Strep-tag II Mutant MBP for Reagentless Fluorescence Sensing

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Abstract: Protein pengikat maltosa (MBP) adalah protein pengikat periplasmik yang boleh dijumpai di dalam bakteria gram negatif. MBP terlibat di dalam proses pengangkutan maltosa serta kemotaksis bakteria; ia mengikat pada maltosa serta maltodekstrin yang mempunyai rangkaian linear α(1-4)-glukosilik polimer glukosa dan rangkaian siklodekstrin α(1-4)-glukosilik. Semasa protein mengikat pada ligan, perubahan struktur konformasi MBP berlaku dari bentuk terbuka ke bentuk tertutup. Pengecaman molecular ini yang menyebabkan transduksi pengikatan ligan kepada bentuk fizikal menjadikan MBP calon yang sesuai sebagai penderia pendaflour tanpa reagen. Di sini, kami akan menerangkan bagaimana membina mutan Strep-tag II MBP untuk dijadikan sebagai penderia pendaflour tanpa reagen. Gen malE, yang mengekodkan MBP telah diamplifikasikan. Residu sistina telah diperkenalkan melalui proses tapak terarah mutagenesis agar label lekatan tunggal pada tapak yang spesifik yang mempunyai kuar pendaflour spesifik-tiol. Fluorofor yang sensitif pada persekitaran (IANBD amide) terlekat secara kovalen pada kumpulan tiol yang diperkenalkan sebelum ini da kemudian dianalisi secara deria pendaflour. Mutan MBP (D95C) telah ditulenkan (saiz molecular, ~42 kDa). Ukuran pendaflour Strep-tag II–D95C yang telah dilabel oleh IANBD di dalam larutan telah menunjukkan perubahan keamatan pendaflour yang memberangsangkan (pemalar penguraian Kd 7.6 ± 1.75 μM). Mutan MBP Berjaya
abstract: maltose-binding protein (mbp) is a periplasmic binding protein found in gram-negative bacteria. mbp is involved in maltose transport and bacterial chemotaxis; it binds to maltose and maltodextrins comprising α(1-4)-glucosidically linked linear glucose polymers and α(1-4)-glucosidically linked cyclodextrins. upon ligand binding, mbp changes its conformation from an open to a closed form. this molecular recognition—transducing a ligand-binding event into a physical one—renders mbp an ideal candidate for biosensor development. here, we describe the construction of a strep-tag ii mutant mbp for reagentless fluorescence sensing. malE, encoding mbp, was amplified. a cysteine residue was introduced by site-directed mutagenesis to ensure a single label attachment at a specific site with a thiol-specific fluorescent probe. an environmentally sensitive fluorophore (ianbd amide) was covalently attached to the introduced thiol group and analyzed by fluorescence sensing. the tagged mutant mbp (D95C) was purified (molecular size, ~42 kDa). the fluorescence measurements of the ianbd-labeled strep-tag ii–D95C in the solution phase showed an appreciable change in fluorescence intensity (dissociation constant, 7.6 ± 1.75 µM). our mutant mbp retains maltose-binding activity and is suitable for reagentless fluorescence sensing.

keywords: maltose-binding protein, strep-tag ii, fluorescence sensing, D95C

introduction

fluorescence sensing is a rapidly developing field of technology for detecting natural and synthetic compounds in different media and living cells. reagentless fluorescence biosensors are emerging as a novel form of fluorescence biosensors. their quantification does not change the composition of the
sensor, unlike enzyme-based competitive assays, in which the analyte is consumed (Marvin and Hellinga 1998). Periplasmic binding proteins (PBPs) are being extensively studied for applications as reagentless fluorescence biosensors because of their general structural form and conformational changes during ligand binding, which can be described as a "Venus flytrap," wherein the two lobes of the protein close on the ligand, completely entrapping it (Hellinga and Marvin 1998).

Maltose-binding protein (MBP) belongs to the PBP superfamily found in gram-negative bacteria (Zhou and Cass 1991). MBP is a monomeric 40,600-Da protein encoded by the malE gene, and it has two distinct globular domains separated by a three-strand hinge region (Scharff and Quicho 1993). Other than binding to maltose, MBP can also be manipulated by protein engineering to bind to metal ions for environmental biosensor applications (Marvin and Hellinga 2001; Shahir 2006). MBP changes its conformation from an open to a closed form upon ligand binding. This molecular recognition which transforms a ligand-binding event into a physical one renders MBP suitable for biosensor development.

MBP lacks cysteine residues; therefore, a unique thiol group can be introduced by oligonucleotide-directed site-specific mutagenesis (Gilardi et al. 1994). Thiol-specific fluorophores are specific to the thiol group and are sensitive to environmental changes. In addition, this fluorophore is detected by fluorescence sensing of the conformational changes of MBP upon ligand binding. This strategy has been used to introduce single fluorophores that respond to ligand binding in MBPs (Dattelbaum et al. 2004; De Lorimier et al. 2002; Gilardi et al. 1994; Marvin and Hellinga 2001; Sohanpal et al. 1993), ribose-binding proteins (Vercillo et al. 2006), glucose-binding proteins (Lyndon et al. 2001), glutamine-binding proteins (De Lorimier et al. 2002), and sulfate-binding proteins (Shrestha et al. 2002). Many types of thiol-reactive fluorophores have been used in PBP fluorescence sensor studies to detect ligand binding of PBPs. Owing to its consistent fluorescence response, IANBD amide has been found to be a suitable fluorophore for covalent attachment to the cysteine residue of recombinant MBP (Dattelbaum et al. 2004; Gilardi et al. 1994; Marvin and Hellinga 1998; Shahir 2006). IANBD amide is an iodoacetamide that forms a thioether bond with a cysteine residue, and its fluorescence emission is highly sensitive to its solvation state (Johnson and Spence 2011).

In this study, the fluorophore was attached to the protein at the allosteric site of the protein, which is at position 95, where aspartic acid is replaced by cysteine (D95C). The allosteric site in the protein
structure is located away from the ligand-binding site, and it undergoes a local conformational change in concert with ligand binding (De Lorimier et al. 2002). Furthermore, the allosteric site has an advantage of no direct interaction between the ligand and the fluorophore; hence, the binding constant is unaffected (Marvin and Hellinga 1998). There are several mutations of MBP at the allosteric site for potential reagentless fluorescence sensing that has been constructed which are D95C, F92C, and I329C (De Lorimier et al. 2002; Sohanpal et al. 1993). In this study, D95C was chosen because its use has been reported in several studies (Marvin et al. 1997; Marvin and Hellinga 2001; Sohanpal et al. 1993) and the binding affinity towards maltose of D95C is often higher than other mutations (Marvin and Hellinga 2001; Wemmer 2003).

However, to our knowledge, all the reported research describing signal transduction by fluorophore-labeled PBPs has been carried out without linkage to solid supports (Brune et al. 1994; Gilardi et al. 1994; Hellinga and Marvin 1998; Marvin and Hellinga 2001;). Affinity tags are widely used in biotechnology to assist purification of recombinant protein. Strep-tag II provides a befitting combination of excellent purification with good yield and moderate cost compared to other affinity tags such as His-tag and GST-tag (Litchy et al. 2005). The mutant MBP was fused to an affinity tag, Strep-tag II, to aid in purification and application in site-directed immobilization of the MBP onto functionalized solid surfaces. This is the first report of reagentless fluorescence sensing of maltose using Strep-tag II-fused mutant MBP. Site-directed mutagenesis was performed on a malE template fused with Strep-tag II to incorporate a single cysteine at the allosteric site of MBP. The cysteine residue of the mutated protein (D95C) was labeled with IANBD amide. The response of the labeled mutant MBP upon binding with maltose was monitored by following the changes in the fluorescence intensity of the probe, and ligand-binding characteristics were also analyzed.

**METHODOLOGY**

**Construction of Plasmids pET-51b (+)- malE**

The malE gene was amplified from the pMAL-c4x plasmid using the polymerase chain reaction (PCR) with flanking primers designed to introduce a BamHI restriction enzyme site before the start codon and a
HindIII site before the stop codon. The primers (First Base Laboratory Pvt. Ltd.) used for isolating \textit{malE} gene were For\textunderscore malE (5’-CCCGGATCCGAAAATCGAAGAAGTGAACTGTC-3’) and Rev\textunderscore malE (5’-CCCAAGCTTTTTAACGTCGCGTCTTTCCGGCG-3’). A 30 cycle PCR was used with the pMAL-c4x plasmid as the template, primers and \textit{Pfu} polymerase (Fermentas, USA). Cycle conditions were 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes and one cycle of 72°C for 5 minutes. Reaction was run on a TAE 1% agarose gel and when the gene was verified, the remaining PCR mixture was treated with \textit{DpnI} (Fermentas, USA) at 37°C for 1 hour to digest the methylated template and was purified with PCR Clean-up System (Promega, USA). The purified DNA was then digested with \textit{BamHI} and \textit{HindIII} restriction enzymes (Fermentas, USA) and ligated using \textit{T4} DNA ligase into pET-51(b) (Novagen, USA) which was digested with the same restriction enzymes. The constructed plasmid pET-51b(+)\textunderscore malE was then transformed into \textit{E. coli} Novablue Singles cells (Novagen, USA) and transformants were selected on LBA plates with 50 µg/mL ampicillin.

\textbf{Construction of Plasmids pET-51b(+)\textunderscore D95C (Mutant MBP plasmid)}

MBP mutant was made containing a single substitution by replacing Aspartic Acid (position 95) with a cysteine. Site-directed mutagenesis was performed by inverse PCR using the \textit{malE} gene (from pET-51b (+)\textunderscore \textit{malE}) as the template and \textit{Pfu} DNA polymerase. The primers used were D95C\_For (5’ – TGC GCA GTACGTTACAACGGC) and D95C\_Rev (5’- CATCTGCGGGATGTTCGGCAT-3’) and was treated with \textit{T4} polynucleotide kinase (Fermentas, USA) prior to use at 37°C for 1 hour. The PCR reaction was one cycle at 92°C for 2 minutes, 15 cycles of 92°C for 20 seconds, 55°C for 1 minute and 68°C for 6 minutes and one final cycle at 62°C for 10 minutes. The plasmid construct was then verified, purified, ligated and transformed as described for the pET-51b (+)\textunderscore malE plasmid construct.

\textbf{Expression of the \textit{malE} gene}

The recombinant plasmid was transformed into an expression host BL21(DE3)pLySs (Novagen, USA) by heat shock transformation. The BL21(DE3)pLySs cells harbouring the recombinant plasmid were inoculated into 1 mL of LB broth containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol and was incubated overnight at 37°C with shaking. Then, 10 mL of overnight culture was inoculated into 200 mL of
fresh LB broth with both of the antibiotics with addition of 1% (v/v) glucose and incubated at 37°C with shaking until the OD is at 600 nm reached ~0.4-0.5. After that, the cells were induced with 1 mM of IPTG for 4 hours at 30°C. Finally, the cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was either immediately lysed or kept at -20°C prior to lysis.

The cell pellets were lysed by the freeze and thaw method. Firstly, the cell pellets were thawed followed by addition of sterile distilled water and benzonase nuclease. One gram of the cell pellet requires 5 mL of sterile distilled water with the addition of 25 U of benzonase nuclease per 1 mL of sterile distilled water. After that, the mixture was incubated in -20°C freezer for 10 to 15 minutes or until the mixture had frozen. Then, the mixture was thawed on ice for 30 minutes until the mixture became liquefied. This freezing and thawing steps were repeated for three times. The host cell contains pLysS with relatively high levels of T7 lysozyme in the cytoplasm that makes it easier for cells to lyse under mild conditions such as freeze/thaw treatment (Mierendorf et al. 1994). The mixture was then centrifuged at 16 000 x g for 20 minutes at 4°C to remove insoluble cell debris. The supernatant was kept at -20°C.

**Recombinant Protein Purification via Strep-Tag II Affinity Purification**

The crude extract of wild type MBP protein and its mutant were purified by affinity chromatography using a 5 mL pre-packed StrepTrap HP™ column (GE Healthcare, UK). Purification was done using Akta Prime Plus (GE Healthcare, UK) with the flow rate of 2 mL/min. The column, buffers and the protein sample were kept on ice during the purification process to lessen degradation of the mutant MBP. Purification of the wild type MBP was done at room temperature because MBP is known to be thermally stable in solution at temperatures up to 50°C (Gilardi et al. 1994).

Firstly, the Akta Prime Plus system was flushed with filtered deionized water prior to purification. The column was then connected to the designated tubing by carefully dripping the buffer from the tubes into the column to prevent air bubbles. The 5 mL StrepTrap HP™ pre-packed column was washed with three column volumes of filtered deionized water before starting the purification process. The column was equilibrated and washed with five column volumes of binding buffer (20 mM NaPO₄, 280 mM NaCl, 6 mM KCl and 10 mM EDTA, pH 7.4). About 20 mL of 6 mg/mL crude lysate (pre-filtered through 0.45 µm nylon membrane) was loaded into the StrepTrap™ Hp column with the flow rate of 2 mL/min. The column was
washed with eight column volumes of the binding buffer. Next, elution was carried out with three column volumes using the same binding buffer plus 2.5 mM d-des thiobiotin. The elution profile was monitored by measuring OD at 280 nm and fractions were subsequently analyzed by SDS PAGE.

**Ultrafiltration of purified protein**

The fractions containing Strep-tag II MBP and Strep-Tag II D95C were desalted and buffer exchanged against 10 mM potassium phosphate buffer, pH 7.4 using a 20 mL Vivaspin concentrator (10 kDa MWCO membrane, Sartorius Stedim, Germany). Prior to use, the vivaspin column was washed by adding 15 mL of 10 mM potassium phosphate buffer, pH 7.4 and then spun at 4000 rpm for 10 minutes. Then, about 5-10 mL of protein was added; and buffer was added to make up to 15 mL and was centrifuged at 4000 rpm for 5 minutes. The filtrate was decanted and buffer was added to make up to 15 mL and was centrifuged again as previously described. This step was repeated and the protein concentration was determined via Bradford Assay based on Beer Lambert Law.

**Intrinsic Tryptophan Fluorescence of Strep-Tag II Wild Type MBP**

Intrinsic tryptophan fluorescent measurement was done on a Perkin-Elmer fluorometer LS 55 with an excitation wavelength of 280 nm. The excitation wavelength was set at 280 nm because the aromatic amino acid (i.e: tryptophan, tyrosine and phenylalanine) absorbs light at that particular wavelength. The intensity of fluorescent was recorded on addition of maltose towards the purified tagged wild type MBP in the quartz cuvette. About 1.0 mL of 1.0 µM purified tagged wild type MBP in 10 mM potassium phosphate buffer pH, 7.4 was prepared. Firstly, an initial fluorescent measurement of the protein solution without addition of maltose was recorded. Titrations of the aliquots of 1 µL maltose ranging from 10 µM to 100 µM were added to the 1 mL protein solution. These steps were repeated three times. Prior to use, the quartz cuvette was treated with 1 M HCl to remove contaminating protein and maltose. The percent changes in fluorescence intensity with increasing ligand concentrations were calculated and the data fitted to the single-site binding equation (Equation 1). The percent changes (%ΔF) were calculated by subtracting the average value of intensity at 0 µM (F₀) from that at i µM (Fᵢ) and dividing average intensity value F₀.
\[ \Delta F = \Delta F^{\text{max}} \frac{[L]}{K_d + [L]} \]  

\(\Delta F\) is the fluorescence intensity change. \(\Delta F^{\text{max}}\) is the maximum attainable changes in fluorescence intensity. \([L]\) is the concentration of the ligand and \(K_d\) is the dissociation constant.

**Labeling of D95C Mutant With Fluorophore Probe**

A thiol-reactive fluorophore IANBD amide was chosen to label the tagged mutant MBP (D95C) via covalently binding to the thiol group of the cysteine residue in the tagged mutant MBP (D95C) protein. About 4.0 mL of 5.0 µM purified tagged mutant MBP in 10 mM potassium phosphate buffer pH, 7.4 was prepared. The protein solution was pre-incubated on ice for 30 minutes with 2-fold molar excess of dithiothreitol, DTT from Fermentas, USA (55 mM stock solution) to reduce intermolecular disulphide bonds. A 5-fold molar excess of the fluorophore (10 mM stock solution in dimethyl sulphoxide (DMSO)) was added and the mixture was incubated at room temperature for 2 hours in ice, protected from light on a rotary shaker. Excess fluorophore was removed from conjugated protein by diafiltration using a Vivaspin concentrator (0.5 mL, 10 kDa MWCO, Sartorius) with 10 mM potassium phosphate buffer in a centrifuge for 10 minutes at 4000 rpm. The diafiltration was repeated three times. The labeling ratio was determined and the labeled protein was then immediately analyzed or stored at 4°C for no more than a week.

**Steady-state Fluorescence Study of the Labeled Mutant Protein**

Fluorescence measurements were performed on a Perkin-Elmer fluorometer LS 55 (USA). The excitation and emission monochromator slit widths were both set at 10 nm. The excitation wavelength used was 480 nm. The intensity of fluorescence emitted was recorded upon addition of maltose to the labeled tagged mutant MBP (D95C) in the quartz cuvette. The labeled protein from was diluted to 1 µM in 1 mL potassium phosphate buffer, pH 7.4. Initial fluorescent measurement of the protein solution without addition of maltose was recorded. Titrations of the aliquots of 1 µL maltose ranging from 10 µM to 100 µM were added to the 1 mL protein solution. These steps were repeated three times. Prior to use, the quartz cuvette was treated with 1 M HCl to remove contaminating protein and maltose. The percent changes in
fluorescence intensity with increasing ligand concentrations were calculated and fit to single-site binding equation as stated in equation 5.1 and SigmaPlot® V 11.0 software was used to fit and stimulate data. To ascertain the specificity of MBP towards maltose, glucose was added instead of maltose. Titrations of the aliquots of 1 µL glucose ranging from 10 µM to 100 µM were added from an appropriate concentration of ligand stock solution (10 mM glucose) to the 1 mL protein solution. Then, the ligand binding of mutant MBP towards glucose was done as previously described.

RESULTS

Purification of Strep-tagged MBP
To construct a MBP for fluorescence sensing, aspartic acid at position 95 of MBP was mutated to a cysteine residue (D95C) by site-directed mutagenesis. The fluorophore was positioned at a location distant from the binding pocket, but it could sense ligand binding indirectly by an allosteric coupling mechanism based on the effect of domain movements (Shahir 2006). Strep-tagged wild-type MBP and mutant MBP (D95C) were purified by affinity chromatography. The elution peak containing Strep-tagged MBP and D95C had high resolution. Pure MBP and D95C proteins were obtained, both being approximately 42 kDa in size. The pooled fractions from the elution peak of D95C were buffer exchanged with potassium phosphate buffer to remove excess salt, detergent (e.g., DTT), and D-desthiobiotin. The pooled protein was concurrently concentrated by buffer exchange. This is showed in Figure 1 where the protein band of the buffer-exchanged sample was thicker than that of the pooled fractions before buffer exchange. This is further supported in Table 1 which shows that after buffer exchange, the protein concentration after buffer exchange is 0.33 mg/mL which is about three folds higher than the protein before, 0.10 mg/mL.

Intrinsic tryptophan of Strep-tagged MBP
Fluorescence of intrinsic tryptophan of the purified wild-type MBP was resolved to determine if the ligand-binding activity was retained in the presence of the tag. Wild-type MBP was excited at 280 nm, and the fluorescence emission at 340 nm was found to be comparable to the emission wavelength of tyrosine and
tryptophan in water at neutral pH (304 nm and 353 nm, respectively) (Lakowicz 1999). From the plot of intensity values against maltose concentrations and subsequent fitting to the single-site binding equation, the apparent dissociation constant \( (K_d) \) value for the MBP was \( 4.3 \pm 5.74 \) µM (Figure 2).

**Fluorescence sensing for maltose-binding ability**

The sulfhydryl group of D95C was individually labeled with the polarity-sensitive fluorophore, IANBD amide, and typical fluorophore-to-protein molar ratios were found to be in the range of 0.8–1.2. The labeled D95C was then analyzed by fluorescence sensing for its maltose-binding ability at increasing maltose concentrations. The extrinsic fluorescence emission of IANBD-labeled D95C labeled was measured, and the results showed that fluorescence intensity increased as the ligand concentration increased. On exciting the labeled D95C at 480 nm, the fluorescence emission maximum was blue shifted by ~10 nm of the actual emission wavelength for IANBD, which is 530 nm. On adding maltose, there was an increase in fluorescence intensity of up to 40%.

The binding curve of percent changes in fluorescence intensity with increasing concentrations of maltose was plotted using SigmaPlot® V 11.0. The plot and subsequent fitting to the single-site binding equation (Figure 2) showed that the data fit the single-site binding curve, with an \( R^2 \) value closer to 1 (0.8981). The apparent \( K_d \) of IANBD-labeled D95C obtained from the plot of percent changes in fluorescence intensity against maltose concentrations was \( 7.6 \pm 1.75 \) µM.

**DISCUSSION**

According to Litchy et al, Strep-tag II is the most sufficient affinity purification and the cheapest; furthermore, the affinity of Strep-tag II towards StrepTactin is higher. This bioaffinity feature could be manipulated for unidirectional immobilization for further biosensor applications. From the results of intrinsic tryptophan of the Strep-tag II of the wild-type MBP, the apparent \( K_d \) value is comparable with the \( K_d \) value of 3.5 µM of non-tagged and non-labeled MBP reported by Miller et al. 1983. Hence, the Strep-tag II of the wild-type MBP does not affect its maltose-binding ability, and the tag at the N-terminal does
not appear to pose steric hindrance. The result is similar to that of another study where lipase enzyme remained functional with a fused Strep-tag II (Hamid et al. 2009). Furthermore, no fluorescence changes were observed on adding glucose. MBP can bind to maltose, maltotriose, and α(1-4)-linked higher maltodextrins, but it does not recognize or bind to glucose (Spurlino and Quicho 1991).

Exciting the labeled D95C at 480 nm, showed an increased in fluorescence intensity that concurs with the results of Dattelbaum et al. 2004, where a large increase in fluorescence was observed upon maltose titration of IANBD-labeled D95C, and a slight blue shift of about 5–6 nm occurred before and upon the addition of maltose. Furthermore, the plot and subsequent fitting to the single-site binding equation indicate that the IANBD-labeled D95C managed to retain its maltose-binding activity with covalently attached IANBD, as well as Strep-tag II at the N terminal. Additionally, the $K_d$ values obtained demonstrate that the affinity of D95C-fused Strep-tag II toward maltose is lower than that of the wild type ($K_d = 3.5 \mu M$) (Marvin and Hellinga 2001). The apparent $K_d$ value obtained is much higher than those of IANBD-labeled D95C as obtained in earlier studies (Dattelbaum et al. 2004; Marvin et al. 1997; Shahir in 2006). The lower affinity obtained could be due to the steric hindrance of Strep-tag II at the N terminal, which causes a conformational change during maltose binding.

In studies by Marvin et al. in 1997, Dattelbaum et al. in 2004, and Shahir in 2006 (Dattelbaum et al. 2004; Marvin et al. 1997), the use of shorter tags such as His$_6$ (six histidine residues) and biotin-tag (a 15-amino acid peptide) yielded much lower $K_d$ values compared to Strep-tag II–D95C. Strep-tag II has an eight-amino acid tag, with an additional set of 14 amino acids that serves as a cleavage site (Litchy et al. 2005). Thus, there is a stretch of 22 amino acids at the N terminal of D95C. It is possible that the larger size of Strep-tag might be causing steric hindrance, thereby lowering the affinity of the tagged D95C toward maltose. However, further studies are needed to confirm this.

No significant increases in fluorescence intensity response were observed upon addition of increasing concentrations of glucose to the labeled protein. The plot of percent changes in fluorescence intensity data against glucose concentrations shows that with a value of 0.0462, the fluorescence response does not fit the single binding site curve with an $R$-squared value greater than 1. This suggested that D95C does not bind to glucose, because the binding site of MBP does not have hydrogen bond acceptors, leaving the donatable hydrogen of the C4 hydroxyl or C1 hydroxyl of glucose unpaired if the
monosaccharide binds to the binding site (Spurlino and Quicho 1991). Hence, tagged D95C still binds specifically to maltose.

Furthermore, the fluorescence response due to maltose-binding activity indicates that the mutation site of D95C is heterotropically cooperative with respect to the specific binding of maltose (Marvin et al. 1997). Heterotropic cooperation implies that the substrate binds to the enzyme at only one site, and a different molecule modifies the reaction by binding to an allosteric site (Marvin et al. 1997). In this sense, the fluorescence of the conjugated protein with the attached fluorophore IANBD changes in a heterotropically cooperative manner with respect to maltose (Marvin et al. 1997). Furthermore, the mutation of D95C is at the allosteric site of the maltose-binding site, and the attached fluorophore IANBD is appropriately placed within the site such that its environment is sufficiently affected by local conformational changes to elicit a change in fluorescence (Marvin et al. 1997). Thus, Strep-tag II–fused D95C could retain its ligand-binding activity and specificity toward maltose.

In summary, a reagentless sensing scheme for maltose was designed and developed using MBP mutants that allow for site-specific labeling with a fluorophore. The detection limit obtained was nearly the same as that reported in several other studies. The slight difference might be due to the possible steric hindrance of Strep-tag II at the N terminal of the protein. The tagged mutant MBP (Strep-tag II–D95C) could retain its ligand-binding activity toward maltose but less tightly. Thus, this engineered mutant MBP could be developed for immobilized biosensor applications.

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**Table 1**: Purification Table. The concentration and total protein of protein samples using protein assay Bradford reagent.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Percent Yield (%)</th>
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<tbody>
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<td>39.40</td>
<td>100</td>
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<td>1.50</td>
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<td>0.33</td>
<td>1.65</td>
<td>4.2</td>
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</tbody>
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Figure 1: SDS-PAGE analysis of Strep-Tag II MBP and D95C using comassie blue staining. Lane 1, Fermentas unstained protein molecular weight marker; Lane 2, crude lysate of the D95C; Lane 3, Pooled of fractions before buffer exchange; Lane 4, Pooled fractions after buffer exchange.
Figure 2: Maltose binding curves of intrinsic tryptophan of wild type MBP and IANBD D95C. Data were fitted to the single site binding equation using SigmaPlot V11.1.