Tropical Life Sciences Research, 27(1), 63–75, 2016

Strep-tag II Mutant Maltose-binding Protein for Reagentless Fluorescence Sensing

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Abstrak: 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; MBP 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 molekular ini yang menyebabkan transduksi pengikatan ligan kepada bentuk fizikal menjadikan MBP calon yang sesuai sebagai penderia pendaflour tanpa deria. Di sini, kami akan menerangkan bagaimana mutan *Strep*-tag II MBP dibina 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 dan kemudian dianalisa secara deria pendaflour. Mutan MBP (D95C) telah ditulenkan (saiz molekular, ~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 K_d 7.6±1.75 μM). Mutan MBP berjaya mengekalkan aktiviti pengikatan kepada maltosa dan sesuai dijadikan sebagai penderia pendaflour tanpa reagen.

Kata kunci: Protein Pengikat Maltose, *Strep*-tag II, Penderia Pendaflour, D95C

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*, which encodes 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 analysed by fluorescence sensing. The tagged mutant MBP (D95C) was purified (molecular size, ~42 kDa). The fluorescence measurements of the IANBD-labelled

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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 used 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 & Hellinga 1998). Periplasmic binding proteins (PBPs) are being extensively studied for applications as reagentless fluorescence biosensors because of their general structural form and their 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 & Marvin 1998).

Maltose-binding protein (MBP) belongs to the PBP superfamily, which is found in Gram negative bacteria (Zhou & 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 (Sharff *et al.* 1993). Other than binding to maltose, MBP can also be manipulated by protein engineering to bind to metal ions for environmental biosensor applications (Marvin & 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 type of fluorophore is detected by fluorescence sensing of the conformational changes in MBP upon ligand binding. This strategy has been used to introduce single fluorophores that respond to ligand binding in MBPs (Sohanpal *et al.* 1993; Gilardi *et al.* 1994; Marvin & Hellinga 2001; De Lorimier *et al.* 2002; Dattelbaum *et al.* 2004), 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 in PBPs. Owing to its consistent fluorescence response, 4-[N-(2-(Iodoacetoxy)ethyl)-N-methylamino]-7-nitrobenz-2oxa-1,3-diazole (IANBD) amide has been found to be a suitable fluorophore for covalent attachment to the cysteine residue of recombinant MBP (Gilardi *et al.* 1994; Marvin & Hellinga 1998; Dattelbaum *et al.* 2004; 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 & Spence 2011).

In this study, the fluorophore was attached to the protein at the allosteric site of the protein, which is located at position 95, where aspartic acid was 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 in that no direct interaction occurs between the ligand and the fluorophore; hence, the binding constant is unaffected (Marvin & Hellinga 1998). Several mutations of MBP have been induced at the allosteric site for potential reagentless fluorescence sensing, and these are D95C, F92C, and I329C (Sohanpal *et al.* 1993; De Lorimier *et al.* 2002). In this study, D95C was chosen because its use has been reported in several studies (Sohanpal *et al.* 1993; Marvin *et al.* 1997; Marvin & Hellinga 2001), and the binding affinity towards maltose of D95C is often higher than that of other mutations (Marvin & Hellinga 2001; Wemmer 2003).

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

MATERIALS AND METHODS

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

The *malE* gene was amplified from the pMAL-c4x plasmid using the polymerase chain reaction (PCR) with flanking primers designed to introduce a *Bam*HI restriction enzyme site before the start codon and a *Hin*dIII site before the stop codon. The primers (First BASE Laboratories Sdn. Bhd., Sri Kembangan, Selangor, Malaysia) used for isolating the *malE* gene were as follows: forward, For_ *malE* (5'-CCCGGATCCGAAAATCGAAGAAGGTAAACTGGTC-3'), and reverse, Rev_ *malE* (5'-CCCAAGCTTTTAAGTCTGCGCGTCTTTCAGGCG-3'). A 30-cycle PCR program was used, with the pMAL-c4x plasmid used as the template and primers and *Pfu* polymerase added to the reaction mix (Fermentas, Massachusetts, USA). The cycle conditions were 95°C for 2 min, followed by 30 amplification cycles of 95°C for 30s, 55°C for 1 min and 72°C for 2 min and then

one final elongation cycle at 72°C for 5 min. Reactions were run on a tris-acetate-ethylenediamine tetraacetic acid (TAE) 1% agarose gel, and when the presence of the gene was verified, the remaining PCR mixture was treated with *Dpn*I (Fermentas, Massachusetts, USA) at 37°C for 1 hr to digest the methylated template, which was then purified with a PCR Clean-up System (Promega, Wisconsin, USA). The purified DNA was digested with *BamH*I and *Hin*dIII restriction enzymes (Fermentas, Massachusetts, USA) and ligated using T4 DNA ligase into pET-51(b) (Novagen, Darmstadt, Germany), which was digested using the same restriction enzymes. The constructed plasmid pET-51b(+)-*malE* was then transformed into *E. coli* NovaBlue Singles cells (Novagen, Darmstadt, Germany), and transformants were selected on Luria Bertani ampicilin (LBA) plates using 50 µg/mL ampicillin.

Construction of pET-51b(+)-D95C Plasmids (Mutant MBP Plasmid)

The MBP mutant plasmid was constructed to contain a single substitution: aspartic acid (position 95) was replaced with a cysteine. Site-directed mutagenesis was performed using inverse PCR by the *Pfu* DNA polymerase and the *malE* gene (from pET-51b (+)-*malE*) used as the template. The primers used were as follows: forward, D95C_For (5'-TGCGCAGTACGTTACAACGGC-3'), and reverse, D95C_Rev (5'- CATCTGCGGGATGTTCGGCAT-3'), and the reaction mix was treated with T4 polynucleotide kinase (Fermentas, Massachusetts, USA) prior to use at 37°C for 1 hr. The PCR reaction was performed as follows: one cycle at 92°C for 2 min, then 15 cycles at 92°C for 20s, 55°C for 1 min and 68°C for 6 min, and then one final cycle at 62°C for 10 min. The plasmid construct was then verified, purified, ligated and transformed as described for the pET-51b (+)-*malE* plasmid construct, above.

Expression of the *malE* Gene

The recombinant plasmid was transformed into an expression host, BL21(DE3)pLySs (Novagen, Darmstadt, Germany), by heat shock transformation. The BL21(DE3)pLySs cells harbouring the recombinant plasmid were inoculated into 1 mL of Luria Bertani (LB) broth containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol, which was then incubated overnight at 37°C with shaking. Then, 10 mL of this overnight culture was inoculated into 200 mL of fresh LB broth containing both of the antibiotics in addition to 1% (v/v) glucose and incubated at 37°C with shaking until the optical density (OD) at 600 nm reached ~0.4–0.5. The cells were then induced with 1 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 30°C. Finally, the cells were harvested by centrifugation at 4,000 rpm for 20 min 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 using the freeze and thaw method. First, the cell pellets were thawed, followed by the addition of sterile distilled water and benzonase nuclease. One gram of cell pellet material required 5 mL of sterile distilled water, and 25 U of benzonase nuclease was added per 1 mL of sterile distilled water. The mixture was then incubated at –20°C in a freezer for 10 to 15 min or until the mixture had frozen. Then, the mixture was thawed on ice for

30 min until the mixture became liquefied. These freezing and thawing steps were repeated three times. The host cells contained pLysS and had relatively high levels of T7 lysozyme in the cytoplasm, which made it easier to lyse the cells under mild conditions such as the freeze/thaw treatment (Mierendorf *et al.* 1994a,b). The mixture was then centrifuged at 16,000 × g for 20 min 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 extracts of wild type MBP protein and its mutant were purified by affinity chromatography using a 5 mL pre-packed StrepTrap HP™ column (GE Healthcare, Buckinghamshire, UK). Purification was performed using Akta Prime Plus (GE Healthcare, Buckinghamshire, UK) with a flow rate of 2 mL/min. The columns, buffers and the protein samples were kept on ice during the purification process to lessen the degradation of the mutant MBP. Purification of the wild type MBP was performed at room temperature because MBP is known to be thermally stable in solution at temperatures up to 50°C (Gilardi *et al.* 1994).

First, the Akta Prime Plus system was flushed with filtered deionised 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 deionised water before starting the purification process. The column was equilibrated and washed with five column volumes of binding buffer (20 mM $NaPO_4$, 280 mM NaCl, 6 mM KCl and 10 mM ethylenediamine tetraacetic acid [EDTA], pH 7.4). Approximately 20 mL of 6 mg/mL crude lysate (pre-filtered through a 0.45 µm nylon membrane) was loaded into the StrepTrap™ Hp column with a flow rate of 2 mL/min. The column was washed with eight column volumes of binding buffer. Next, elution was performed using three column volumes of the same binding buffer plus 2.5 mM d-desthiobiotin. The elution profile was monitored by measuring OD at 280 nm, and fractions were subsequently analysed 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 molecular weight cut-off [MWCO] membrane, Sartorius Stedim, Goettingen, 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 4,000 rpm for 10 min. Approximately 5–10 mL of the protein solution was added, then buffer was added to make the volume 15 mL, and the solution was centrifuged at 4,000 rpm for 5 min. The filtrate was decanted, buffer was added to make the volume 15 mL, and the solution was centrifuged again as previously described. This step was repeated and the protein concentration was determined via Bradford Assay based on Beer-Lambert's Law.

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

Intrinsic tryptophan fluorescent measurement was performed on a Perkin-Elmer fluorometer LS 55 (Massachusetts, USA) with an excitation wavelength of 280 nm. The excitation wavelength was set at 280 nm because the aromatic amino acids (i.e.: tryptophan, tyrosine and phenylalanine) absorb light at that particular wavelength. The intensity of the fluorescence was recorded on the addition of maltose towards the purified tagged wild type MBP in the quartz cuvette. Approximately 1.0 mL of 1.0 µM purified tagged wild type MBP in 10 mM potassium phosphate buffer, pH 7.4, was prepared. First, an initial fluorescence measurement was recorded for the protein solution without added maltose. Titrations of 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 any contaminating protein or maltose. The percent changes in fluorescence intensity that were observed with increasing ligand concentrations were calculated, and the data was fitted to a single-site binding equation (equation [Eq.] 1). The percent changes (%ΔF) were calculated by subtracting the average value of the intensity at 0 µM (F_0) from the average value at *i* µM (F_i) and then dividing by average intensity value F_0.

$$\Delta F = \Delta F^{\max} \frac{[L]}{K_d + [L]} \tag{Eq. 1}$$

where ΔF is the fluorescence intensity change, ΔF^{max} is the maximum attainable change in fluorescence intensity, [L] is the concentration of the ligand and K_d is the dissociation constant.

Labelling of the D95C Mutant with Fluorophore Probes

The thiol-reactive fluorophore IANBD amide was chosen to label the tagged mutant MBP (D95C). The fluorophore was covalently bound to the thiol group of the cysteine residue in the tagged mutant MBP (D95C) protein. Approximately 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 a 2-fold molar excess of dithiothreitol (DTT) from Fermentas, Massachusetts, USA (55 mM stock solution) to reduce intermolecular disulphide bonds. A 5-fold molar excess of the fluorophore (10 mM stock solution in dimethyl sulfoxide [DMSO]) was added, and the mixture was incubated at room temperature for 2 hr in ice, while protected from light, on a rotary shaker. Excess fluorophore was removed from the conjugated proteins by diafiltration using a Vivaspin concentrator (0.5 mL, 10 kDa MWCO, Sartorius, Goettingen, Germany) with 10 mM potassium phosphate buffer in a centrifuge at 4,000 rpm for 10 min. Diafiltration was repeated three times. The labelling ratio was determined, and the labelled protein was then immediately analysed or stored at 4°C for no more than one week.

Steady-state Fluorescence Study of the Labelled Mutant Protein

Fluorescence measurements were performed on a Perkin-Elmer fluorometer LS 55. 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 the addition of maltose to the labelled tagged mutant MBP (D95C) in the quartz cuvette. The labelled protein was diluted to 1 μM in 1 mL potassium phosphate buffer, pH 7.4. An initial fluorescent measurement of the protein solution prior to the addition of maltose was recorded. Titrations of 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 a single-site binding equation, as stated in Eq. 1, and SigmaPlot® V 11.0 (California, USA) software was used to fit and simulate the data. To determine the specificity of MBP binding with maltose, glucose was added instead of maltose. Titrations of aliquots of 1 μL glucose, ranging from 10 μM to 100 μM, were added from a ligand stock solution at an appropriate concentration (10 mM glucose) to the 1 mL protein solution. Then, the specificity of ligand binding of the mutant MBP with glucose was analysed as previously described.

RESULTS

Purification of *Strep*-tagged MBP

To construct a MBP for fluorescence sensing, the aspartic acid at position 95 of MBP was mutated to a cysteine residue (D95C) using site-directed mutagenesis. The fluorophore was positioned at a location that was distant from the binding pocket but at which ligand binding could be sensed indirectly using an allosteric coupling mechanism based on the effect of domain movements (Shahir 2006). *Strep*-tagged wild type MBP and mutant MBP (D95C) were purified using affinity chromatography. The elution peak containing *Strep*-tagged MBP and D95C had a high resolution. Pure MBP and D95C proteins were obtained, with 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 shown in Figure 1, where the protein band of the buffer-exchanged sample is observed to be thicker than the band of the pooled fractions before buffer exchange. This finding is further supported in Table 1, which shows that after buffer exchange, the protein concentration is 0.33 mg/mL, which is about three-fold higher than the protein concentration before exchange (0.10 mg/mL).

Intrinsic Tryptophan of *Strep*-tagged MBP

The fluorescence of the intrinsic tryptophan of the purified wild type MBP was resolved to determine if 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 a plot of intensity values against maltose concentrations and subsequent fitting to the single-site binding equation, the apparent dissociation constant (K_d) value of the MBP was 4.3±5.74 μM (Fig. 2).

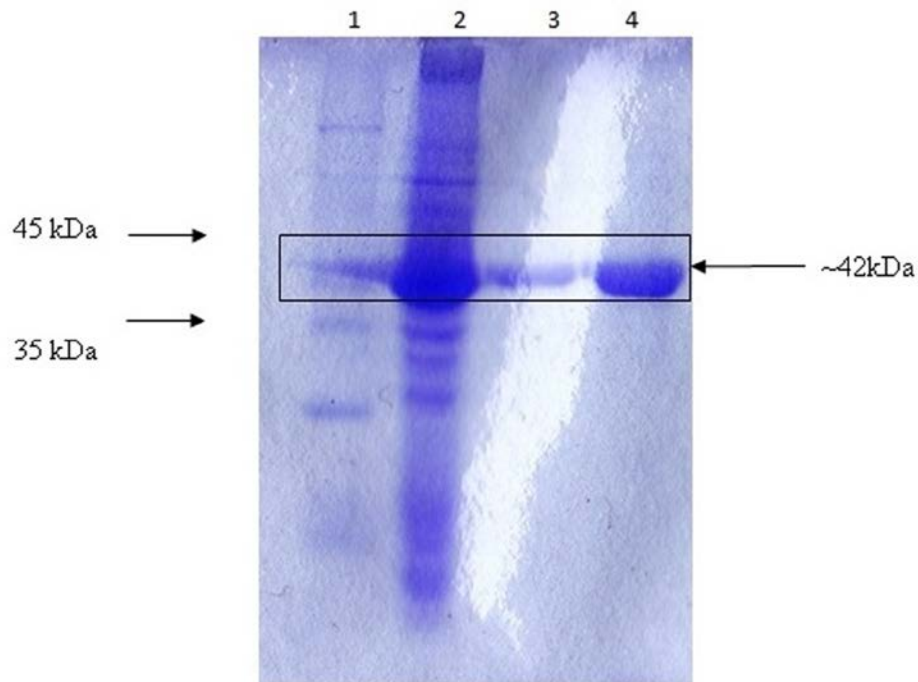

Figure 1: SDS-PAGE analysis of *Strep*-Tag II MBP and D95C using coomassie blue staining.

Notes: Lane 1 - Fermentas unstained protein molecular weight marker; lane 2 - crude lysate of D95C; lane 3 - pooled fractions before buffer exchange; lane 4 - pooled fractions after buffer exchange.

Table 1: Purification table. The concentrations of and total protein in the protein samples, as determined using protein Bradford assay reagents.

Step	Total volume (mL)	Concentration (mg/mL)	Total protein (mg)	Percent yield (%)
Break cell (crude lysate)	20	1.97	39.40	100.0
Affinity purification	15	0.10	1.50	3.8
Ultrafiltration (buffer exchange)	5	0.33	1.65	4.2

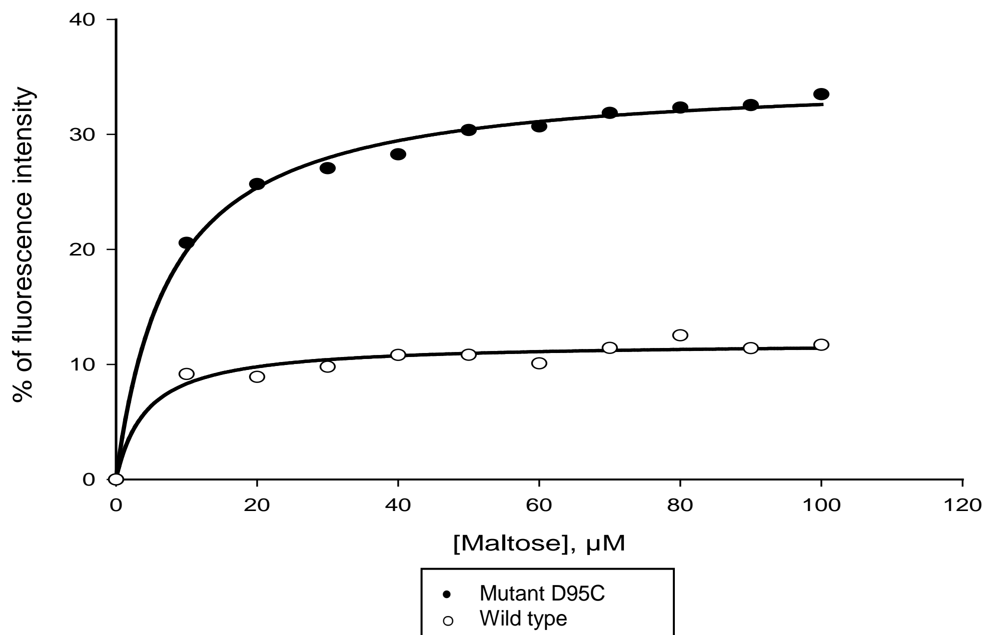

Figure 2: Maltose-binding curves for the intrinsic tryptophan of wild type MBP and IANBD D95C. Data were fitted to the single-site binding equation using SigmaPlot V 11.0.

Fluorescence Sensing for Maltose-binding Ability

The sulfhydryl group of D95C was individually labelled with a polarity-sensitive fluorophore, IANBD amide, and typical fluorophore-to-protein molar ratios were observed in the range of 0.8–1.2. The labelled D95C was then analysed by fluorescence sensing for its maltose-binding ability at increasing maltose concentrations. The extrinsic fluorescence emission of IANBD-labelled D95C was also measured, and the results showed that the fluorescence intensity increased as the ligand concentration increased. On exciting the labelled 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 the fluorescence intensity of up to 40%.

The binding curve of the 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 (Fig. 2) showed that the data fit the single-site binding curve, with an R^2 value close to 1 (0.8981). The apparent K_d of IANBD-labelled D95C that was obtained from the plot of percent changes in fluorescence intensity against maltose concentrations was 7.6±1.75 µM.

DISCUSSION

According to Litchy *et al.* (2005), *Strep*-tag II is the most efficient and the cheapest system for affinity purification; furthermore, the affinity of *Strep*-tag II towards StrepTactin is higher. This bioaffinity feature could be manipulated for unidirectional immobilisation for further biosensor applications. From the results of our intrinsic tryptophan assay of the *Strep*-tag II of the wild type MBP, its apparent K_d value is comparable to the K_d value of 3.5 μM of non-tagged and non-labelled MBP that was reported by Miller *et al.* (1983). Hence, the *Strep*-tag II on wild type MBP does not affect its maltose-binding ability, and the tag at the N-terminal did not appear to pose a steric hindrance. These results are similar to those in 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 recognise or bind to glucose (Spurlino *et al.* 1991).

Exciting the labelled D95C at 480 nm resulted in an increase in fluorescence intensity that was in agreement with the results of Dattelbaum *et al.* (2004), where a large increase in fluorescence was observed upon maltose titration of IANBD-labelled D95C, and a slight blue shift of approximately 5–6 nm occurred before and following the addition of maltose. Furthermore, the plot and subsequent fitting to the single-site binding equation indicated that the IANBD-labelled D95C managed to retain its maltose-binding activity with the covalently attached IANBD and with the *Strep*-tag II attached at the N terminal. Additionally, the K_d values that we 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 μM) (Marvin & Hellinga 2001). The apparent K_d value that was obtained was much higher than the values for IANBD-labelled D95C that were obtained in earlier studies (Marvin *et al.* 1997; Dattelbaum *et al.* 2004; Shahir 2006). The lower affinity obtained in this study could be due to steric hindrance by the *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, 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 serving as a cleavage site (Litchy *et al.* 2005). Thus, there is an extra stretch of 22 amino acids at the N terminal of D95C. It is possible that the larger size of the *Strep*-tag might cause steric hindrance, which may thereby lower the affinity of the tagged D95C toward maltose. However, further studies are needed to confirm this.

No significant increases in fluorescence intensity responses were observed upon the addition of increasing concentrations of glucose to the labelled protein. The plot of the percent changes in fluorescence intensity data against glucose concentrations shows that at 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 suggests that D95C does not bind to glucose because the binding site of MBP does not have hydrogen bond acceptors, which leaves the

donatable hydrogen of the C4 hydroxyl or the C1 hydroxyl of glucose unpaired if the monosaccharide binds to the binding site (Spurlino *et al.* 1991). Hence, tagged D95C still binds specifically to maltose.

Furthermore, the fluorescence response observed with maltose-binding activity indicates that the mutation site at 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 that 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 is changed 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 retains its ligand-binding activity and specificity toward maltose.

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

ACKNOWLEDGEMENT

We would like to thank the Ministry of Education Malaysia for funding this research under the Fundamental Research Grant Scheme Vote number 78309. We are grateful to Universiti Teknologi Malaysia for providing research facilities.

REFERENCES

Brune M, Hunter J L, Corrie J E and Webb M R. (1994). Direct, real-time measurement of rapid inorganic phosphate release using novel fluorescent probe and its application to actomysin subfragment 1 ATPase. *Biochemistry* 33(27): 8262–8271.

Dattelbaum J D, Looger L L, Benson D E, Sali K M, Thompson R B and Hellinga H W. (2004). Analysis of allosteric signal transduction mechanisms in an engineered fluorescent maltose biosensor. *Protein Science* 14(2): 284–291.

De Lorimier R M, Smith J J, Dwyer M A, Looger L L, Sali K M, Paavola C D, Rizk S S *et al.* (2002). Construction of fluorescent biosensor family. *Protein Science* 11(11): 2655–2675.

Gilardi G, Zhou L Q, Hibber L and Cass A E. (1994). Engineering the maltose binding protein for reagentless fluorescence sensing. *Analytical Chemistry* 66(21): 3840–3847.

Hamid T H T A, Eltaweel M A, Rahman R N Z R A, Basri M and Salleh A B. (2009). Characterization and solvent stable features of Strep-tagged purified recombinant lipase from thermostable and solvent tolerant *Bacillus sp.* Strain 42. *Annals of Microbiology* 59(1): 111–118.

Hellinga H W and Marvin J S. (1998). Protein engineering and the development of generic biosensor. *Trends in Biotechnology* 16(4): 183–189.

Johnson L and Spence M T Z. (2011) *Molecular probes® handbook*: *A guide to fluorescent probes and labeling technologies*, 11th ed. Massachusetts, USA: Invitrogen Life Technologies Corporation.

Lakowicz J R. (1999). *Principles of fluorescence spectroscopy*, 2nd ed. New York: Springer.

Litchy J L, Malecki J L, Agnew H D, Michelson-Horowitz D J and Tan S. (2005). Comparison of affinity tags for protein purification. *Protein Expression and Purification* 41(1): 98–105.

Lyndon L E, Salins R A W, Ensor C M and Daunert S. (2001). A novel reagentless sensing system for measuring glucose based on the galactose/glucose-binding protein. *Analytical Biochemistry* 294(1): 19–26.

Marvin J S, Corcoran E E, Hattangadi N A, Zhang J V, Gere S A and Hellinga H W. (1997). The rational design of allosteric interactions in a monomeric protein and its applications to the construction of biosensors. *Proceedings of the National Academy of Sciences USA* 94(9): 4366–4371.

Marvin J S and Hellinga H W (2001). Manipulation of ligand binding affinity by exploitation of conformational coupling. *Natural Structure of Biology* 8(9): 795–798.

______. (1998). Engineering biosensors by introducing fluorescent allosteric signal transducers: Construction of a novel glucose sensor. *Journal of American Chemistry Society* 120(1): 7–11.

Mierendorf R C, Morris B B, Hammer B and Novy R E. (1994a). Expression and purification of recombinant proteins using the pET system. In R Rapley (ed.). *The nucleic acid protocols handbook*, vol. 13. Totowa, NJ, USA: Humana Press Inc., 257–292.

Mierendorf R, Yeager K and Novy R E. (1994b). The pET system: Your choice for expression. *InNOVAtions* 1(1): 1–3.

Miller D M 3rd, Olson J S, Pflugrath J W and Quiocho F A. (1983). Rates of ligand binding to periplasmic proteins involved in bacterial transport and chemotaxis. *Journal of Biological Chemistry* 258 (22): 13665–13672.

Shahir S. (2006). *Engineering the maltose binding protein for metal ion sensing*. PhD diss., Imperial College London.

Sharff A J, Rodseth L E and Quiocho F A. (1993). Refined 1.8-Å structure reveals the mode of binding of p-cyclodextrin to the maltodextrin binding protein. *Biochemistry* 32(40): 10553–10559.

Shrestha S, Salins L L E, Ensor C M and Dauner S. (2002). Rationally designed fluorescently labeled sulfate-binding protein mutants: Evaluation in the development of a sensing system for sulfate. *Biotechnology and Bioengineering* 78(5): 517–526.

Sohanpal K, Watsuji T, Zhou L Q and Cass A E G. (1993). Reagentless fluorescence sensor based upon specific binding proteins. *Sensors and Actuators* 11(1–3): 547–552.

Spurlino J C, Lu G Y and Quicho F A. (1991). The 2.3 Å resolution structure of the maltose or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis. *Journal of Biological Chemistry* 266(6): 5202–5219.

Vercillo N C, Herald K J, Fox J M, Der B S and Dattelbaum J D. (2006). Analysis of ligand binding to a ribose biosensor using site-directed mutagenesis and fluorescence spectroscopy. *Protein Science* 16(3): 362–368.

Wemmer D E. (2003). The energetic of structural change in maltose binding protein. *Proceedings of the National Academy of Sciences USA* 100(22): 12529–12530.

Zhou Q L and Cass A E G. (1991). Periplasmic binding protein based biosensors: preliminary study of maltose binding protein as sensing element for maltose biosensor. *Biosensors and Bioelectronics* 6(1): 445–450.