BAP-Fusion: A Versatile Molecular Probe for Biotechnology Research

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Abstract

Bacterial alkaline phosphatase (BAP) is a useful enzyme for detection in biotechnological researches. There are vast arrays of commercial available substrates, which can be converted to soluble or precipitated products for either colorimetric or chemiluminescent detection. This research article describes the application of bacterial alkaline phosphatase fusion protein as a convenient and versatile molecular probe for direct detection of different molecular interactions. Short peptide, protein binding domain, or single chain variable fragment (scFv) of monoclonal antibody were fused to bacterial alkaline phosphatase and used as one step detection probe for the study protein-protein or antibody-antigen interactions. The BAP-fusion could be generated by cloning a gene of interest in frame of BAP gene in an Escherichia coli expression vector. The fusion protein contained N-terminal signal peptide for extracellular secretion and could be induced for over-expression with isopropyl-β-D- thiogalactopyranoside (IPTG), allowing simple harvesting from culture broth or periplasmic extract. The BAP-fusion that was tagged with poly-histidine could be further purified by nickel affinity chromatography. This one step detection probe generated a specific and robust signal, suitable for detection in various formats as demonstrated on microtiter plate, dot blot, or western blot. In addition, it could also be used for an estimation of binding affinity by competitive inhibition with soluble ligand.

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Introduction

One of the important experiments in biotechnological research is the detection of molecular interactions. These include the interactions between protein-protein and protein with other type of molecules such as DNA, lipid, carbohydrate, small chemical compounds, metals, etc. These studies are required for the understanding of biological systems, which are essential for efficient drug development, or the development of affinity reagents for various biotechnological applications. Typically, basic techniques for the detection of protein interaction with various targets are done via a secondary probe, such as specific antibody that is linked to enzyme or fluorescent probe and are used for the detection of molecular interactions in various formats, such as enzyme-link immuno assay (ELISA), western blot, dot blot, or staining of tissue or cells [1]. The limitation of these assays is the availability of specific antibody to the protein of interest.

One solution to this problem is the construction of alkaline phosphatase fusion protein, which can be used as one-step detection probe for the study of the interaction of protein of interest with various targets, as demonstrated in numerous publications. The enzyme alkaline phosphatase can be obtained from various sources such as human placenta [2, 3], calf intestine [4], or bacteria [5, 6]; however, the most common type of the enzyme is Escherichia coli alkaline phosphatase (BAP) [5, 7] or its derivatives [8]. A BAP-fusion protein can be obtained by simply cloning the gene of the protein of interest into an appropriate fusion vector and over express in Escherichia coli expression system. Normally the protein of interest is fused to the N-terminus of the enzyme, as it is far from active site and will not interfere with its catalytic activity [9]. In many cases, the fusion protein is linked to the N-terminal signal peptide for secretion, thus it can be conveniently harvested from culture broth or periplasmic extracts [10]. In addition, many BAP is linked with poly-histidine tag, allowing convenient purification with one-step immobilized metal affinity chromatography (IMAC).

In addition to basic experiment in biotechnological research, BAP-fusions are also useful for high-throughput screening of potential drug leads because of its robust and highly specific interaction [11-14] [15] [16-18]. It also has potential for the development of useful biosensor [19-23].

This research provides evidences for the versatile use of BAP-fusion protein in various biotechnological applications. The BAP-fusion vector for E. coli expression system, which contains signal peptide for the secretion of the fusion protein and 10xHistidine tag at the C-terminus for affinity purification by IMAC, has been constructed. The genes of interest were fused to the N-terminus of the BAP by cloning into the multiple cloning site that comprises recognition sequences for five restriction enzymes. Over expression of BAP-fusions can be induced with isopropyl-β-D-thiogalactopyranoside (IPTG).

The genes encoding short peptide, protein binding modules, and single chain variable fragment (scFv) of human antibody, have been cloned into this vector and produced as BAP-fusion proteins. Detection of molecular interactions in various formats with these constructs has been demonstrated.
Material and Method

Construction of BAP-Fusion Vector: pMY201

The BAP-fusion vector used in this study was modified from pFLAG-CTS (Sigma). The construction was done in two steps, first DNA insert containing the multiple cloning site and sequence encoding ten histidine was ligated into Hind III and Sal I sites of pFLAG-CTS. This DNA insert was generated by annealing of two oligonucleotides; pHisFLAG1up: AGC TTC GCT CGA GGA ATT CGG ATC CGG TAC CAG ATC TGT CGA CCA CCA TCA CCA TCA CCA TCA CCA TCA CCA TCA TCA CC; and pHisFLAG1dn : TCG AGG TGA TGA TGG TGA TGG TGA TGG TGA TGG TGG TAC TTT CAG CCC CAG AGC. The two oligonucleotides were slowly assembled in a thermo cycler machine by mixing equal molar of each strand at 95°C for 5 min, and reduce the temperature slowly to room temperature. The insert was then ligated into pFLAG-CTS that has been cut and purified by agarose gel extraction. This vector was designated as p10HisFlag. Then the gene for \(E.\ coli\) alkaline phosphatase was amplified by using specific primers containing Kpn I and Sal I site. These are APfwKpnI: CTG TGC GGT ACC ATG CCT GTT CTG GAA AAC CGGG and APrvSalI: CTGTGC GTC GAC CGG TAC TTT CAG CCC CAG AGC. The template for amplification of BAP was from a previously published AP-fusion vector; pMY101 [14, 24]. The 100 µl of PCR reaction consisted of 0.5 µM of primers, 0.2 mM dNTP, 3 units of \(Pfu\) DNA polymerase (Promega), and 10X the reaction buffer, provided by the manufacturer. The amplifications were done as follows: initial DNA denaturation at 95°C for 2 min.; 30 cycles of denaturation at 95°C for 45 sec., annealing at 55°C for 1 min., extension at 72°C for 3 min., and a final extension at 72°C for 10 min. PCR products were purified using PCR purification kits (Qiagen GmbH, Germany) and then cut with appropriate restriction enzymes (Kpn I and Sal I) and ligated into p10HisFlag that has been cut with corresponding enzymes. The ligation reaction was transformed into \(E.\ coli\) DH5α. The DNA sequence and the integrity of the construct were determined by automated DNA sequencing (Macrogen, Korea).

Construction of BAP-Fusion Proteins

All BAP-fusion constructs were created by PCR amplification of interested genes, using specific primers that were flanked by appropriate restriction sites, and cloned into pMY101[14] or pMY201 expression vector that has been cut with corresponding restriction enzymes. The integrity of all constructs was determined by automated DNA sequencing (Macrogen, Korea).

Expression and Purification of BAP-Fusion Proteins

Freshly transformed \(E.\ coli\) harboring BAP-fusion construct was inoculated into 5-10 ml of LB broth containing 100 µg/ml of ampicillin at 37°C for 16 hr. Then, the overnight culture
was inoculated into 250-1000 ml of LB broth containing 100 µg/ml ampicillin and grown at 37°C until the optical density (O.D.) at 600 nm reached 0.9-1.2. Then, IPTG was added into the culture broth to a final concentration of 1 mM. The culture was then incubated with vigorous shaking (200 rpm) at 26°C (room temperature) for 4-6 hr. Then, the culture was collected and chilled in an ice box for 5 min and centrifuged at 8,000 rpm for 10 min at 4°C to collect cells and supernatant. To extract the periplasmic content, the cells were resuspended in 2.5 ml of cold (4°C) spheroplast buffer [100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 µg/ml phenylmethylsulfonyl fluoride (PMSF)]. After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 8000g at 4°C for 10 min and re-suspended in 1-2 ml of ice-cold sterile water supplemented with 1 mM MgCl₂ and incubated on ice for 5 minutes with frequent shaking. The supernatant of nearly 1-2 ml was then collected by centrifugation at 8000g at 4°C for 15 min as the periplasmic fraction. To extract the cell lysate, the precipitated cells from the previous step were washed once with lysis buffer (50mM Tris-HCl + 0.5 mM EDTA), resuspended in 1-2 ml of lysis buffer, and sonicated (Ultrasonic Processor; 60 amplitude, pulser 6 sec, for 2 minutes) on ice. The cell debris was then spun down at 10,000xg and the supernatant was collected as the cell lysate.

**SDS-PAGE Analysis**

Denaturing sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [25], in a 12% (w/v) polyacrylamide. The protein samples were briefly heat at 100°C in a heat block (Eppendorf) for 3 mins in the loading buffer. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight markers were from Biorad.

**Detection on Microtiter Plate (ELISA)**

Target molecules were immobilized onto wells of a microtiter plate by incubating 1-10 μg of target molecules in 0.1M NaHCO₃ (pH8.5) at 4°C, overnight. The wells were then washed with TBS [25 mM Tris-HCl (pH 7.5), 145 mM NaCl, 3 mM KCl] containing 0.1% (v/v) Tween 20. To eliminate nonspecific binding, 5-10 μg of BSA in 100 μl TBS were added into each well and incubated for an additional hour. The wells were then washed and incubated with 150 μl of culture media or TBS containing appropriate BAP-fusion proteins for 1 hour at room temperature. Then, the wells were washed 5 times with TBS + 0.1% (v/v) Tween 20. The amount of bound BAP fusions was measured by adding 150 μl of p-Nitrophenyl Phosphate, pNPP (Sigma Fast) and quantifying the absorbance with a microtiter plate reader at 405 nm. To screen for peptides that can inhibit the binding of BAP fusions to target proteins, various concentration of peptides were added with BAP fusions into each well and processed as described above.
Dot Blot Analysis

Seven amino acid-long peptides were immobilized on cellulose membrane using Spot-Synthesis method [26]; whereas target proteins were dot-blotted onto Immobilon P nylon membrane strips (Millipore) with dot blot apparatus (Biorad). The membrane was first blocked with 10% BSA in TBS for 1 hour at room temperature. The membrane was then washed 3 times with TBS containing 0.1% (v/v) Tween 20 and incubated in culture media containing an appropriate BAP fusion for 1 hour at room temperature, or overnight at 4°C. The membrane was then washed 5 times with TBS + 10% (v/v) Tween 20 and incubated with Nitro Blue Tetrazolium and 5-Bromo-4-Cholor-3-Indolyl Phosphate (NBT/BCIP, Sigma Fast) for 10 minutes and washed with de-ionized water. For chemiluminescent detection of the fusion proteins, the membrane was incubated with CSPD chemiluminescent substrate (Applied Biosystem, Foster city, CA, USA) and the signal was detected with Fluor-S Multimager (BioRad, Hercules, USA)

Western Blot Analysis

Cell lysate or GST fusion proteins were electrophoresed on a 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp.). The membrane was blocked in blocking solution (SuperBlock™, Pierce, Rockford, IL) at 4°C overnight, then transferred into a plastic bag containing 15 ml of culture supernatant containing appropriate BAP fusions and incubated at room temperature for 2 hours. The membrane was then washed with 1xTBS, Tween20 three times for 15 minutes each at room temperature, and incubated with CSPD chemiluminescent substrate (Applied Biosystem, Foster city, CA, USA). The signal was detected by exposure to BioMax Light-1 film (Eastman Kodax).

Result

Construction of BAP-Fusion Vector

The new BAP-fusion vector was constructed based on the fusion vector that has been previously reported (pMY101) [14]. This new vector contains five restriction sites in the multiple cloning site (MCS), allowing the generation of fusion protein to the N-terminus of bacterial (E. coli) alkaline phosphatase (BAP) gene. The C-terminus of the BAP gene is linked to DNA encoding ten histidine followed by a FLAG epitope (DYKDDDK). Normally, 6histidine is sufficient for purification with Nickel affinity chromatography; however, in some case, we found that 10xHis was a better tag for affinity purification, thus 10xHis was
used instead of 6xHis. The expression of BAP fusion proteins were under the control of tac promoter, which is a combination of -35 region of trp promoter to end of lacI binding region. The vector contain lac I gene which encodes lac repressor, which is over produced from lacI promoter and allow induction of the BAP-fusion construct with IPTG. The vector contains ampicillin resistance gene for cloning and maintenance of the recombinant plasmid. All of the fusion proteins are fused to the E. coli secretory signal peptide, ompA at the N-terminus, allowing the secretion of the fusion proteins into the periplasmic space as well as the culture media [10]. Amino acid sequence analysis of the BAP-fusion constructs can be done by using; forward sequencing primer, N-26: 5’ CAT CAT AAC GGT TCT GGC AAA TAT TC 3’; and reverse sequencing primer, C-24: 5’ T GTA TCA GGC TGA AAA TCT TCT CT 3’.

Figure 1. Map of the bacterial alkaline phosphatase fusion vector (BAP-fusion vector), pMY201. The coding region of the E. coli ompA signal/leader peptide, the multiple cloning site (MCS), and the first four amino acid of E. coli alkaline phosphatase (BAP) gene is shown. The c-terminus of BAP is linked to ten histidine tag and FLAG epitope, allowing purification with IMAC. The 6663 base-pair (bp) vector was derived from pFLAG-CTS vector. The vector also carries genes for ampicillin resistance (amp) and the lac repressor (lacI), which is over produced from lacI promoter and represses tac promoter. The over-expression of BAP fusion protein can be induced with IPTG.

Expression and Purification of BAP Fusion Proteins

All BAP fusion constructs used in this study could be induced for over expression by 1mM IPTG. However, we found that the proteins could also be produced without IPTG. This may because the tac promoter is relatively leaky. The levels of expression of different constructs were varied, depending on the size and the amino acid composition of the fusion protein. Figure 2 on the left panel illustrated a representative of the expression of one BAP-fusion (peptide-BAP). The cells were grown in LB media containing 100 μg/ml ampicillin until the optical density at 600 nm (OD600) reached 1.2, before 1mM IPTG was added (0 hr). At this point, we could find some of the proteins accumulated in the periplasmic space. After
two hours of induction by IPTG, the protein was found in culture broth, periplasm, as well as cell lysate. There was no significant different in the level of protein expression at other time points of induction. Interestingly, more protein was accumulated in the cell lysate after overnight induction. Nevertheless, it is recommended that optimization of induction condition for different protein should be performed.

Figure 2. Expression and purification of BAP fusion proteins. An example of the expression of a BAP fusion is demonstrated in the left panel. The peptide-BAP fusion construct was generated in pMY201 and over-expressed in *E. coli* Top10. The bacteria was grown in LB medium containing 100 μg/ml ampicillin until the OD_{600nm} reach 1.2 before 1mM IPTG was added. The samples were collected at 2, 4, 6, and 20 (overnight, o/n) hours after induction. Culture broth, periplasmic extract, and cell lysate were prepared for each time point of induction, and resolved by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Equal volume of the samples was loaded onto each lane. The volume of culture broth was ten times more than the periplasm and cell lysate extracts. The mixture of periplasm and cell lysate were further purified by IMAC and resolved in 10% SDS-PAGE as shown in the middle panel. The last panel on the right illustrates the purification of scFv-BAP fusion, which was equally pure, but had much less yield. I: input, F: flow through, w1-3; first, second, and third wash, E1and2: first and second elution.

Even if all the BAP fusions used in this study were fused with the ompA secretory signal peptide, significant amounts of BAP fusion constructs were found to accumulated in the periplasmic space as well as in the cytoplasm, as shown in Figure 2, left panel. Thus, both periplasmic and cytoplasmic extracts were used for subsequent affinity purification step. Since all the BAP fusion constructs that were generated from pMY201 contains 10xHis, all of these constructs could be purified by affinity column chromatograph with Ni-NTA agarose beads. The result of the purification of the peptide-BAP construct, as seen in the left panel, was shown in Figure 2, middle panel. *E. coli* cell lysate was incubated with Ni-NTA agarose beads before washing and eluted using column chromatography. More than 90% of purified proteins can be obtained. We found that the amounts of purified BAP-fusions from one-liter culture media were varied greatly, depending on the level of expression of different BAP constructs. As seen in the Figure 2, right panel, when scFv-BAP construct was purified using the same protocol, much less protein could be obtained.

**Stability and Specificity of BAP**
Routinely, BAP-fusion prepared from culture broth or crude periplasmic extract could be used directly for most experiments. This is because the fusion protein is linked to the ompA signal peptide, which can direct the secretion of the protein into periplasm and eventually leaked out into the culture broth [10]. Even if only a fraction of the fusion protein were present in the culture broth, it is sufficient for most detection assay [15, 24]. The residual activity of alkaline phosphatase in different conditions was measured to determine its stability as shown in Figure 3, upper panel.

Figure 3. Stability and specificity of bacterial alkaline phosphatase. **Upper panel** illustrates the residual activity of BAP after storage in different conditions at different durations (7, 28, 60, 90, and 120 days). BAP activity was estimated by checking OD_{405} after incubation with pNPP, a chromogenic substrate for BAP. The protein was kept at 4°C in culture supernatant (4), purified and stored at -20°C (-20), or left dry at room temperature on microtiter plate wells (dry). The Intersectin SH3 domain-BAP fusion was used in this study. Different result might be obtained with other constructs. **Bottom panel** illustrates the specificity of the binding of peptide-BAP fusion compared with other formats. Various GST-SH3 fusion proteins (Src, Yes, p53BP2, Abl, and Grb2) were immobilized onto triplicate wells of a microtiter plate and incubated with different formats of p53BP2 SH3 peptide ligand i.e., BAP-fusion, (BAP-peptide), N-terminal fusion to the minor coat protein III of bacteriophage M13 (Phage-peptide), and multivalent biotinylated peptide, pre-complexed with SA-AP (Biotin-peptide). Bound BAP fusion and biotinylated peptides were detected by colorimetric assay with 8 mM pNPP (p-Nitrophenyl Phosphate), whereas bound bacteriophage particles were detected by ELISA [70]. The average OD_{405}
values and standard errors are shown. The synthetic peptide ligand of p53BP2 was SGSGYDASSAPQRPLPVRKSRPGG, whereas the peptide ligand that fused to BAP and phage coat proteins was WVVDSRDPILRRSLP.

We found that less than half of the enzyme activity was left after keeping the enzyme in crude culture broth at 4 °C for 2 weeks, and most of the enzyme activity was completely demised after one month. However, if the enzyme was purified and kept at -20°C, more than 90% of relative activity could be detected after storage for 3 months. Surprisingly, if the enzyme is left dry at room temperature, approximately 80% of the activity was still remained after 3 months. This experimental data was observed when the enzymes were left dry on plastic pins, or on wells of microtiter plate. Thus, bacterial alkaline phosphatase is a relatively stable enzyme, suitable for various assays. It is worthwhile to note that the enzyme is sensitive to buffer containing phosphate [9], thus Tris-buffered saline (TBS) is preferred instead of phosphate-buffered saline (PBS).

Bacterial alkaline phosphatase is a homodimeric enzyme [7, 9, 27]. Its N-terminus protrudes away from the globular body of the protein; therefore, many protein segments or peptides can be fused to the N-terminus of BAP without interfering with the catalytic activity of the enzyme. The homodimeric structure of the bacterial alkaline phosphatase suggested that the peptide or protein that displays as BAP-fusion will interact with its partner in a dimeric form as well. This format allows robust and specific interaction with its partners as demonstrated in Figure 3, low panel. The peptide ligands of p53BP2-SH3 domains (WVVDSRDPILRRSLP) [28] were tested for its binding properties with various GST-fusion SH3 domains. The binding specificity was the highest when the peptide ligand was fused to BAP (BAP-peptide) because it didn’t cross-react with other SH3 domains. When the same peptide ligand was displayed on the minor coat protein (pIII) of bacteriophage M13 (Phage-peptide), its binding was less specific, as it also cross-reacted with Src and Yes SH3 domain. The least binding specificity was observed when the peptide was chemically synthesized as biotinylated peptide and pre-complexed with streptavidin-conjugated alkaline phosphatase (Biotin-peptide). In this format, the peptide was found to react with most of the SH3 domains tested. Thus, our results suggested that BAP-fusion could be used for specific and robust detection of molecular interactions.

Detection on Microtiter Plate and Estimation of Binding Affinity with BAP Fusions

One of the most common assays to detect molecular interaction is on microtiter plate. The well-known assay on microtiter plate is enzyme-linked immunosorbent assay (ELISA). This technique allows detection of a small amount of a large number of samples at the same time, suitable for high-throughput application. Both qualitative and quantitative analysis can be performed. As demonstrated in Figure 4, application of BAP-fusion for detection of protein-protein interaction on microtiter plate was reported. On the left panel, various peptide ligands of Eps15 homology (EH) domain [29, 30] that have been isolated from phage-displayed peptide library [31] were tested against different GST-fusion proteins as BAP-fusion peptides. Detection of BAP activity could be done by using soluble substrate such as
pNPP. Many assays could be done at the same time on microtiter plates. The binding signal is very strong as seen from the OD_{405} values (2.0-4.0), and the background was very low. This allows confirmation of the binding of the peptide ligand that has been isolated from phage library.

Figure 4. Detection on microtiter plate and estimation of binding affinity of BAP fusions. Left panel illustrates the binding characteristic of selected Intersectin EH domain-peptide ligand-BAP fusions. Various GST fusion proteins and the anti-FLAG monoclonal antibody were immobilized onto triplicate wells of a microtiter plate and incubated with different EH domain peptide ligand-BAP fusions. Bound BAP-fusions were detected by colorimetric assay with pNPP. The sequences of the peptides fused to the N-terminal of BAP are shown on the left of each histogram. The target proteins were also detected by ELISA using HRP-conjugated anti-GST antibody and ABTS substrate to demonstrate that equal amounts of proteins were immobilized onto the wells. The average OD_{405} and standard errors are shown. Right panel illustrates the estimation of binding affinity by competition of synthetic peptide ligands with the binding of a BAP fusion to the EHα, and EHβ domains of Intersectin. Microtiter plate wells, coated with approximately two micrograms of GST fusion protein to EHα (EH-a, bottom) or EHβ (EH-b, top), were incubated with a peptide (DCTNPFRSCWR)-BAP fusion in the presence of various concentrations of competitor peptides (μM). After one hour of incubation, the BAP fusion retained in the wells after washing was detected by a colorimetric assay with pNPP. Optical density at 405 nm of the average of triplicate samples and standard deviations are shown. cyclic NPF sequence is DCTNPFRSCWR (with intramolecular disulfide bond), NPFL-COOH and NPFL-COONH2 correspond to the C-terminus (aa 327-335) of RAB/Rip, vssNPFmtg: is the internal NPF motif of RAB/Rip (aa 310-318), and Src-ligand is SGSGILAPPVPRNTR.

In addition to confirmation of the interaction, BAP-fusion can also be used to estimate the binding affinity as demonstrated in Figure 4, right panel. The EH domain peptide ligand BAP fusion (cyclic-NPF-AP) [31] were added to the wells of microtiter plate that had been coated with GST fusion to two Intersectin EH domains, EH-a and EH-b [31], in the presence
of various concentration of different soluble peptides. The binding affinity could be estimated by determination of the concentration of soluble peptide that can inhibit 50% of the binding of BAP fusion, IC50. As shown in our results, the IC50 of cyclic NPF to EHa and EHB is approximately, 3 μM and 9 μM, respectively. This analysis also suggested that cyclic NPF can bind with the highest affinity to EH domains, followed by NPFL-COOH motif, whereas the interactions of an internal NPF with EH domain is relatively weak. In addition, it also suggested that the carboxylate group at the C-terminus is involved in the binding, as the IC50 of NPFL-COONH2 is higher than that of NPFL-COOH.

**Dot Blot/Spot Analysis with BAP-Fusions**

BAP fusion proteins can also be used for detecting protein-protein interaction by dot blot analysis. This research demonstrated the application of SH3 domain peptide ligand-BAP fusion [28] and Intersectin EH domain-BAP fusions [31] for the detection of molecular interaction using dot-blot/spot format. GST fusions of various SH3 domains were dot-blotted onto PVDF membrane and incubated with various peptide ligand BAP fusions. The signal can be detected by using precipitated colorimetric or chemiluminescent substrates such as NBT/BCIP or CSPD, respectively.

![Figure 5. Dot Blot/Spot analysis with BAP fusions.](image)

Left panel illustrates the binding of different BAP-fusion peptides (peptide ligands of SH3 domains of Src (type I and II), Abl, p53BP2, Cortactin, Crk, and 300 C-terminal amino acids of the mouse Sos protein, cSos) to different GST-SH3 fusion proteins were dot-blotted onto membrane strips. One microgram of each GST-SH3 fusion proteins (Grb2-SH3, Abl-SH3, p53BP2-SH3, Yes-SH3, and Src SH3) was immobilized onto PVDF membrane using dot blot apparatus, cut, and strips were probed with the seven different BAP fusions. The amount of BAP fusion protein bound to the membrane strips was visualized with the chromogenic reagents 5-Bromo-4-Cholor-3-Indolyl Phosphate (BCIP) and Nitro Blue Tetrazolium (NBT). Right Panel illustrates the binding of Intersectin EH domain-BAP fusions to synthetic peptide immobilized on membrane. Seven-amino acid long-peptide were synthesized onto the cellulose membrane by Spot-Synthesis method, and probed with BAP fusions to Intersectin EHa, EHb, or EHaandb. The membrane could be re-used several time, as shown in this figure, the membrane was first probe with EHa, washed, and re-probe with the second and the third BAP constructs. The results showed the binding to ninety six peptides; peptide 1-8 corresponds to alanine scan of TTNPFLL (C-terminus of epsin2/Ibp2, MP90); peptide 9-16 corresponds
to alanine scan of NTNPFLL (C-terminus of epsin1, Ibp1); peptide 17-92 corresponds to 19-amino acid replacement of TTNPFLL; peptide 93-94 corresponds to internal NPF motif of epsin2/Ibp2; and peptide 95-96 corresponds to internal NPF motif of epsin1/Ibp1. The bound BAP fusions on membrane were detected with CSPD chemiluminescent substrate and the signal was detected with Fluor-S Multimager.

As shown in Figure 5, left panel, specific interaction of SH3 peptide ligand-BAP with different SH3 domains could be observed by dot-blotted analysis, similar to the result obtained from microtiter plate assay. The type I peptide ligand of Src SH3 domain can interact with both SH3 domains of Src and Yes, because their structures are highly similar [28]. On the contrary, peptide ligand of SH3 domains of Abl or p53BP2 did not cross-react with other SH3 domains. This is because the Abl and p53BP2 are relatively distinct [28]. Src-SH3-peptide ligand type II (SrcII) and cSos were relatively promiscuous in their binding activities, as they were able to cross react with almost every SH3 domains.

In addition to detecting GST fusion protein immobilized on PVDF membrane, BAP fusion could also be efficiently used to detect synthetic peptides that were immobilized on the membrane. As demonstrated in Figure 5, right panel, a large number of different seven-amino acid-long peptides were synthesized onto the cellulose membrane using Spot-Synthesis method [26]. With this method, as much as 425 peptides could be spotted onto one cellulose membrane, allowing rapid and extensive analysis of various molecular interactions between ligand and its target. The membrane is quite durable, as it can be washed with mild detergent (1% TBS + 0.1% Triton X-100) and re-used for a couple of more times. As seen in this report, alanine scan, and peptide mapping for the study of EH domain-ligand interaction could be done using this assay.

Figure 6. Western blot analysis of BAP fusion. **Left panel** illustrates the detection of GST fusion to different Intersectin EH domains with two BAP fusion probes. GST fusions were resolved by 12% SDS-PAGE and the proteins were blotted onto PVDF membrane before incubating with culture supernatant containing EH-ligand-BAP fusion (CyclicNPF-BAP), or scFv from biopanning of Phage display library with EHα domain (scFv-BAP). The BAP fusion was detected with chemiluminescent substrate (CSPD) and exposed to BioMax Light-1 film (Kodak). The cyclicNPF sequence is DCTNPFSCWR. **Right panel** illustrates western blot analysis of COS-7 cell lysate with BAP fusion to
three different scFvs that have been isolated from Phage display library. COS cell lysate was resolved in 12% SDS-PAGE, blotted onto PVDF membrane and probed with 50ng of different scFv-BAP that have been purified by IMAC. The BAP fusion was detected with chemiluminescent substrate (CSPD) and exposed to BioMax Light-1 film (Kodak).

**Western Blot Analysis with BAP Fusions**

Western blot analysis is commonly used in molecular cell biology research to detect denatured recombinant proteins or proteins from cellular extracts. In this research we demonstrated that BAP-fusion can be used as a convenient one-step detection probe for western blot analysis. EH domain peptide ligand (cyclicNPF-BAP) [31] and single chain variable fragments of monoclonal antibody (scFvs) isolated from Phage display antibody library were used to detect GST-EH domain fusion proteins (Figure 6, left panel) or cell lysate (Figure 6, right panel). CyclicNPF-BAP, which is the EH domain peptide ligand [29] could interact with all GST-EH domain fusions; the signal from the binding is robust with no background. When using BAP-fusion to scFv, which has been affinity selected from Phage display library against EHa, only the bands of GST fusion to EHa and EHaandEHb were positive. The result on the right panel illustrated western blot analysis of whole cell lysate using three different scFv-BAPs as probes. Different patterns of protein bands with different scFv-BAPs could be detected. The background remained clear after long exposure (>15min). These results suggested that BAP-fusion is also an efficient reagent for western blot analysis.

**Discussion**

Numerous examples of application of bacterial alkaline phosphatase (BAP) fusion protein in different aspects of molecular biotechnology research have been reported [2, 3, 13, 14, 16-20, 22, 23, 32-51]. This article demonstrated that BAP-fusion can be used as a versatile molecular probe for detection of molecular interaction in various formats. These include detection on microtiter plate, dot blot, and western blot analysis.

The signal from the detection is strong and the background is very low. The binding specificity is high, and can be used to estimate binding affinity in a competitive inhibition experiment. In addition to the types of assay reported in this study [32, 36, 42, 47, 52-54], there have been previous reports on other formats of detection such as direct cell staining [34], characterization of receptor ligand interaction [13], or protein localization study [2].

The type of protein that is mostly used to fuse with BAP is a variable region of monoclonal antibody; such as scFv or Fab fragments [2, 6, 11, 12, 33, 34, 38-41, 43-46, 48-51, 53-61]. This protein is about 250 amino acid-long. In addition, BAP fusions to peptides [24, 62, 63], and protein domains have also been reported [15, 24]. In this research, peptides ranging from 9 amino acid to 300 amino acid-long (C-terminal of mouse Sos) have been successfully expressed as BAP fusion proteins. Thus, BAP fusion is a flexible system for an expression of a wide variety of proteins.

The *E. coli* alkaline phosphatase is a homodimeric enzyme with its N-terminus protruding out of the globular body of the proteins [5, 64, 65], thus peptide or protein as large as 300 amino acid-long can be fused to the N-terminus of the enzyme without interfering
with the enzymatic activity. Moreover, the ten histidine tag and FLAG epitope at the C terminus does not interfere with its catalytic activity, as demonstrated in this report and from previously published work [20], even if the C-terminus of the enzyme doesn’t protrude out. These tags are useful for one-step affinity purification, or immuno-detection with anti-FLAG antibody. It has been shown that 6xhistidine tag is sufficient for one-step affinity purification; however we found that in some case, 10xhistidine is more efficient. Even though BAP fusion is a dimer, it can be used to detect specific molecular interactions and estimate the binding affinity. However, in case it is necessary to determine the binding affinity with monomeric form, such as in BiAcore measurement, a monomeric form of BAP with mutations that inhibit the interaction at the dimeric interface [66] could be used instead.

Bacterial alkaline phosphatase (BAP) that is commonly used came from *E. coli*; however in some case, fusion with human placental alkaline phosphatase has also been reported [2, 23, 45, 62]. BAP is a relatively stable enzyme. As seen in our report that it can be left dry at room temperature without losing much activity. However, if the enzyme is left in culture broth, it can be kept only for 1 or 2 weeks, this may be the result of proteolysis by some proteases. Thus, fresh sample of culture broth should be prepared every time if it will be used directly for detection.

As seen from this study, the binding signal from an assay on microtiter plate is very strong, OD₄₀₅ values of 2.0-4.0, suggesting that BAP fusion is a very sensitive probe. Recently, there has been a report on the improving *E. coli* alkaline phosphatase efficacy by two additional mutations inside and outside the catalytic pocket (D330N/D153G). This mutant is highly thermostable and the catalytic efficiency (kcat/Km) value was increased by a factor of two, relative to that of the wild type [67]. This mutant should be useful for the assay that requires a very sensitive detection. Further improvement of BAP property such as acid tolerant, solvent, or detergent resistant should also be useful for certain biotechnological applications. Recently, a moderately thermostable alkaline phosphatase from *Geobacillus thermodenitrificans* T2 have been reported [6].

For most binding experiments, BAP fusions in crude culture broth, periplasmic extract, or cell lysate could be used directly as one-step detection probe without further purification. The level of expression and the secretion efficiency depends greatly on the structure of the fused peptide [68]. Thus, it will be beneficial for the researcher to optimize the induction of protein expression in order to obtain the highest amount of the protein. Even if the BAP-fusion is fused to the ompA signal peptide, in most case a large amount of protein was found to accumulate in the cytoplasm. This could be the result of high level of expression of the protein by the tac promoter, resulting in the saturation of *E. coli* secretion machinery. Thus, normally, if the BAP fusion will be further purified by IMAC, the whole cell lysate should be used for purification. Purified BAP fusion is useful for long-term storage or certain type of assay. There are a number of BAP-fusion vectors available for generation of BAP fusion constructs [2, 11, 35, 38, 43, 46, 49, 51, 52, 54, 55, 57, 59, 62, 69]. These vectors are different in the multiple cloning site, type of promoter, strains of *E. coli* for expression, and the presence of tag. Recently, a vector for over expression of genes from phage display library using ligation independent cloning (LIC) has been developed [12]. The vector pMY101 that has been used in this study was generated specifically for the expression of BAP fusion of peptide from phage display library with *Xho*I and *Xba*I cloning site, while the
vector pMY201 from this study contains larger multiple cloning site and two tags for affinity purification.

Thus it can be concluded that BAP fusion is a versatile molecular probe for detection of various interactions in biotechnological research. It is convenient, and sufficiently sensitive and specific for binding assay in many formats. It is suitable not only for the study of a particular interaction, but highly adaptable for high-throughput analysis.

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