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# Noncompetitive Detection of Low Molecular Weight Peptides by Open Sandwich Immunoassay

Shean-Lee Lim,<sup>†</sup> Hiroko Ichinose,<sup>‡</sup> Tatsuya Shinoda,<sup>‡</sup> and Hiroshi Ueda<sup>\*,†,§,||</sup>

Department of Medical Genome Sciences, Graduate School of Frontier Sciences, and Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan, Kyowa Medex Co. Ltd., 600-1, Minami-Ishiki, Nagaizumi-cho, Sunto-Gun, Shizuoka, 411-0932, Japan, and PRESTO, JST, 5-Sanbancho, Chiyodaku, Tokyo, 102-0075, Japan

Small peptides with less than 1000 in molecular weight are not considered amenable to sandwich immunoassays due to their difficulty of simultaneous recognition by two antibodies. As an alternative, we attempted noncompetitive detection of small peptides by open sandwich enzyme-linked immunosorbent assay (OS-ELISA) utilizing the antigen-induced enhancement of antibody  $V_H/V_L$  interaction. Taking fragments of human osteocalcin (BGP), a major non-collagen peptide produced in bone, as model peptides, OS immunoassay was performed using the cloned  $V_H$  and  $V_L$  cDNAs from two anti-BGP monoclonal antibodies either recognizing the N- or C-terminal fragment, respectively. When the clones were used for OS-ELISA with immobilized  $V_L$  fragment and phage-displayed  $V_H$  fragment, enhanced  $V_H/V_L$  interaction upon BGP addition was observed. Especially the clone for the C-terminal fragment showed a superior detection limit as well as a wider working range than those of competitive assay. The result was reproduced with purified  $V_H$ -alkaline phosphatase and MBP- $V_L$  fusion proteins, where the latter was directly immobilized onto the microplate wells. The minimum detectable fragment was the hexamer including the C-terminus. This simple approach with a single monoclonal antibody with a short measurement time may prove a useful tool in immunodiagnostics as well as in proteomics research.

Peptides are the family of short molecules formed from the linking, in a defined order, of various amino acids, and play fundamental roles in our biosphere including the human body. Peptides have been receiving prominence in molecular biology for several reasons. Probably, the most important reason is that peptides allow the creation of antibodies in animals without the need to purify the protein of interest. One can simply make antigenic peptides of sections of the protein of interest and immunize animals as a conjugate with carrier proteins.

As a use of such antibodies, enzyme immunoassay is one of the most popular methods for the specific detection and quantification of various target molecules and is routinely utilized in clinical diagnostics. Generally, a noncompetitive immunoassay such as a sandwich enzyme-linked immunosorbent assay (ELISA) is a preferred method whenever possible, because the assay has several merits such as higher sensitivity and specificity, wider dynamic range, and lower background than competitive assay can perform.<sup>1</sup> However, conventional sandwich immunoassay has a fundamental limitation that the antigen to be measured must be large enough to have at least two epitopes to be captured. To our knowledge, the minimum peptide noncompetitively measured by sandwich immunoassay was octapeptide angiotensin II, with the use of an anti-metatype antibody<sup>2</sup> that can recognize an antigen-antibody complex.<sup>3</sup> However, because of the difficulty of making metatype antibodies, the number of reports based on this approach including that for non-peptide haptens is quite limited.<sup>4–6</sup> Another type of noncompetitive assay utilizing two types of anti-idiotypic antibodies (idiometric assay) is also reported, but the assay needs more steps and has not been applied for peptide detection.<sup>7,8</sup>

As a way to circumvent these limitations, previously we proposed a new immunoassay approach called the open sandwich immunoassay (OS-IA).<sup>9,10</sup> Briefly, this assay exploits the reassociation of the generally weak antibody variable region  $V_H$ - $V_L$  complex by a bridging antigen. With the use of immobilized  $V_L$  and enzyme-tagged  $V_H$  fragments, one can measure less than 10 ng/mL antigen in a shorter time period than by a conventional sandwich assay, due to the omission of an incubation/washing cycle. Also, the assay was found to be compatible with a number

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\* Corresponding author. E-mail: hueda@chembio.t.u-tokyo.ac.jp. Tel: +81-3-5841-7362. Fax: +81-3-5841-7362.

<sup>†</sup> Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo.

<sup>‡</sup> Kyowa Medex Co. Ltd.

<sup>§</sup> Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo.

<sup>||</sup> PRESTO, JST.

**Table 1. List of Primers**

MH1Back	5'-SARGTNMAGCTGSAGSAGTC-3'
MH2Back	5'-SARGTNMAGCTGSAGSAGTCWGG-3'
MH2Back-Sfi	5'-GTCCTCGCAACTGCGGCCAGCCGCCATGGCCSARGTNMAGCTGSAGSAGTCWGG-3'
MH2Back-RV	5'-GGGGGGATATCSARGTNMAGCTGSAGSAGTCWGG-3'
mulgVH3'-2	5'-CCCAAGCTTCCAGGGGCCARKGGATARACIGRTGG-3'
VH1For2	5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC-3'
VH1For2-Hind	5'-GGGGGAAGCTTGTCTCGAGACGGTGACCGTGGT-3'
Vk4BkFl2	5'-GACGACGATTAAGGAGATATCATATGGAYATTGTGMTSACMCARWCTMC-3'
MKCFor	5'-GGATACAGTTGGTGCAGCATC-3'
MkBack	5'-GAYATTGTGMTSACMCARWCTMCA-3'
MkBack2A	5'-GCGCAAGCTCAGTCGACGGAYATTGTGMTSACMCARWCTMCA-3'
MJK2FONX	5'-CCGTTTATTTCCAGCTTGGTGCC-3'
JK2NOT10	5'-GAGTCATTCTGCGGCCGCCCGTTTATTTCCAGCTTGGTGCC-3'
OlinkBack2	5'-GGGGCCAAGGGACACGGTCACCGTCTCGAGCGGTGCTGCTGACTAC-3'
LinkForA	5'-TCCGTCGACTGAGCTTGCACAAC-3'

of anti-hapten antibodies and could attain a similar or lower detection limit as well as a wider working range than attained with the corresponding competitive assay.<sup>11–14</sup> However, the applicability of OS-IA to short peptides has not been examined to date.

To seek the possibility of further expanding the scope of OS-IA, we attempted the detection of model peptides derived from human osteocalcin (also known as bone glutamic acid residue (Gla)-protein or BGP), a 49-amino acid peptide that is the major non-collagen protein of bone.<sup>15</sup> The mature osteocalcin peptide consists of two antiparallel  $\alpha$ -helical domains (residues 16–25 and 30–41) connected by a  $\beta$ -turn (residue 26–29) and stabilized by a Cys<sub>23</sub>–Cys<sub>29</sub> disulfide bond. It has three carboxylated Gla at positions 17, 21, and 24 that are known to mediate strong binding of osteocalcin to hydroxyapatite.<sup>16,17</sup> Osteocalcin level in blood is considered to reflect new protein synthesis, while the level of its fragments might be derived from bone resorption, and therefore, its measurement provides a valuable tool for assessing skeletal metabolism. The elevated levels of certain fragments in the serum also reflect various bone-related diseases.<sup>15</sup> However, due to its rather labile structure, the half-life of osteocalcin and its fragments in blood serum is relatively short. Therefore, it is important to have a rapid and sensitive immunoassay to diagnose bone-related diseases.

## MATERIALS AND METHODS

**Materials.** Mouse hybridoma KTM-219 and KTM-223 secreting anti-human BGP (hBGP) IgG were established by the fusion of murine myeloma P3-X63-Ag8-U1 with the thymocytes of the mice repeatedly immunized with a N-terminal or a C-terminal hBGP peptide conjugated with keyhole limpet hemocyanin. Clone KTM-223 was raised against the Cys-added N-terminal peptide

(NH<sub>2</sub>-YLYQWLGAHPVPPYPC-COOH) conjugate, whereas KTM-219 was against the C-terminal peptide (NH<sub>2</sub>-FQEAYRRFYGPV-COOH) conjugate. Synthetic intact hBGP (residues 1–49) was purchased from Princeton Biomolecules (Langhorne, PA), and the N-terminal and the C-terminal peptides were either from the Peptide Institute Inc. (Osaka, Japan) or Qiagen (Tokyo, Japan). Other hBGP C-terminal peptides were synthesized by Genscript (Piscataway, NJ).

*Escherichia coli* strain TG1 (GE Healthcare, Tokyo, Japan) was used for phage production and also for the production of MBP–V<sub>L</sub> protein, HB2151 (GE Healthcare) for V<sub>H</sub>-displaying phage/V<sub>L</sub> production, XL-10 Gold (Stratagene, La Jolla, CA) for recombinant DNA preparation, and BL21(DE3, pLysS) (Novagen, San Diego, CA) for the production of V<sub>H</sub>–AP fusion protein. The oligonucleotides synthesized by Texas Genomics (Tokyo, Japan) used in this study are listed in Table 1.

**Cloning of Antibody V-Genes.** The cDNAs for the antibody variable regions were amplified from total mRNA derived from mouse hybridomas using one-step RT-PCR kit (Qiagen) according to manufacturer's instructions. Primers MH1Back, MH2Back, and mulgVH3'-2 were used for the amplification of V<sub>H</sub> fragments, whereas Vk4BkFl2 and MKCFor were used for the amplification of V<sub>L</sub> fragments. The reaction conditions were 50 °C for 30 min, 94 °C for 15 min, and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by final extension at 72 °C for 6 min. The cDNAs were then gel purified using Wizard SV gel and PCR Clean-Up System kit (Promega, Madison, WI) and cloned into pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA) for sequence determination. Plasmids from several positive clones for each preparation were determined using Big Dye Termination v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and 3100 Genetic Analyzer (Applied Biosystems). Subsequently, nested PCR was carried out with the plasmid having target V-gene as a template using primers M13RV and VH1-For2 for V<sub>H</sub> fragments whereas, MkBack2A and JK2Not10 for V<sub>L</sub> fragments. PCR conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified V<sub>H</sub> and V<sub>L</sub> fragments were gel purified and stored at –20 °C until use. All PCR was performed using *Ex-Taq* DNA polymerase (Takara Bio, Shiga, Japan) unless otherwise indicated.

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**Construction of Split-Fv Fragment** The spFv linker was amplified from phagemid pKST2(HyHEL-10)<sup>11</sup> using primers OlinkBack2 and LinkForA. The gel-purified V<sub>H</sub>, V<sub>L</sub>, and spFv linker fragments were then assembled through overlap extension PCR at the ratio of 1:1:0.5–1 in the two separate cycles. The PCR conditions for the first cycle (excluding primers) were 94 °C for 3 min, 8 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min, and final extension at 72 °C for 5 min. The second cycle with extension primers was carried out at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 1.5 min, and final extension at 72 °C for 5 min. The assembled spFv fragments were then digested with restriction enzymes *Sfi*I and *Not*I, gel purified and ligated with phagemid pKST2 digested with the same using ligation high (Toyobo, Kyoto, Japan) as manufacturer's instructions. Either TG1 or HB2151 strain was transformed with the ligation products and plated on 2YAG agar (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.2 supplemented with 100 µg/mL ampicillin, 1% glucose, and 1.5% agar) plates overnight at 37 °C. For the subsequent splice overlap extension (SOE) PCR, the gel-purified bands were reamplified with primers MH1BackSfi<sup>11</sup> and VH1For-2 for V<sub>H</sub>, and with Mkbck2 and JK1/2/4/5Not10.<sup>18</sup> The gel-purified fragments were then assembled by SOE PCR using a split Fv linker including gene 9 that was preamplified with primers OlinkBack2 and LinkForA essentially as described.<sup>11</sup>

The purified spFv fragments were then digested with restriction enzymes *Sfi*I and *Not*I, gel purified again, and ligated with phagemid pKST2 digested with the same using ligation high (Toyobo, Osaka, Japan). The transformed TG1 or HB2151 cells were grown on 2YTAG agar plates overnight at 37 °C.

**Split-Fv Phage Display.** Single colonies were inoculated into 3 mL of 2YTAG and incubated overnight at 37 °C. The overnight culture was then 100-fold diluted with 2YTAG, incubated at 37 °C until OD<sub>600</sub> reached ~0.5, and then helper phage M13KO7 added to moi of 20. The cells were left undisturbed for 30 min at 37 °C and then centrifuged at 2500g for 15 min. The cells were resuspended in 2YTAK containing 100 µg/mL ampicillin and 50 µg/mL kanamycin and incubated overnight with shaking at 30 °C. For TG1, the overnight culture was centrifuged at 10800g for 10 min, and the supernatant containing spFv displayed phages was precipitated with 1/5 v/v PEG solution (20% PEG6000 and 2.5 M NaCl) after 1-h incubation on ice, at 11500g for 15 min. The pellet was resuspended in PBS, centrifuged at 10800g for 10 min to pellet cell debris, and the supernatant was stored at 4 °C. In the cases of using HB2151, the overnight culture containing soluble V<sub>L</sub>- and V<sub>H</sub>-displayed phages was centrifuged at 10800g for 10 min and supernatant was stored at 4 °C.

**Split-Fv Phage ELISA.** Falcon 353914 (BD Biosciences) 96-well microplate was incubated with 100 µL per well of either 5000-fold diluted anti-FLAG M2 monoclonal antibody (Sigma), 1 µg/mL of anti-myc 9E10 monoclonal antibody (Sigma), or various concentrations of intact hBGP in PBS for 1 h at 37 °C, and 25% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in PBS (BPBS) for 1 h at 37 °C, followed by 3 washes with PBS containing 0.1% Tween-20 (Sigma) (PBST). Then 100-µL samples of 50-fold diluted spFv phages (from TG1) in 10% BPBS were added to each well and incubated at 25 °C for 2 h, washed three times with PBST,

and incubated with 100 µL of 5000-fold diluted HRP-conjugated mouse anti-M13 monoclonal antibody (GE Healthcare) in 10% BPBS for 1 h at 25 °C. The microplate was then washed 6 times with PBST and developed with 100 µL of substrate solution (100 µg/mL 3,3',5,5'-tetramethylbenzidine (Sigma) and 0.04 µL/mL H<sub>2</sub>O<sub>2</sub> in 100 mM NaOAc, pH 6.0). The reaction was stopped with 50 µL of 1 M H<sub>2</sub>SO<sub>4</sub> after incubation between 5 and 30 min, and the absorbance was read at 450 nm with a reference at 655 nm using a microplate reader model 680 (Biorad, Tokyo, Japan).

**Indirect Competitive ELISA.** The 96-well microplate pre-coated with or without 100 µL per well of 2 µg/mL of intact hBGP for 1 h at 37 °C was blocked with 25% BPBS for 1 h at 37 °C and washed 3 times with PBST. Dilutions of C-terminal peptide were prepared in 10% BPBS, and 200 µL of each dilution was transferred into a 1.5-mL tube. Then 10-µL samples of spFv phages from TG1 or intact KTM-219 antibody (final 0.2 µg/mL) were added to respective tubes, and after a gentle mix, the tubes were left undisturbed at 25 °C for 30 min. Then 100 µL of the mixture was added into the respective wells in duplicate and incubated for 2 h at 25 °C. They were washed 3 times with PBST, and 100 µL of 5000-fold diluted HRP-conjugated mouse anti-M13 monoclonal antibody in 10% BPBS added to each well and incubated for 1 h at 25 °C. The microplate was then washed 6 times with PBST and developed as mentioned above. Dose–response curves were fitted to Hill equation to give IC<sub>50</sub> using Kaleida Graph 4.0 (Synergy Software, Reading, PA).

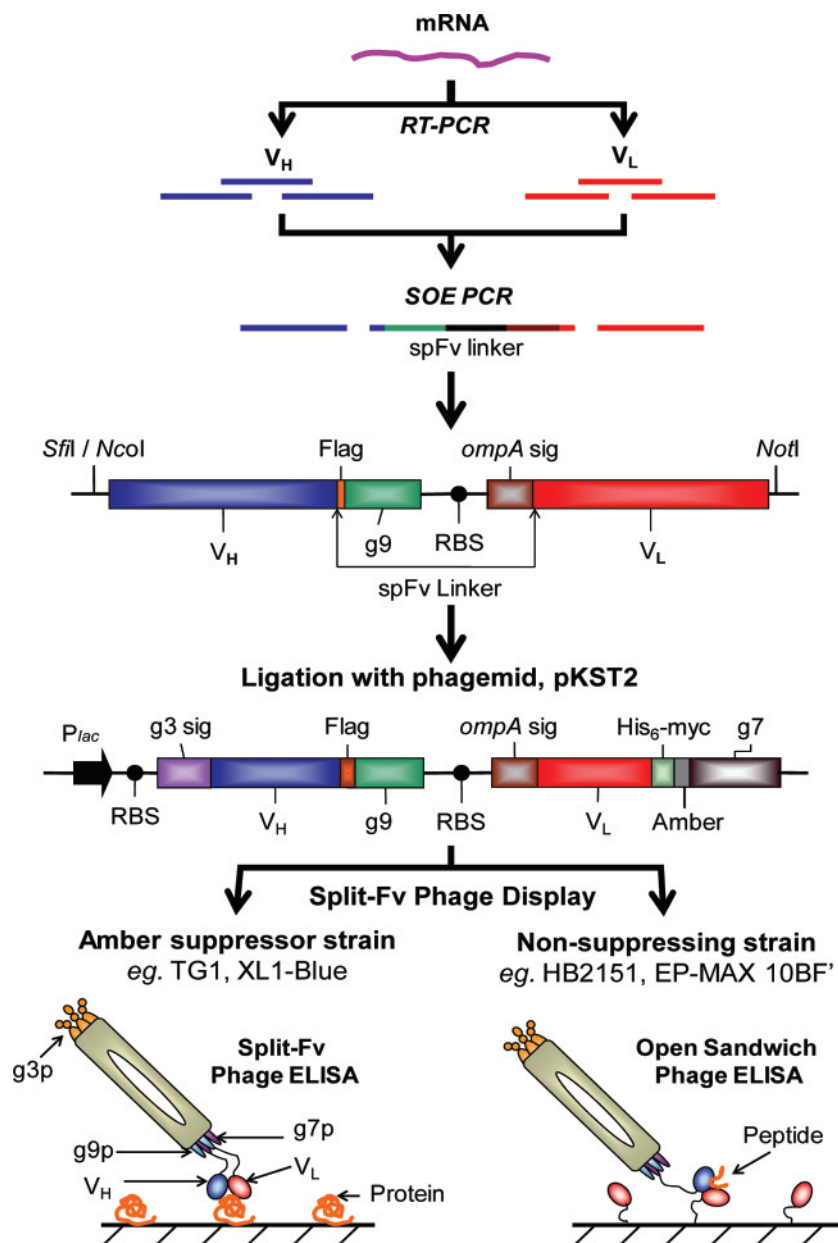
**Open Sandwich Phage ELISA.** The 96-well microplate was incubated with 100 µL per well of 1 µg/mL mouse anti-myc 9E10 monoclonal antibody (Sigma) or 0.5 µg/mL Penta-His monoclonal antibody (Qiagen), overnight at 4 °C, and blocked with 25% BPBS for 2 h at 25 °C. The microplate was then washed once with PBST, and 100-µL samples of intact hBGP or its N-/C-terminal peptide at various concentrations diluted in 2% MPBS were added to respective wells. Then 10 µL of culture supernatant from HB2151 was added to the respective wells and gently mixed. The microplate was incubated for 2 h at 25 °C and washed 3 times with PBST, and bound phages were detected as mentioned above.

**Expression and Purification of MBP–V<sub>L</sub> Fusion Protein.** The V<sub>L</sub>219 gene was obtained by the direct digestion of phagemid pKST2-KTM219 with *Sal*I and *Not*I. The purified V<sub>L</sub>219 fragment was ligated with pMAL-VL(ZEA) plasmid<sup>14</sup> digested with the same enzymes. *E. coli* TG1 was transformed with the ligation product, plated on 2YTAG agar, and incubated overnight at 37 °C. Several colonies were picked and plasmids were extracted for sequence determination.

Single colony with correct sequence was picked up and cultured in 4 mL of LBAG medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5 supplemented with 50 µg/mL ampicillin and 1% glucose) overnight at 30 °C. Subsequently, the culture was inoculated to 500 mL (2 × 250 mL) of enrichment medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 2 g/L glucose; pH 7.0) containing 100 µg/mL ampicillin and incubated at 30 °C until OD<sub>600</sub> reached 0.6–0.8. The expression of MBP–V<sub>L</sub> fusion protein was induced by addition of IPTG (Wako, Osaka, Japan) to a final concentration of 1 mM and incubated further for 5 h at 27 °C. The culture was centrifuged at 6,000 rpm, 4 °C for 10 min, and the bacterial pellet was resuspended with 50 mL of osmotic shock solution (30 mM Tris-

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**Figure 1.** Schematic flowchart showing the construction of spFv and spFv-phage display. The V-genes were amplified from total RNA by RT-PCR and assembled into split-Fv with split-Fv linker through overlap extension PCR, cloned into phagemid vector pKST2, and transformed into *E. coli* TG1 or HB2151 strain for phage display.

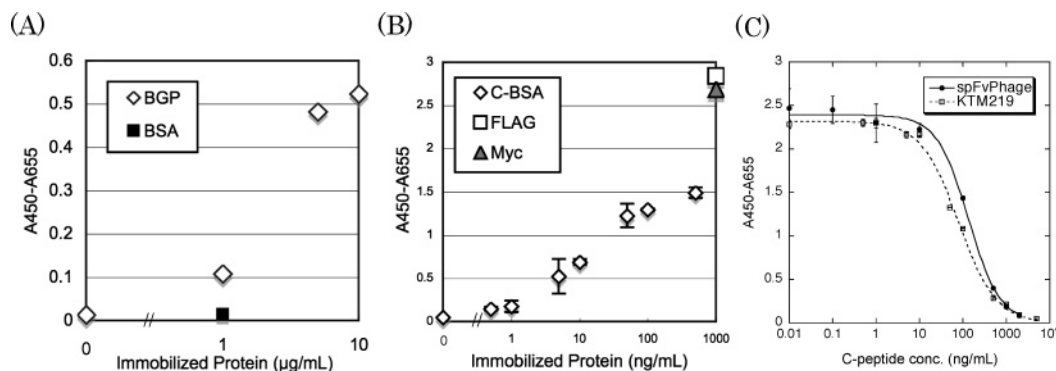
HCl, 20% sucrose, 1 mM EDTA, pH 8.0). The suspension was incubated at 25 °C for 10 min with frequent shaking followed by centrifugation at 8000g, 4 °C for 10 min. Supernatant was discarded, and 15 mL of ice-cold 5 mM MgSO<sub>4</sub> was added immediately to resuspend the cells. The suspension was incubated for 20 min at 4 °C or on ice with frequent shaking and centrifuged at 8000g, 4 °C for 15 min. The supernatant obtained was then dialyzed against column buffer (10 mM Tris-HCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, pH 7.4). The dialyzed periplasmic extracts were purified using 1 mL of Talon IMAC (Clontech, Takara-Bio) and eluted in 0.5-mL fractions using elution buffer (column buffer containing 150 mM imidazole). Eluates containing target protein were pooled and exchanged for its buffer using a PD-10 column (GE Healthcare) with TBS (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl, pH 7.4). The protein concentration was determined using

Bradford reagent (Biorad) and stored in small aliquots at -80 °C. The proteins were analyzed by 10% SDS-PAGE.

**Expression and Purification of V<sub>H</sub>-AP Fusion Protein.** The V<sub>H</sub> 219 gene was PCR amplified from phagemid pKST2(KTM-219) using primers MH2BackEcoRV and VH1For2Hind. The fragment was digested with *EcoRV* and *HindIII*, gel purified and ligated with pVH-PhoA(D101S),<sup>19</sup> which had been digested with the same. The ligation product was used to transform XL10-Gold and plated on LBAG plate, overnight at 37 °C. Several colonies were picked and plasmids were extracted for sequence determination.

*E. coli* BL21(DE3, pLysS) strain was transformed with a plasmid with correct sequence and plated on LBAC (10 g/L

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**Figure 2.** Binding of split-Fv phages displaying Fv of (A) KTM-223 and (B) KTM-219 to immobilized proteins indicated. Intact BGP and the C-peptide-BSA conjugate were immobilized as antigen (shown in open diamonds) for the phages displaying KTM-223 and KTM-219 Fv, respectively. Detected by HRP-labeled anti-M13. (C) Competitive ELISA of KTM-219 either displayed on phage as spFv (filled circle) or as an intact IgG (open square). Averages of two samples with 1SD are shown.

tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5 containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol) agar plate. After overnight incubation at 37 °C, a single colony was picked and inoculated into 4 mL of LBAC medium, overnight at 30 °C. Subsequently, 3 mL of the overnight culture was inoculated into 300 mL (2 × 150 mL) of LBAC medium and incubated at 30 °C until OD<sub>600</sub> reached 0.6–0.8. The expression of V<sub>H</sub>-AP fusion protein was induced with 0.2 mM IPTG, and the culture was incubated further for 18–24 h at 20 °C. The culture was centrifuged at 6000 rpm, 4 °C for 10 min. The bacterial pellet was resuspended with 40 mL of osmotic shock solution (30 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0) and incubated at 25 °C for 10 min with gentle shaking followed by centrifugation at 8000g, 4 °C for 10 min. Supernatant was discarded and 10 mL of ice-cold 5 mM MgSO<sub>4</sub> added to resuspend the cells. The suspension was incubated for 20 min at 4 °C or on ice with occasional shaking and centrifuged at 8000g, 4 °C for 15 min. The supernatant was then dialyzed against column buffer and purified with Talon IMAC as mentioned above.

**Open Sandwich ELISA Using MBP-V<sub>L</sub> and V<sub>H</sub>-AP Proteins.** The 96-well microplate was incubated with 100 μL of 5 μg/mL MBP-V<sub>L</sub> protein, overnight at 4 °C, and blocked with 25% Block Ace in TBS (BTBS) for 2 h at 25 °C. The microplate was washed once with TBS containing 0.05% Tween-20 (TBST), and 100-μL samples of hBGP or C-terminal peptide at various concentrations diluted in 10% BTBS were added to respective wells. Then 2 μL of 250 μg/mL purified V<sub>H</sub>-AP protein was added to each well. The microplate was incubated for 2 h at 25 °C, washed 3 times with TBST and 100 μL of substrate solution (1 mM *p*-nitrophenyl phosphate in 1 M Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 μM ZnCl<sub>2</sub>, pH 9.5) added to each well, and incubated for up to 30 min at 25 °C before being measured at its absorbance 405 nm.

In the case of chemiluminescent detection, a white 96-well microplate (Maxisorp, Nunc, Denmark) was incubated with 100 μL of 2.5 μg/mL MBP-V<sub>L</sub> protein in PBS, overnight at 4 °C, and blocked with 25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) in PBS (BPBS) for 1 h at 25 °C. The microplate was washed 3 times with PBST, and 50 μL of 2× sample at various concentrations diluted in PBS and 50 μL of 5 μg/mL purified V<sub>H</sub>-AP protein in 25% BPBS were added to each well. The microplate was incubated for 90 min at 25 °C, washed 5 times with PBST, once with CDP star buffer, and 100 μL of CDP star substrate

solution (New England Biolabs) added to each well and incubated for 30 min at 25 °C in dark, before being measured for chemiluminescence for 5 s using a microplate luminometer AB-2100 (ATTO, Tokyo, Japan).

## RESULTS AND DISCUSSION

### Cloning of Anti-hBGP Antibody Variable Region cDNA.

First, we cloned the gene for anti-BGP peptide antibody variable regions. We used two anti-hBGP monoclonal antibodies (IgG1, k) each recognizing its N-terminal (1–13) and C-terminal (38–49) peptides, respectively. Since the isotype of the antibodies was known, the V<sub>H</sub> and V<sub>k</sub> cDNAs were amplified using reverse-transcribed mRNA extracted from the hybridoma cells using leader/FR1 and constant region-specific primers.

The V<sub>H</sub> and V<sub>L</sub> genes of the anti N- and C-terminal peptide of osteocalcin antibodies were amplified from mRNA samples. However, since hybridoma cells often express more than one each of V<sub>H</sub>/V<sub>L</sub> genes often due to the fusion partner, screening of functional V<sub>H</sub>/V<sub>L</sub> genes is inevitable. To select for functional V<sub>H</sub>/V<sub>L</sub> genes, a phage display system that is suitable for both antigen-binding ELISA and OS ELISA was employed (Figure 1). Briefly, the system (split Fv, spFv system) uses M13 gene 9 and gene 7 proteins (g9p and g7p) for the display of V<sub>H</sub> and V<sub>L</sub>, respectively. In the system, an amber codon was placed between V<sub>L</sub> and gene 7; hence, when we use a suppressor *E. coli* strain such as TG-1 to produce phage particles, functional Fv is displayed on the phage. On the contrary, when a nonsuppressing strains such as HB2151 is used for phage production, V<sub>H</sub>-displaying phage and his-myc-tagged soluble V<sub>L</sub> fragment are produced.

To display V<sub>H</sub>/V<sub>L</sub> fragments on the phage, the genes were assembled to a split-Fv linker encoding gene 9 and inserted into a phagemid encoding gene 7 to display both V<sub>H</sub> and V<sub>L</sub> proteins on g9p and g7p, respectively. The ligated phagemid was used to transform TG-1, and phages displaying V<sub>H</sub>/V<sub>L</sub> were prepared. First, the display of V<sub>H</sub> and V<sub>L</sub> fragments on the g9p and g7p of the phage particles was determined by using anti-Flag and anti-myc tag antibodies immobilized on the microplate. The results show that both V<sub>H</sub> and V<sub>L</sub> fragments of KTM-223 and KTM-219 were properly displayed on the tip of the phage as high signals were observed from the wells coated with the two antibodies (Figure 2B for KTM-219, not shown for KTM-223). To clarify whether the displayed V<sub>H</sub> and V<sub>L</sub> fragments are functional and retain sufficient

**A**

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1  GAGGTACAGCTGGAGGAGTCTGGGGCTGAGTTTGTGAAGGCTGGGGCTTCAGTGAAGCTG  60
   E V Q L E E S G A E F V K A G A S V K L
61  TCCTGCAAGACTTCTGGCTACACCTTCAACAAACTACTGGATTCACTGGGTCAAACAGAGC  120
   S C K T S G Y T F N N Y W I H W V K Q S
121 CCAGGACAAGGCCTTGAATGGATCGGAGAAATTGATCCCTCTGATGGTTATTCTAACTAC  180
   P G Q G L E W I G E I D P S D G Y S N Y
181 AATCAAAATTCAAGGCAAGGCCACATTGACTGTGGACAAGTCCTCCAGCACTGCCTAC  240
   N Q K F K G K A T L T V D K S S S T A Y
241 ATGCACCTCAACAGTCTGACTTCTGAGGACTCTGCGGTCTATTATTGTACAAGCAGCACT  300
   M H L N S L T S E D S A V Y Y C T S S T
301 TCCGTAGGAGGTTCTGGGGCCAAGGGACCACGGTCACCGTCTCGAGC  348
   S V G G S W G Q G T T V T V S S

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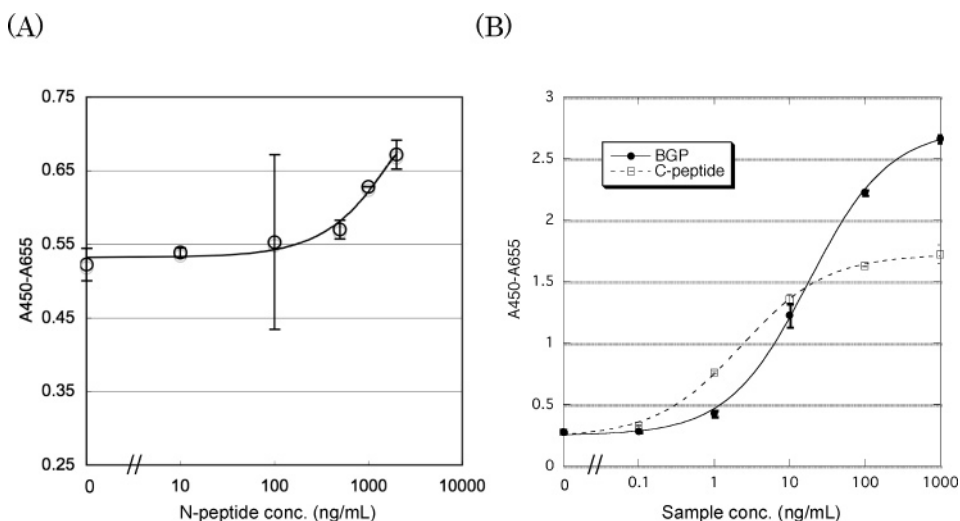
**B**

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1  GACATTGAGCTCACCCAGTCTCCACTCTCCCTGCCTGTCACTTCTGGAGATCAAGCCTCC  60
   D I E L T Q S P L S L P V S L G D Q A S
61  ATCTCTTGCACATCTAGTCAGAGCCTTCTACACAGTAATGGAGACACCTATTACATTGG  120
   I S C T S S Q S L L H S N G D T Y L H W
121 TACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACACACTTCCCAACCGTTT  180
   Y L Q K P G Q S P K L L I Y T L S N R F
181 TCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCGGGACAGATTTCACACTCAAGATC  240
   S G V P D R F S G S G S G T D F T L K I
241 AGCAGAGTGGAGGCTGCGGATCTGGGAATTTATTTCTGCTCTCAAACTACTCATGTTCCG  300
   S R V E A A D L G I Y F C S Q T T H V P
301 TACACGTTCGGAGGGGGACCAAGCTGGAAATAAAACGG  339
   Y T F G G G T K L E I K R

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**Figure 3.** Nucleic acid and amino acid sequences of V<sub>H</sub> (A) and V<sub>L</sub> (B) of KTM-219. Complementarity determining regions are shown in italics.



**Figure 4.** Dose–response curves for phage-based open sandwich ELISA with (A) KTM-223 for the N-peptide and (B) KTM-219 for hBGP and the C-peptide. Phages are detected with HRP-anti-M13. Averages of two samples with 1SD are shown.

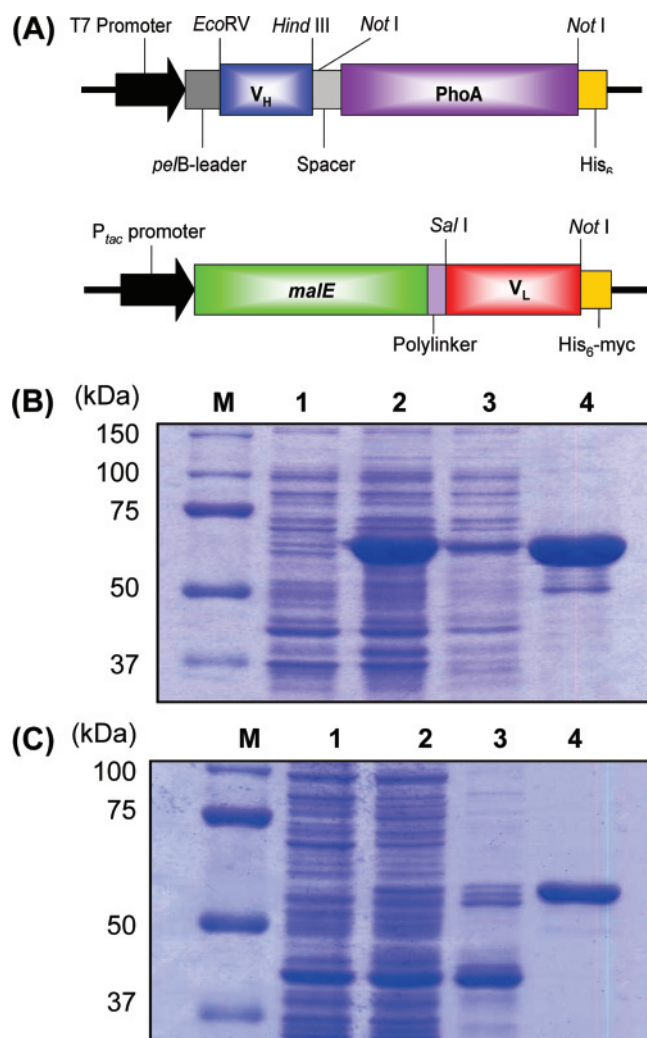
binding affinity toward their target antigen, phage ELISA was performed for the two split-Fv display phages. The results show positive correlations between the concentration of the immobilized antigen and the signal attained (Figure 2A and B), implying their functionality. However, phages displaying KTM-219 gave a considerably stronger binding signal to a smaller amount of antigen than KTM-223 phages.

**Indirect Competitive ELISA with KTM-219.** To further validate the cloned variable regions, indirect competitive phage ELISA for KTM-219 spFv was performed. Using phage-displayed spFv, competition between hBGP in sample and immobilized hBGP was probed with HRP-labeled anti-M13 antibody. As a comparison, detection was performed with KTM-219 and HRP-labeled anti-mouse IgG. As a result, almost the same detection limit and working range in competitive ELISA were obtained for both cloned spFv phage and IgG antibody (Figure 2C). The IC<sub>50</sub> and the detection range obtained were 135 ng/mL with ~30–1000 ng/mL for spFv phage, and 79 ng/mL with ~10–300 ng/

mL for IgG, respectively. Since the IgG gave almost the same competition curve with similar ED<sub>50</sub> concentration, we concluded that at least the cloned genes for the anti C-terminal KTM-219 variable regions were functional. The nucleotide and the deduced amino acid sequences for KTM-219 V<sub>H</sub>/V<sub>L</sub> are shown (Figure 3).

**Open Sandwich Phage ELISA.** By taking advantage of the split-Fv system, open sandwich ELISA was performed using the phage culture supernatant prepared with the HB2151 strain. To catch the secreted V<sub>L</sub> fragment in the culture supernatant, either anti-myc or anti-his antibody was coated on the microplate wells. The amount of phage particles bound to the wells through the V<sub>H</sub>/V<sub>L</sub> interaction in the presence of hBGP in the sample was evaluated using HRP-labeled anti-M13 antibody. For the anti N-terminal peptide antibody KTM-223 Fv, the result showed a modest N-terminal peptide dose-dependent increase in signal (Figure 4A). However, the sensitivity attained was relatively low, with high background and fluctuation in the signal probably due to strong interaction between V<sub>H</sub> and V<sub>L</sub> fragments in the absence

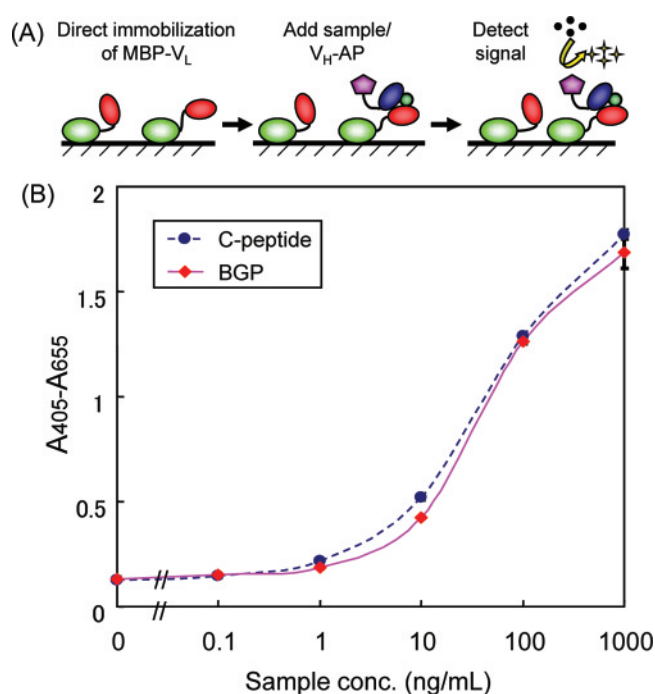




**Figure 5.** (A) Schematic representation of pET-V<sub>H</sub>-PhoA(D101S) and pMAL-V<sub>L</sub> expression vectors. (B) SDS-PAGE analysis for V<sub>H</sub>-219-AP and (C) that for MBP-V<sub>L</sub>219 protein. Lane M, marker; lane 1, total cell lysates prior induction with IPTG; lane 2, total cell lysate after IPTG induction; lane 3, periplasmic extract; and lane 4, TALON IMAC purified fusion proteins (V<sub>H</sub>-AP ~61 kDa, MBP-V<sub>L</sub> ~60 kDa).

of antigen. In contrast, for the anti C-terminal KTM-219 Fv, the results showed much lower background and clearer antigen-dependent increase in signal, for both intact hBGP and its C-terminal peptide as an antigen (Figure 4B). The lowest measurable concentration for both antigens was between 0.1 and 1 ng/mL, which was lower than that of the competitive ELISA for the C-terminal peptide (Figure 2C). The result clearly showed the suitability of KTM-219 Fv for OS-ELISA and also the advantages of the assay over competitive ELISA for its higher sensitivity, broader working range, and shorter measurement time. Therefore, we focused on this clone for further evaluations.

**Open Sandwich ELISA Using MBP-V<sub>L</sub> and V<sub>H</sub>-AP Proteins.** Though the phage-based system was convenient for the screening of a large number of clones, it was not a defined system for detection purposes, since one cannot either control the amount of individual protein produced or fully rule out the effect of unknown substances in the culture supernatant. To perform the assay in a more defined condition, OS-ELISA with purified proteins (one for the detection, the other for the immobilization) was attempted. For the detection of the V<sub>H</sub> fragment, the protein was



**Figure 6.** OS-ELISA with MBP-V<sub>L</sub> and V<sub>H</sub>-AP detected with pNPP. Averages of two samples with 1SD are shown.

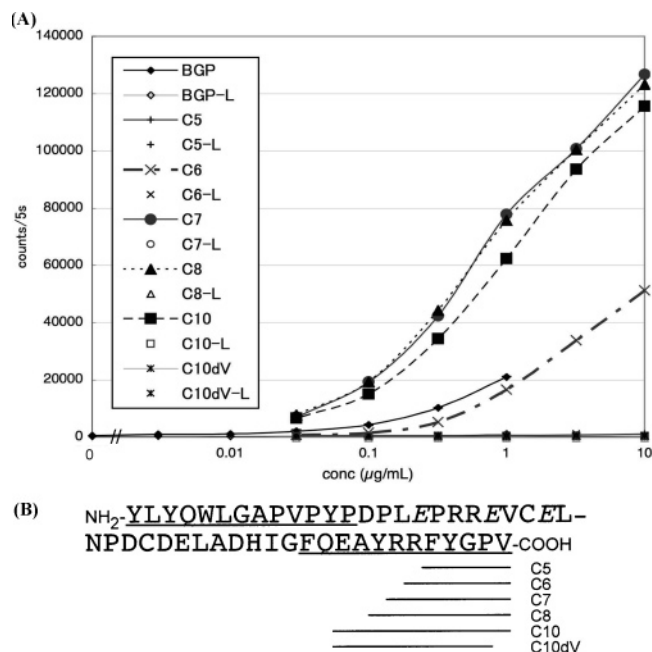
genetically fused with *E. coli* alkaline phosphatase mutant with higher  $V_{\max}$  than the wild-type enzyme (D101S),<sup>19</sup> and expressed in *E. coli* BL21(DE3, pLysS) (Figure 5A). To express the V<sub>L</sub> fragment, *E. coli* maltose binding protein (MBP) was chosen as a fusion partner. MBP has been widely used as a fusion tag for both cytoplasmic and periplasmic/secretory expression of various proteins.<sup>20</sup> Fusion with MBP has been known to enhance the solubility of the protein and also enhance the folding of scFv even in reductive cytoplasm.<sup>21</sup> Beside this, we decided to use MBP not only for the ease of production but also for passive immobilization to a solid surface. This was considered favorable to avoid possible interference of an anti-tag antibody to the target V<sub>H</sub>/V<sub>L</sub> interaction, which had been observed for the OS-ELISA with some antibody clones. Also, direct immobilization of MBP-V<sub>L</sub> was expected to result in overall reduction in assay time and steps. These proteins were expressed and purified by an IMAC, to almost homogeneity, with approximate yields of 5 mg/L culture for V<sub>H</sub>-AP and 1.4 mg/L culture for MBP-V<sub>L</sub> (Figure 5B and C). The resultant dose-response curve using the purified immobilized MBP-V<sub>L</sub> and V<sub>H</sub>-AP together with detection with colorimetric substrate *p*-nitrophenyl phosphate (pNPP) is shown in Figure 6. The results attained are comparable to those attained by OS phage ELISA with minimum detectable concentration between 0.1 and 1 ng/mL for both hBGP and C-peptide. Thus, the results clearly indicate the applicability of the fusion proteins in the OS-ELISA that can greatly reduce the incubation time, washing steps, and production cost, compared to conventional sandwich or competitive ELISA.

**Antigen Specificity.** To test the specificity of the Fv in OS-ELISA format, several C-terminal peptides were synthesized and

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**Figure 7.** (A) OS-ELISA with MBP-V<sub>L</sub> and V<sub>H</sub>-AP detected by chemiluminescence. Either intact hBGP in triplicate, C-terminal peptides of different lengths (C5–C10), or the C10 peptide without C-terminal valine (C10dV) shown in (B) were added together with V<sub>H</sub>-AP. Signals from the wells without immobilized MBP-V<sub>L</sub> are also shown to be negligible. (B) Amino acid sequences of hBGP and its peptides. The sequences for the immunized peptide are underlined, and the three glutamic acids in the middle (shown as E in italics) are γ-carboxylated.

their dose responses were examined. Using a white microplate immobilized with MBP-V<sub>L</sub>, sample and V<sub>H</sub>-AP were mixed and incubated for 90 min, before chemiluminescent detection of well-bound AP activity was performed. As shown in Figure 7, the signal detected by CDP star increased clearly with increased amounts of the C-terminal peptides of more than hexamer in length. All the peptides including intact hBGP, containing the C-terminal 7 residues (NH<sub>2</sub>-Arg-Arg-Phe-Tyr-Gly-Pro-Val-COOH, Mr: 894.03, C7) showed almost identical reactivity. Interestingly, the nonamer encoding the C-terminal 10 residues except the C-terminal Val (C10dV) showed negligible binding, showing the crucial importance of C-terminus in the recognition. According to the crystal structure of porcine BGP, the C-terminus of BGP is forming a hapten-like structure.<sup>17</sup> It is possible that recognition of the compact structure buried in the cavity between V<sub>H</sub> and V<sub>L</sub> made the OS-IA possible, like other antibodies capable of OS-IA that recognize low molecular haptens.

It is worth noting that the obtained background signal without antigen was very low ( $619 \pm 16$ ,  $n = 2$ ), compared with the signal

without immobilized MBP-V<sub>L</sub> ( $314 \pm 14$ ,  $n = 2$ ). Since the maximum value obtained for 10 μg/mL C7 was  $1.27 \times 10^6$ , the calculated dynamic range in signal was very high (>400-fold), marking the highest obtained for OS immunoassays to date. Also, all the peptides that exhibited a positive signal showed a wide measurable concentration range of more than 300-fold, showing the superiority of the noncompetitive assay.

## CONCLUSIONS

Using the cloned V<sub>H</sub>/V<sub>L</sub> cDNA fragments of an anti-hBGP C-terminus antibody, noncompetitive detection of C-terminal peptides as short as six residues was successfully performed with OS-ELISA. The obtained detection limit and working range were superior than those obtained with conventional competitive ELISA, covering the clinically relevant BGP concentration range (2.5–500 ng/mL).<sup>15</sup> Since the obtained working range was ideal for clinical tests, the assay might find its use in quicker detection of hBGP and its fragments.

As an application of the developed assay, selective detection of the proteins with the C-terminal BGP “tag” by the methods based on the OS-IA principle will be possible. Moreover, the present result also poses a possibility that OS-ELISA can be conducted for many other peptides, especially using antibodies recognizing the C-terminus of the peptides. Antibodies recognizing the free carboxy terminus of the peptide are relatively easy to obtain.<sup>22</sup> In addition, a structure of an antibody recognizing the C-terminus of the His<sub>6</sub> tag peptide in a deep pocket between the V domains has been reported, which is a common recognition mode of anti-hapten antibodies.<sup>23</sup> If we can generalize these and our results, OS-IA is not only useful for diagnosing certain peptides of clinical relevance but also could be a powerful tool in a range of proteomics research fields.

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