslinking reagents (*1, 2*). However, these procedures may yield randomly cross-linked molecules, and require several steps to obtain heterogeneous immuno-enzyme conjugates. Constructing genetically engineered fusion proteins of enzymes and antibodies is one of the attractive alternatives to prepare the immuno-enzyme conjugate, and the construction of bifunctional protein using alkaline phosphatase (homo-dimeric enzyme) has been extensively attempted (*3-5*).

In this study, we have designed and constructed a bacterial expression system for a fusion protein of alkaline phosphatase and anti-NP antibody. Anti-NP (4-hydroxy-3-nitrophenyl-acetyl) antibody has been reported to function as a very efficient affinity tag (*6, 7*). In addition, it can used as a regulator of the enzymatic function (*8*). So far, eukaryotic expression of bifunctional proteins of anti-NP antibody and enzyme have been reported (*8-10*). Although these hybrid enzymes can be purified easily on an NP-Sepharose column without loss of activity, the production cost has been comparatively high due to the low expression level of the protein in the expensive media. Another drawback is the large molecular weight, which hampers their use as efficient tagging counterparts. To overcome these problems, we constructed single-chain antibody Fv (ScFv) which comprises only V_L and V_H domains, but contains the complete antigen binding site. ScFv is a recombinant molecule in which the V_L and V_H fragments are connected

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Abbreviations: BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IgE, immunoglobulin E; IgG, immunoglobulin G; IPTG, isopropyl β-D-thiogalactoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; NIP, 3-iodo-4-hydroxy-5-nitrophenyl acetyl; NP, 4-hydroxy-3-nitrophenyl acetyl; PBS, phosphate-buffered saline; ScFv, single-chain Fv, V_H and V_L domains of antibody variable fragments connected with peptide linker.

by a peptide linker (*11-13*). Because ScFvs can bind cognate antigens or haptens with an affinity approaching to those of their parent antibodies, many applications have been studied using this small molecules. For example, to generate bifunctional fusion proteins, ScFvs have been successfully fused to various heterologous protein moieties, such as *Pseudomonous* exotoxin A (*14*), or beta-lactamase (*15*).

In this study, we conjugated V_H and V_L fragments with a helix-formed peptide linker 205 (*16*), between the C-terminus of V_L and the N-terminus of V_H, then the ScFv fragment was inserted into the N-terminus of alkaline phosphatase mono-domain. The ScFv(NP)-PhoA fusion protein was thus designed, expressed in *Escherichia coli* with good yield, and purified efficiently by the use of affinity resin. The antigen-binding ability and enzymatic activity of the recombinant fusion protein were almost identical to those of parent molecules. Our system represents an advance in the preparation of PhoA-labeled ligands, and the fusion protein was shown to have a useful bifunctional properties, which enabled direct colorimetric detection of nanogram amount of NP-BSA in microtitration plate wells.

MATERIALS AND METHODS

Materials—Oligonucleotides were synthesized using a 380A automated DNA synthesizer (Applied Biosystems, Tokyo). All amplifications of DNA were done using *Pyrococcus furiosus* (*Pfu*) DNA polymerase obtained from Stratagene (La Jolla, CA, USA). Polymerase chain reaction (PCR) was performed in a DNA Thermal Cycler PV-2000 (Perkin-Elmer, Tokyo). The strain of *E. coli* used for propagation of DNA was XL1-blue (Stratagene). DNA sequences were determined using a Hitachi DNA sequencer SQ-5500 (Hitachi, Tokyo). Cell densities and alkaline phosphatase activity was measured using a Hitachi spectrometer U-2000 (Hitachi). The chemicals and other reagents, unless otherwise indicated, were obtained from Sigma (St. Louis, MO, USA) or Kanto Chemicals (Tokyo).

Cloning of λ1 cDNA—Poly(A) containing RNA was isolated from J558L mouse myeloma cell line (*17*) by the guanidinium isothiocyanate method and with oligo dT-latex (Takara, Kyoto), and this was used for first strand λ1 cDNA synthesis following a standard protocol (*18*). Two primers used for PCR amplification were pLC5 (5′-GCG TCG ACT TAT GGC CTG GAT TTC ACT T-3′) and pLC3 (5′-TCC CCG GGC CGC GTG GGT AAA ATG AAG GTT AG-3′). Fifty picomoles each of the primers, 10 ng of cDNA, 10 mM dNTPs, 20 mM Tris-HCl pH 8.2, 10 mM KCl, 2 mM $MgCl_2$, 6 mM $(NH_4)_2SO_4$, 0.1% Triton® X-100, 10 ng/ml nuclease-free BSA (Pharmacia, Tokyo), and 2.5 units of *Pfu* DNA polymerase in 100 ml of reaction mixture were used for PCRs. The reaction was carried out for 30 cycles of denaturation (1 min at 92°C), annealing (2 min at 37°C), and extension (3 min at 72°C). The amplified fragment was digested with *Xho*I and *Not*I, cloned into Bluescript KS+ (Stratagene), and designated pBSλ1.

The plasmid was sequenced by the dideoxy method (US Biochemicals, USA).

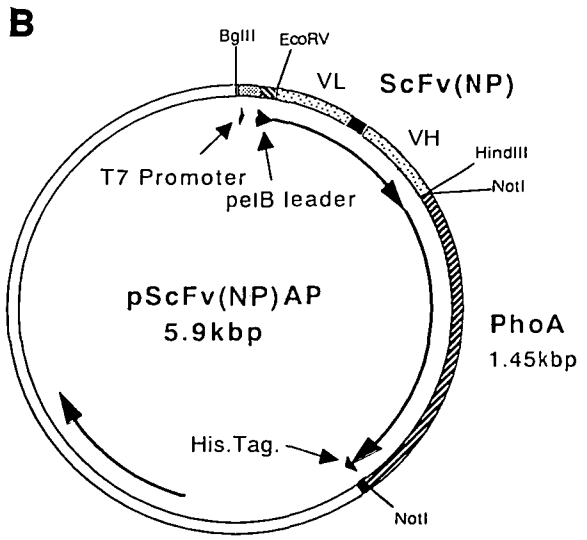
Assembly of the ScFv[NP(4-Hydroxy-3-Nitrophenyl-Acetyl)] Genes—Type 205 linker (5′-SSADDAKKDDAK-KDDAKKDDAKKDG-3′) (*16*) was used for connecting V_H and V_L domains. PCR primers encoding this region were synthesized and the linker fragment was introduced into both the V_H and V_L domains in separate PCR reactions. Primers SCAVHFor (5′-CTT AAG GAC TCA CCT GAG G-3′) and SCAVHBack (5′-GCA AAG AAG GAT GAT GCT AAA AAA GAC GAC GCT AAA AAA GAC GGA CAG GTC CAA CTG CAG CAG C-3′) were used for amplification of the V_H domain with pSV-Vm1 plasmid, encoding the V_H region, as the template. Primers SCAVLFor (5′-ATC ATC CTT CTT TGC GTC GTC TTT TTT AGC GTC GTC AGC AGA AGA TAG GAC AGT CAG TTT GGT TC-3′) and SCAVLBack (5′-CGC CAT GGC CTG GAT TTC ACT T-3′) were used for amplification of the V_L domain with pBSλ1 as the template. PCR was carried out for 25 cycles under the same conditions as above. Both gel-purified amplified fragments were taken and another PCR was carried out for 7 cycles without primers, and with primers SCAVHFor (5′-CTT AAG GAC TCA CCT GAG G-3′) and SCAVLBack (5′-CGC CAT GGC CTG GAT TTC ACT T-3′) for 25 cycles. The amplified fragment of 0.8 kb was cloned into pGEM-T vector (Promega, WI) and designated pGEMSCA.

Preparation of Expression Plasmid for E. coli Alkaline Phosphatase—The chromosomal DNA of *E. coli* XL1-blue was extracted by the standard procedure (*18*). Using this DNA as the template, the 1,450 bp fragment encoding alkaline phosphatase subunit (the gene: PhoA, EC 3.1.3.1) was amplified by PCR. *Not*I sites were introduced into both termini using oligonucleotides PhoAD5Not (5′-CCG CGG CCG CGG GTA CCC CAG AAA TGC CTG TTC TAG AAA-3′), and PhoAD3Not (5′-AAG CGG CCG CCT TAA GCC CCA GAG CGG C-3′) as PCR primers. The PCR was carried out for 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (2 min at 72°C), with 10 ng of chromosomal DNA of *E. coli* XL1-blue as the template in 100 ml of reaction mixture containing 50 pmol each of the primers, and 2.5 units of *Taq* DNA polymerase (Takara). The obtained fragment of the desired length was gel-filtrated, digested with *Not*I, and purified by agarose gel electrophoresis. The expression plasmid pET20b (Novagen, WI, USA) was digested with *Not*I, treated with the phosphatase from bovine small intestine (Takara), and ligated with the alkaline phosphatase DNA fragment, resulting in plasmid pPhoA. The pET20b (Novagen, WI, USA) contains an IPTG inducible T7 promoter and the 5′ coding sequence specifies the *pelB* signal peptide, followed by a sequence encoding 6×His affinity tag, which allows the isolation of recombinant protein *via* metal-based affinity resins.

Assembly of the ScFv(NP) and ScFv(NP)-PhoA Gene—The 791bp ScFv(NP) fragment was obtained by PCR with VLNPRVBack (5′-TTT GAT ATC CAG GCT GTT GTG ACT CAG GAA-3′) and VHNPHindFor (5′-AAA TTC GAA CCT GAG TGG ACT CCT CTG ACA-3′), having *Eco*RV and *Hin*dIII sites, respectively, as primers, and pGEMSCA as the template. The PCR was carried out for 35 cycles under the same conditions as for pPhoA. After digestion with *Eco*RV and *Hin*dIII restriction enzymes, the 777 bp ScFv(NP) fragment was inserted into plasmid pET20b (Novagen, WI, USA) at the *Eco*RV/*Hin*dIII sites, resulting in plasmid pScFv(NP). The ScFv(NP) fragment was also inserted into plasmid pPhoA at the *Eco*RV/*Hin*dIII sites, resulting in pScFv(NP)-PhoA. Figure 1 shows a schematic structure of pScFv(NP)-PhoA.

Expression and Purification of ScFv(NP)-PhoA and

Fig. 1. **Schematic representation of the pScFv(NP)-PhoA expression plasmid.** A: Nucleotide and amino acid sequences of the ScFv(NP) fragment in pScFv(NP)-PhoA. The fragment was cloned into pPhoA at the N-terminus of alkaline phosphatase with restriction enzyme sites *Eco*RV and *Hin*dIII. B: Construction of the expression plasmid. The gene encoding ScFv(NP)-PhoA is under the control of the IPTG inducible T7 promoter. The plasmid pET20b has a coding sequence specifying the 5′-*pelB* signal peptide and 3′-6×His affinity tag.



ScFv(NP) Protein—The *E. coli* strain used as the host was BL21(DE3)LysS, which has T7 RNA polymerase on its genome, and contains the pLysS plasmid, which codes for T7 lysozyme (*19*). The pScFv(NP) and pScFv(NP)-PhoA were transformed into the host cells by the calcium chloride method, and the transformed cells were cultivated at 37°C overnight in LB medium (*18*) containing 1.5% agar and antibiotics (50 mg/liter ampicillin and 34 mg/liter chloramphenicol). A single colony was picked and grown overnight at 28°C in 5 ml of LB medium containing antibiotics (50 ml/liter ampicillin and 34 mg/liter chrolamphenicol), and the cells were collected by centrifugation. The collected cells were resuspended again in 50 ml of fresh LB medium containing antibiotics (50 mg/liter carbenicillin and 34 mg/liter chloramphenicol) and cultivated for 3 to 4 h at 28°C. Then the culture were transferred to 1 liter of fresh LB medium containing the same antibiotics. When the cell density reached at A_{600} of 0.3-0.4, 0.1 mM of IPTG was added to induce the expression of the ScFv(NP)-PhoA and ScFv(NP) proteins. The cells were cultured overnight, and 20 ml of the culture liquid was used for SDS-polyacrylamide electrophoresis (SDS-PAGE) in order to confirm the expression of target protein.

Cells were collected by centrifugation and lysed by sonication (output; approximately 60 W, duty cycle per second; 0.5 s exposure and 0.5 s non-exposure, carried out on ice) in 20 ml sonication buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 100 mM NaCl: pH 8.0). Lysates were cleared by centrifugation (15,000 rpm, 20 min, 4°C), and the supernatant containing soluble ScFv(NP)-PhoA and ScFv(NP) was collected and applied to an NP-cap-Sepharose column (*8*) of 2 ml bed volume. The column was washed with 20 ml of wash buffer (0.1 M Tris-HCl, pH 7.5, 0.2 M NaCl), and bound proteins were eluted with 12 ml of elution buffer [0.5 mg/ml NP-cap-OH (Cambridge Research Biochemicals) in 1 M Tris-HCl, pH 8.0] in 1 ml fractions. The fractions containing ScFv(NP) and ScFv(NP)-PhoA were pooled in 18 mm cellulose dialysis tubing (Sanko Junyaku, Tokyo) and powdered PEG6000 was placed around the tube to concentrate the solution. The fractions were concentrated to 1-2 ml, then subjected to the gel filtration on a 50 ml Sephadex G-100 column (Pharmacia). The proteins were separated with Tris buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl) and collected in 2 ml fractions. Fractions containing ScFv(NP) and ScFv(NP)-PhoA were pooled in 18 mm cellulose dialysis tubing, and concentrated as above. Then the fraction was dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.0, and 50 mM NaCl. The yield of the purified proteins from 1 liter LB medium were approximately 800 mg for ScFv(NP) and 750 mg for ScFv(NP)-PhoA fusion proteins.

Expression and Purification of PhoA-His6 Protein—Plasmid pPhoA was transformed into *E. coli* BL21(DE3)-LysS and protein was expressed by the same method as in

the case of ScFv(NP) and ScFv(NP)-PhoA. Cells were collected by centrifugation and lysed by sonication in 20 ml of sonication buffer with under the conditions mentioned above. The lysate was cleared by centrifugation, and the supernatant containing soluble proteins was collected and applied to a Talon™ metal affinity column (Clontech, CA, USA) of 2 ml bed volume. The column was washed with 20 ml of the sonication buffer, and bound proteins were eluted in 1 ml fractions with elution buffer (50 mM NaH_2PO_4, 20 mM MES, 100 mM NaCl, pH 5.0). The fractions containing PhoA-His6 were pooled in 18 mm cellulose dialysis tubing and concentrated as above. Then the protein was dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Final concentration of PhoA-His6 was 247 mg/ml.

Expression and Purification of Anti-NP IgG2b Monoclonal Antibodies—Preparation of transfectoma Jγ2b-11: The cell line J558L, which is mouse plasmacytoma, transfected with anti-NP IgG2b expression vector pSV-Vγ2b that produces anti-NP IgG2b monoclonal antibodies, will be described elsewhere. The cells were cultured in RPMI-1640 medium containing 5% (v/v) FCS in a humidified 5% CO_2 incubator at 37°C. After cell growth, the culture supernatant was collected and applied directly to the NP-cap-Sepharose column prepared as above. The column was washed with 20 ml of washing buffer (0.1 M Tris-HCl, pH 7.5, 0.2 M NaCl), and bound proteins were eluted in 1 ml fractions with elution buffer [0.5 mg/ml NP-cap-OH (Cambridge Research Biochemicals, Cambridge, UK) in 1 M Tris-HCl, pH 8.0]. The fractions containing IgG2b were pooled in dialysis tubing and concentrated. Then the fraction was dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and used for the binding constant determination.

Molecular weight determination: To estimate the molecular weight of ScFv(NP)-PhoA fusion protein, we analyzed the protein by FPLC with a Superose 12 HR 10/30 column (Pharmacia). Lysozyme (MW = 14 kDa), carbonic anhydrase (MW = 30 kDa) and the High Molecular Weight marker (Pharmacia) were used as standards. Approximately 100 μg of the purified protein was applied to the column with the buffer containing 0.1 M Tris-HCl and 0.05 M NaCl, pH 7.5, at the flow rate of 0.5 ml/min, and the elution profile was monitored in terms of the UV absorbance at 214 nm.

Enzymatic Activity and Kinetic Parameters of the PhoA-His6 and the ScFv(NP)-PhoA Protein—Alkaline phosphatase activity was assayed as the rate of the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP, Wako, Osaka) to *p*-nitrophenol. The reaction was monitored by following the increase in absorbance at 410 nm ($E = 1.62 \times 10^4\ M^{-1} \cdot cm^{-1}$) in a solution of *p*-nitrophenyl phosphate, 1 M Tris-HCl, 10 mM $MgSO_4$, 5×10^{-5} M $ZnSO_4$, pH 8.0, 25°C. Determination of the kinetic parameters was performed by monitoring initial reaction velocities at several substrate concentrations from 5×10^{-4} to 5×10^{-5} M. The values of V_{max} and K_m were determined by using Hanes-Woolf (s/v-s plot) analysis. All the assays were performed according to the method described by Tyler-Cross *et al.* (*20*).

Binding Affinities—The binding affinity of *E. coli*-expressed ScFv(NP)-PhoA protein to NP was roughly estimated using microtitration plates. Individual wells of a microtitration plate were coated with 100 ml of 100 mg/ ml NP-BSA (*8*), and the plate was incubated at 37°C for 1 h, washed three times with PBS and blocked overnight at 4°C with blocking solution (2% skim milk in PBS). After removal of the blocking solution, a dilution series of fusion protein in PBS was added to the plate in dual sets of wells and the plate was incubated at 30°C for 2 h. It was then washed three times with PBS, and alkaline phosphatase activity was assayed with 50 ml of 1×10^{-3} M *p*-NPP in 1 M Tris-HCl, 10 mM $MgSO_4$, 5×10^{-5} M $ZnSO_4$, pH 8.0. The color development was measured with a Sjeia auto reader Model ER-8000 (Sanko Junyaku, Tokyo) at 410 nm.

Fluorescence Quenching—Binding affinity of the ScFv-(NP)-PhoA fusion protein was measured by fluorescence quenching (*21*) using a Hitachi fluorescence spectrometer 850, and the binding constant was determined. Binding constants of IgG2b and ScFv(NP) were also determined. The excitation and emission wavelength were 295 and 340 nm respectively, and the temperature was maintained at 25°C. Since the fluorescence of tryptophan residues at the antigen-binding site of antibodies quenches by antigen-binding to antibody, and a decrease in the fluorescence of antibodies was taken as being proportional to NP-cap-OH (or NIP) occupancy of the antigen-binding sites.

Antibody solutions were diluted to 100 nM in PBS, filtered with a 0.45-mm pore size cellulose acetate membrane filter, and titrated with hapten (NP-cap-OH or NIP) in the range of 0.2–50 mM. Titration was carried out by adding hapten stock solution to antibody solution (2.5 ml) in a quartz cuvette. As a negative control, purified human IgG (Zymed, CA, USA) was titrated in parallel.

Application to ELISA—In order to confirm that the fusion protein ScFv(NP)-PhoA can be used as an immunoassay tool, Sandwich ELISA was performed. Individual wells of a microtitration plate were coated with 1 mg/ml of chicken anti-BSA monoclonal antibody (Immunsystem, Sweden). The plate was incubated at 37°C for 1 h, washed three times with phosphate-buffered saline containing 0.2% (v/v) Tween 20 (PBS-T), and blocked overnight at 4°C with 2% skim milk in PBS. After removal of the blocking solution, 50 ml/well of serially diluted NP-BSA was added to triplicate sets of wells, and the plate was incubated at 37°C for 1 h. It was then washed three times with PBS-T, and 50 ml of 4 mg/ml ScFv(NP)-PhoA was added to each well. The plate was incubated at 37°C for another 1 h, and washed three times with PBS-T. Alkaline phosphatase activity was assayed with 50 ml of *p*-nitrophenyl phosphate disodium salt (1 mg/ml) in 1 M Tris-HCl, pH 8.0, containing 0.5 mM $MgCl_2$. The color development was measured at 410 nm.

RESULTS

Fusion proteins of two useful functions, such as antibody and enzyme, have been drawing many attractions as bifunctional immunological tools. We noticed the effective functions of anti-NP antibody as efficient affinity tag, as well as alkaline phosphatase is a sensitive reporter enzyme, and constructed the bifunctional fusion protein which has both antigen-binding ability and alkaline phosphatase enzymatic activity.

Expression of PhoA-His6, ScFv(NP), and ScFv(NP)-PhoA—In order to obtain the target protein, the expression plasmids pPhoA, pScFv(NP), and pScFv(NP)-PhoA were

transformed into the *E. coli* strain BL21(DE3)LysS (see "DISCUSSION"). Single colonies were grown, protein expression was induced with IPTG, and bacterial lysates were prepared. To confirm the expression of the target proteins, SDS-PAGE analysis was performed for total bacterial proteins. Figure 2A shows the Coomassie Blue-stained gel of the whole proteins expressed under induced and non-induced conditions. A prominent band was visible on the gel even under non-induced conditions for PhoA-His6 and ScFv(NP). These proteins were able to be expressed in the absence of IPTG, presumably, since the T7 promoter is strong and leaky. In the case of ScFv(NP)-PhoA fusion protein, no prominent protein was visible under non-induced conditions, while a strong band of approximately 80 kDa was visible under induced conditions. Judging from the molecular weight, this band seemed to be the target fusion protein ScFv(NP)-PhoA. Since the expression plasmid pET20b contains a sequence encoding 6×His affinity tag, each expressed protein should have six histidines at the 3′ terminal end, and we refer to the expressed alkaline phosphatase as PhoA-His6, in order to distinguish it from the native enzyme.

To confirm that ScFv(NP)-PhoA protein retains both antigen-binding ability and enzymatic activity, a cell lysate was incubated in microtitration plates coated with NP-BSA. Alkaline phosphatase activity of bound protein was revealed by using *p*-nitrophenyl phosphate (*p*-NPP). Specific color development was observed, which implied that the protein has the two independent activities (data not shown). PhoA-His6 and ScFv(NP) were also confirmed to retain their own biological functions. The cell lysate containing PhoA-His-6 was mixed with equivalent amount of 1 mM *p*-NPP solution (1 mM in 1 M Tris-HCl, pH 8.0 containing 0.1 mM $MgCl_2$), and significant color development was observed compared with that of BL21(DE3)LysS without plasmid, implying that PhoA-His6 retains its intrinsic enzymatic activity. NP binding affinity of ScFv-(NP) and IgG2b(NP) was roughly confirmed by ELISA using anti-mouse lambda chain conjugated with horseradish peroxidase as a secondary antibody. Apparent absorbance was observed for both proteins, suggesting that ScFv(NP) and IgG2b(NP) also retain their binding abilities (data not shown).

Purification of PhoA, ScFv(NP), and ScFv(NP)-PhoA Proteins—Based on the observation that the ScFv(NP)-PhoA fusion protein retained binding affinity to NP, NP affinity column was utilized for the protein purification. Fusion protein was purified using an NP-Sepharose column, and two major bands (one of them approximately 80 kDa, and the other, less than 30 kDa) were visualized in Coomassie Blue-stained gel on SDS-PAGE after purification (Fig. 2B, lanes 3 and 4). Since the expected size of the ScFv(NP)-PhoA fusion protein was 77 kDa, gel filtration was performed to eliminate the smaller band. Subsequent SDS-PAGE analysis showed one distinct band corresponding to ScFv-(NP)-PhoA, on the Coomassie Blue-stained gel (Fig. 2B, lanes 5 and 6). The same purification procedure was employed for ScFv(NP) protein and a distinct band of 27 kDa was visible on the Coomassie Blue-stained gel on SDS-PAGE after purification. PhoA-His6 protein was purified to homogeneity using a Talon™ metal affinity column, and the purified protein of 50 kDa was confirmed to be homogeneous by SDS-PAGE (data not shown).

Since native alkaline phosphatase is active in dimeric form (*22, 23*), it is natural to think that ScFv(NP)-PhoA also forms a dimer. The molecular weight of ScFv(NP)-PhoA was estimated as 151 kDa by size exclusion gel chromatography (Fig. 3). Since the calculated molecular weight of ScFv(NP)-PhoA monomer is 77 kDa, we can conclude that ScFv(NP)-PhoA is a dimer.

Enzymatic Activity—In order to evaluate the enzymatic activity of the alkaline phosphatase, the kinetic parameters of both ScFv(NP)-PhoA and PhoA-His6 proteins were determined. The initial velocity of the reaction at different substrate concentrations were measured by color development, and kinetic parameters K_m and V_{max} were calculated

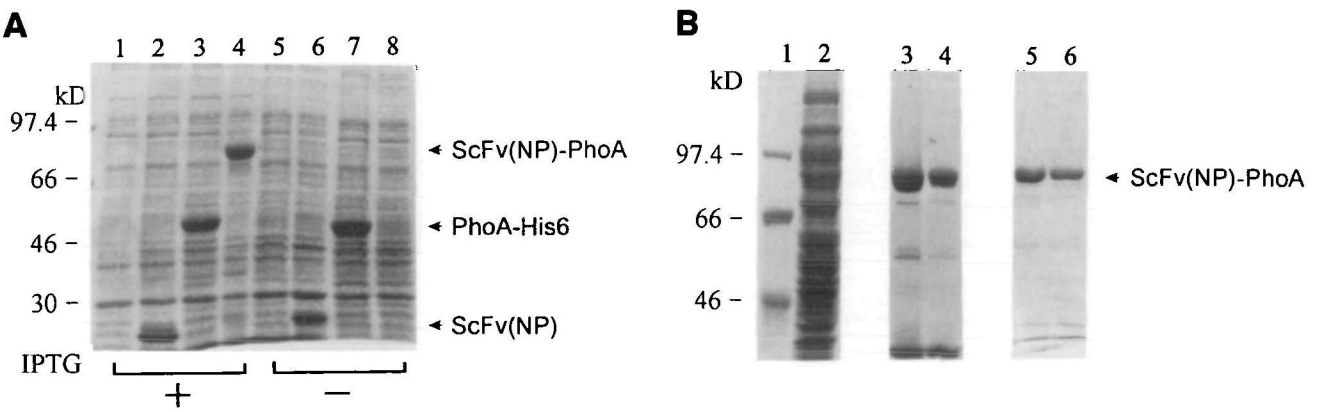


Fig. 2. **Expression and purification of pScFv(NP)-PhoA fusion protein.** A: Analysis of the expressed proteins by SDS-PAGE. All cells from 20 μl of overnight culture were directly subjected to SDS-PAGE. Cells were collected by centrifugation, boiled for 2 min with 2-mercaptoethanol and SDS, and subjected to 7.5% polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue (CBB-R250). Lanes: 1-4, induced with 0.1 mM IPTG; 5-8, without IPTG; 1 and 5, BL21(DE3)pLysS, pET20b; 2 and 6, BL21(DE3)-pLysS, pScFv(NP); 3 and 7, BL21(DE3)pLysS, pPhoA; 4 and 8, BL21(DE3)pLysS, pScFv(NP)-PhoA. The positions of the molecular weight markers are indicated. B: CBB-stained band from SDS-PAGE shows fractions during the purification process of ScFv(NP)-PhoA. ScFv(NP)-PhoA was purified to homogeneity by NP-affinity purification and gel filtration. Conditions of the SDS-PAGE were the same as above. Lanes: 1, low molecular weight marker (Pharmacia); 2, total soluble protein; 3 and 4, affinity-purified protein on a NP-Sepharose column (eluted fractions); 5 and 6, elutes from gel filtration.

from an s/v-s plot based on Michaelis-Menten steady-state kinetics (Fig. 4). The k_{cat} values were calculated from these parameters. The data are summarized in Table I. The V_{max} for ScFv(NP)-PhoA (40.1 mmol/min/mg) is 30% lower than that of PhoA-His6 (57.3 mmol/min/mg), but this is mainly because of their molecular weight difference [ScFv(NP)-PhoA: 77.0 kDa×2, PhoA-His6: 50.3 kDa×2). ScFv(NP)-PhoA exhibits a higher apparent affinity for the substrate than PhoA-His6, as is reflected in the K_m value, which is slightly lower for ScFv(NP)-PhoA (14.8 mM) than for PhoA-His6 (22.8 mM), but no apparent difference was observed in k_{cat} values between PhoA-His6 (96.1 s^{-1}) and ScFv(NP)-PhoA (103 s^{-1}). The results show that alkaline phosphatase enzymatic activity was not affected by the PhoA protein fused to ScFv(NP).

Binding Affinity—The equilibrium binding constants of the ScFv(NP)-PhoA fusion protein with two different haptens, NP-capronate and NIP, was evaluated by a fluorescence quenching technique (Table II). Binding constants of anti-NP IgG2b monoclonal antibody and monomeric single chain antibody ScFv(NP) were also determined as controls.

The reported equilibrium constants of mouse anti-NP IgE for NP-cap and NIP-cap are 8.3×10^5 (M^{-1}) and 5.0×10^7 (M^{-1}), respectively (*24*). In our study, the equilibrium constants of IgG2b(NP) were 3.3×10^5 (M^{-1}) for NP-cap and 1.98×10^6 (M^{-1}) for NIP. The values for NP-cap are similar, but for NIP (or NIP-cap), the value of IgG2b(NP) was almost 30-fold lower than that of IgE. The ScFv(NP), and ScFv(NP)-PhoA proteins had similar affinities for either hapten (NP-cap or NIP), which were 30-50% lower than that of IgG2b(NP). Affinities of these proteins for NIP were 4-5 times higher than for NP-cap, and this tendency

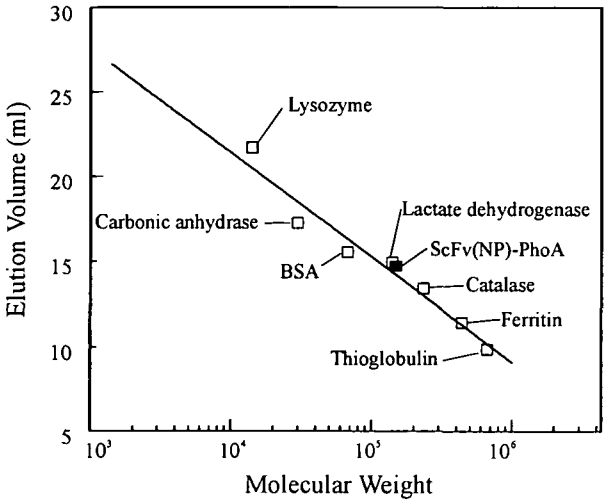


Fig. 3. **Size estimation of ScFv(NP)-PhoA.** The ScFv(NP)-PhoA fusion protein was eluted at a position corresponding to 151 kDa, reflecting dimerization of the fusion protein. Size markers used were thioglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), BSA (67 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa).

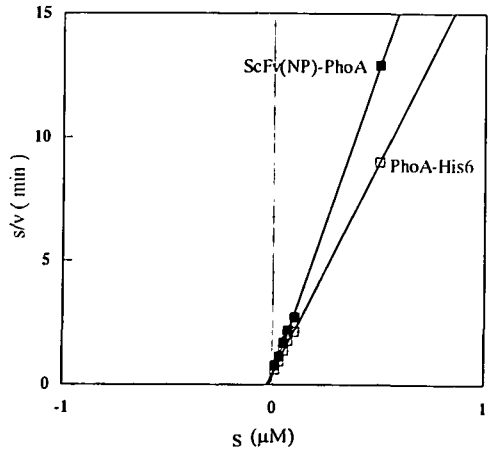


Fig. 4. **Hanes-Woolf (s/v-s plot) analysis of alkaline phosphatase activity.** (■) ScFv(NP)-PhoA fusion protein, (□) PhoA-His6. The initial velocities [v] were obtained from the reaction curves of each substrate concentration [s]. The enzymatic reaction was performed as described under "MATERIALS AND METHODS."

TABLE I. **Kinetic parameters of alkaline phosphatases.** Kinetic parameters V_{max}, K_m, k_{cat} were calculated based on Michaelis-Menten steady-state, kinetics by using the s/v-s plot shown in Fig. 3.

	V_{max} (μmol/min/mg)	K_m (μM)	k_{cat} (1/s)
phoA-His6	57.3	22.8	96.11
ScFv(NP)-PhoA	40.1	14.8	103

TABLE II. **Equilibrium binding constants of IgG2b, ScFv(NP), and ScFv(NP)-PhoA for the haptens NP-capronate and NIP.** The affinities were determined by fluorescence quenching with excitation at 295 nm and emission observed at 340 nm.

	K_a (NP-cap) (1/M)	K_a (NIP) (1/M)
ScFv(NP)-PhoA	2.20×10^5	1.00×10^6
ScFv(NP)	2.70×10^5	1.00×10^6
IgG2b	3.03×10^5	1.98×10^6

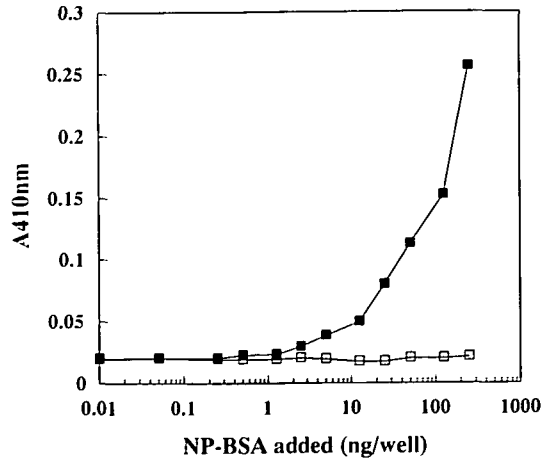


Fig. 5. **Sandwich ELISA of NP-BSA.** Chicken anti-BSA antibody was absorbed on a microtitration plate, and increasing concentrations of NP-BSA were added. Subsequently, 50 μl of 4 μg/ ml ScFv(NP)-PhoA fusion protein was added. The bound protein was assayed by measuring alkaline phosphatase activity. Assays were carried out in triplicate and the values shown are means: (■) in the presence of NP-BSA, (□) in the absence of NP-BSA.

is similar to that in the case of IgG2b(NP).

Application to ELISA—In order to confirm that the ScFv(NP)-PhoA fusion protein can be used as an immunoassay tool, sandwich ELISA was performed using NP-BSA as a detection sample, and ScFv(NP)-PhoA as the detection reagent. Because the fusion protein contains intrinsic alkaline phosphatase activity, no enzyme-conjugated secondary antibody was required in the ELISA. Nanogram amounts of NP-BSA could be detected by *p*-NPP color development, as shown in Fig. 5.

DISCUSSION

Fusion proteins have been drawing many attractions because of their potential applications. Antibody-enzyme conjugates are the best-studied bifunctional proteins, and are used mainly as immunological tools (*3-5, 25, 26*). However, it is not always the case that both moieties of the fused protein retain their biological functions. We have shown here that a fusion protein consisting of a single-chain antibody Fv and *E. coli* alkaline phosphatase, retains both biological activities when expressed in *E. coli*. Similar gene-fusion protein with alkaline phosphatase activity and antigen binding activity have been reported. Ducancel *et al.* reported the preparation of Fab fragment-alkaline phosphatase fusion protein [F(ab)2-PhoA] using an IgG2a monoclonal antibody, Mα2-3 (*3*), while Weiss *et al.* have reported the efficient preparation of a similar fusion protein of Fab fragment and alkaline phosphatase (Fab-PhoA) using an IgG1 monoclonal antibody recognizing human tumor necrosis factor α (*4*). Wang *et al.* have also reported the fusion of a single Fc binding domain with alkaline phosphatase (*5*). All those fusion proteins retained both antigen binding ability and enzymatic activity, as did our conjugate. However there are a few respects in which our fusion protein [ScFv(NP)-PhoA] differs from the others.

First, we chose NP as the antigen of the ScFv. NP is an inorganic compound, which can be conjugated to either nucleic acids or proteins. Therefore, by labeling targeted protein or nucleic acid with NP, facile detection of the target molecule is made possible by detecting the NP label. We have demonstrated that ScFv(NP)-PhoA fusion protein can be applied to sandwich ELISA, as shown in Fig. 5. This fusion protein should also be applicable to Northern (or Southern) hybridization as a detection tool of the probed DNA (or RNA).

Another point is that we used V_L-V_H type ScFv as the antibody domain. Generally single-chain antibody (ScFv) is composed of antibody light and heavy chain variable domains tethered by a designed peptide linker. Because of their small size and ease of extraction from bacterial expression systems. ScFvs are attractive in various types of physicochemical research (*12, 27, 28*). Recently Tsumoto *et al.* constructed two types of ScFvs of anti-lysozyme monoclonal antibody (HyHEL-10), which were V_H-V_L and V_L-V_H in series. They reported that ScFv of V_L-V_H showed a dramatically higher expression level in *E. coli* than that of V_H-V_L. They also reported that interaction between ScFv and antigen was also more favorable in V_L-V_H (*29*). Bird *et al.* and others have previously constructed hapten-specific V_L-V_H type ScFvs and various linker peptides were designed for these ScFvs by computational methods and computer graphics (*11, 16, 30*). The 205 type peptide linker, which was used in our study, is a helix-forming linker with 25 amino residues, and was reported to be the most stable among those examined in terms of binding affinity, stoichiometry, and conformational stability (*16*). Thus, the ScFv we have constructed was successfully expressed and retained its intrinsic affinity.

E. coli BL21 competent cells are hosts specifically designed for use in high-level protein expression, and three different strains that vary in repression level are available (*31*). At first, we used the strain BL21(DE3) as a host, but ScFv(NP) and ScFv(NP)-PhoA fusion protein were not expressed in this strain, while PhoA was expressed strongly. We concluded that ScFv could to be rather toxic within *E. coli*, and for the tight control of expression and prevention of leaky expression, we used BL21(DE3)LysS, which contains pLysS coding for T7 lysozyme (*19*). By using this strain, the proteins were successfully expressed in good yields.

From Fig. 2b, it is clear that affinity-purified ScFv(NP)-PhoA fusion protein is resolved into two major bands (approx. 77 and 27 kDa) by SDS-PAGE analysis. Since the calculated molecular weights of PhoA-His6 and ScFv(NP) are approximately 50 and 27 kDa, respectively, it is probable that the 77 kDa band is ScFv(NP)-PhoA fusion protein and the other band (27 kDa) is ScFv(NP). It is natural to think that some of the fusion protein may be cleaved by protease during purification, affording the two major bands observed in SDS-PAGE analysis. The yield of the purified ScFv(NP)-PhoA fusion protein from the soluble fraction was approximately 1 mg per 1 liter culture, and this is quite high compared with that of anti-NP antibody and enzyme fusion proteins expressed in mammalian cells (*9, 10*).

E. coli alkaline phosphatase is a periplasmic protein, which is active in a dimeric form (*22, 23*). The result of size exclusion gel chromatography showed that ScFv(NP)-PhoA fusion protein also intrinsically forms a dimer (Fig. 3). Several examples of truncated antibody-alkaline phosphatase fusion proteins have been already reported, and alkaline phosphatase genes are generally inserted from residues +7 of the polypeptide chain. In the case of our ScFv(NP)-PhoA fusion protein, the full-length alkaline phosphatase was inserted. It has been reported that, though alkaline phosphatase cleaved between residues +9 and +10 still retains its specific activity almost similar to that of the native enzyme (*20*), but still, there was a 6% activity reduction of the modified enzyme. In our study, the alkaline phosphatase was inserted at full length with no loss of specific activity. Furthermore, ScFv(NP)-PhoA fusion protein has a 6×His affinity tag at its C-terminal end. Addition of the extra polypeptide to the phoA might inhibit the enzymatic activity, but in our case, the addition didn't alter the specific activity (as shown in Table II). We evaluated alkaline phosphatase enzymatic activity by determination of kinetic parameters. Several standard plots have been proposed based on Michaelis-Menten steady-state kinetics, and we chose the Hanes-Woolf (s/v-s) plot for extracting the kinetic parameters. No significant difference was observed between the k_{cat} values of ScFv(NP)-PhoA (103 s^{-1}) and PhoA-His6 (96.1 s^{-1}), showing that the alkaline phosphatase activity was unaffected by the extra molecule fused to its end.

The binding affinity of the ScFv(NP)-PhoA fusion protein

to NP-cap and NIP was evaluated in terms of equilibrium binding constants. Although the molecular sizes and stable pairing of V_H and V_L chains were reduced, the NP binding affinities of ScFv(NP) and ScFv(NP)-PhoA were not significantly decreased compared with the recombinant anti-NP IgG-2b (Table II). Jones *et al.* have reported that both the V_H and V_L domains of anti-NP antibody have a role in determining the affinity of the hapten and the class of constant domains does not affect binding to the hapten (*24*). This is consistent with our results, and we could also say that there was no significant loss of binding affinity, even though the V_H and V_L domains were truncated. The ScFv(NP) was inserted at the N-terminus of PhoA, but stability of the hapten binding was still observed. The K_a value of ScFv(NP)-PhoA was slightly lower than that of the parent antibody, but almost the same as that of ScFv(NP). In our study, the NIP binding constants were 4-5 times higher than those for NP, which are approximately 1/10 of that in Jones' report. This could be caused by the differences of the hapten, since we used NIP as the hapten, while they used NIP-capronate.

Antibodies, which are chemically coupled to enzymes are most commonly used as ligands in immunoassays. In this study, we have shown that it is possible to use recombinant ScFv(NP)-PhoA fusion protein to replace enzyme-conjugated secondary antibodies in biological assays based on the immuno-detection of NP (or NIP).

REFERENCES

1. Halow, E. and Lane, D.P. (1988) *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York
2. Ternynck, T., Portsmann, T., and Avrameas, S. (1987) Enzyme immunoassay using a monoclonal antibody against 5-bromo-2-deoxyuridine for the assessment of lymphoid cell proliferation. *Methods Enzymol.* **150**, 117-129
3. Ducancel, F., Gillet, D., Carrier, A., Lajeunesse, E., Menez, A., and Boulain, J.C. (1993) Recombinant colorimetric antibodies: construction and characterization of a bifunctional F(ab)2/alkaline phosphatase conjugate produced in *Escherichia coli. Biotechnology* **11**, 601-605
4. Weiss, E., Chatellier, J., and Orfanoudakis, G. (1994) In vivo biotinylated recombinant antibodies: construction, characterization, and application of a bifunctional Fab-BCCP fusion protein produced in *Escherichia coli. Protein Exp. Purif.* **5**, 509-517
5. Wang, C.L., Huang, M., Wesson, C.A., Birdsell, D.C., and Trumble, W.R. (1994) A single Fc binding domain—alkaline phosphatase gene fusion expresses a protein with both IgG binding ability and alkaline phosphatase enzymatic activity. *Protein Eng.* **7**, 715-722
6. Nakayama, T., Arata, Y., and Shimada, I. (1993) A multinuclear NMR study of the affinity maturation of anti-NP mouse monoclonal antibodies: comparison of antibody combining sites of primary response antibody N1G9 and secondary response antibody 3B62. *Biochemistry* **32**, 13961-13968
7. Mizutani, R., Miura, K., Nakayama, T., Shimada, I., Arata, Y., and Satow, Y. (1995) Three-dimensional structures of the Fab fragment of murine N1G9 antibody from the primary immune response and of its complex with (4-hydroxy-3-nitrophenyl) acetate. *J. Mol. Biol.* **254**, 208-222
8. Ueda, H., Kikuchi, M., Yagi, S., and Nishimura, H. (1992) Antigen responsive antibody-receptor kinase chimera. *Biotechnology* **10**, 430-433
9. Williams, G.T. and Neuberger, M.S. (1986) Production of antibody-tagged enzymes by myeloma cells: application to DNA polymerase I Klenow fragment. *Gene* **43**, 319-324
10. Casadei, J., Powell, M.J., and Kenten, J.H. (1990) Expression and secretion of aequorin as a chimeric antibody by means of a mammalian expression vector. *Proc. Natl. Acad. Sci. USA* **87**, 2047-2051
11. Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., and Whitlow, M. (1988) Single-chain antigen-binding proteins [published erratum appears in *Science* 1989 Apr 28;244(4903): 409]. *Science* **242**, 423-426
12. Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. *et al.* (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85**, 5879-5883
13. Raag, R. and Whitlow, M. (1995) Single-chain Fvs. *FASEB J.* **9**, 73-80
14. Brinkmann, U., Lee, B.K., and Pastan, I. (1993) Recombinant immunotoxins containing the VH or VL domain of monoclonal antibody B3 fused to *Pseudomonas* exotoxin. *J. Immunol.* **150**, 2774-2782
15. Goshorn, S.C., Svensson, H.P., Kerr, D.E., Somerville, J.E., Senter, P.D., and Fell, H.P. (1993) Genetic construction, expression, and characterization of a single chain anti-carcinoma antibody fused to beta-lactamase. *Cancer Res.* **53**, 2123-2127
16. Pantoliano, M.W., Bird, R.E., Johnson, S., Asel, E.D., Dodd, S.W., Wood, J.F., and Hardman, K.D. (1991) Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in *Escherichia coli. Biochemistry* **30**, 10117-10125
17. Oi, V.T., Morrison, S.L., Herzenberg, L.A., and Berg, P. (1983) Immunoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA* **80**, 825-829
18. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning Ver. 2. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
19. Studier, F.W. (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **219**, 37-44
20. Tyler-Cross, R., Roberts, C.H., and Chlebowski, J.F. (1989) Proteolytic modification of *Escherichia coli* alkaline phosphatase. *J. Biol. Chem.* **264**, 4523-4528
21. Azuma, T., Sakato, N., and Fujio, H. (1987) Maturation of the immune response to (4-hydroxy-3-nitrophenyl)-acetyl (NP) haptens in C57BL/6 mice. *Mol. Immunol.* **24**, 287-296
22. Gettins, P. and Coleman, J.E. (1983) 31P nuclear magnetic resonance of phosphoenzyme intermediates of alkaline phosphatase. *J. Biol. Chem.* **258**, 408-416
23. Kim, E.E. and Wyckoff, H.W. (1991) Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. *J. Mol. Biol.* **218**, 449-464
24. Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., and Winter, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522-525
25. Wels, W., Harwerth, I.M., Zwickl, M., Hardman, N., Groner, B., and Hynes, N.E. (1992) Construction, bacterial expression and characterization of a bifunctional single-chain antibody-phosphatase fusion protein targeted to the human erbB-2 receptor. *Biotechnology* **10**, 1128-1132
26. Sun, S. and Lew, A.M. (1992) Chimaeric protein A/protein G and protein G/alkaline phosphatase as reporter molecules. *J. Immunol. Methods* **152**, 43-48
27. Reiter, Y., Brinkmann, U., Lee, B., and Pastan, I. (1996) Engineering antibody Fv fragments for cancer detection and therapy: Disulfide-stabilized Fv fragments. *Biotechnology* **14**, 1239-1245
28. Davis, G.T., Bedzyk, W.D., Voss, E.W., and Jacobs, T.W. (1991) Single chain antibody (SCA) encoding genes: one-step construction and expression in eukaryotic cells. *Biotechnology* **9**, 165-169
29. Tsumoto, K., Nakaoki, Y., Ueda, Y., Ogasahara, K., Yutani, K., Watanabe, K., and Kumagai, I. (1994) Effect of the order of antibody variable regions on the expression of the single-chain HyHEL10 Fv fragment in *E. coli* and the thermodynamic analysis of its antigen-binding properties. *Biochem. Biophys. Res. Commun.* **201**, 546-551
30. Bedzyk, W.D., Weidner, K.M., Denzin, L.K., Johnson, L.S., Hardman, K.D., Pantoliano, M.W., Asel, E.D., and Voss, E.W., Jr. (1990) Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody. *J. Biol. Chem.* **265**, 18615-18620
31. Grodberg, J. and Dunn, J.J. (1988) ompT encodes the *Escherichia coli* outer membrane protease that cleaves T7 Rna polymerase during purification. *J. Bacteriol.* **170**, 1245-1253