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Efficient function of Signal Peptidase 1 of *Escherichia coli* is partly determined by residues in the mature N-terminus of exported proteins.

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Abstract

Exported proteins require an N-terminal signal peptide to direct them from the cytoplasm to the periplasm. Once the protein has been translocated across the cytoplasmic membrane, the signal peptide is cleaved by a signal peptidase, allowing the remainder of the protein to fold into its mature state in the periplasm. Signal peptidase I (LepB) cleaves non-lipoproteins and recognises the sequence Ala-X-Ala. Amino acids present at the N-terminus of mature, exported proteins have been shown to affect the efficiency at which the protein is exported. Here we investigated a bias against aromatic amino acids at the second position in the mature protein (P2'). Maltose binding protein (MBP) was mutated to introduce aromatic amino acids (tryptophan, tyrosine and phenylalanine) at P2'. All mutants with aromatic amino acids at P2' were exported less efficiently as indicated by a slight increase in precursor protein *in vivo*. Binding of LepB to peptides that encompass the MBP cleavage site were analysed using surface plasmon resonance. These studies showed peptides with an aromatic amino acid at P2' had a slower off rate, due to a significantly higher binding affinity for LepB. These data are consistent with the accumulation of small amounts of preMBP in purified protein samples. Hence, the reason for the lack of aromatic amino acids at P2' in *E. coli* is likely due to interference with efficient LepB activity. These data and previous bioinformatics strongly suggest that aromatic amino acids are not preferred at P2' and this should be incorporated into signal peptide prediction algorithms.

Keywords:

N-terminal mature residues; signal peptide processing; evolution; aromatic amino acids

1. Introduction

For all cells to survive and function efficiently it is essential for proteins to be expressed in the correct location, which may involve the utilisation of a secretion system. Most secretion systems follow the typical pathway of (i) targeting a protein to the membrane, (ii) translocating the protein across and lastly, (iii) releasing the protein from the membrane [1]. Exported proteins often contain an N-terminal signal peptide, which assists in directing them to the membrane via either co-translational pathway via direct binding of signal recognition particle [2], or post translationally via the molecular chaperone SecB [3]. To release the protein from the membrane, the signal peptide is cleaved by signal peptidase (SPase) allowing the protein to either be incorporated into the membrane or released into the periplasmic space.

Signal peptides are defined by three conserved regions (i) a positively charged N-terminus, (ii) a hydrophobic core, and (iii) a C-terminal hydrophilic region containing the SPase cleavage site [1]. A bias for small, uncharged residues at positions 1 and 3 before the cleavage site (P1 and P3) lead to the definition of the Ala-X-Ala rule as the cleavage specificity of SPase I, where X stands for any amino acid as no bias is seen at P2 [4].

The addition of a signal peptide to a cytoplasmic protein does not (always) lead to successful secretion [5], suggesting that residues on the mature protein side of exported proteins play a role in this process. The large majority of the research into the mature protein side of exported proteins has been focused on the presence of negatively charged residues [6, 7], as well as analysis of amino acid frequencies [8]. The presence of a proline at P1' in maltose binding protein (MBP) [9, 10] completely blocks signal peptide removal by LepB (SPase I). In PhoE, addition of arginine at P2' [6, 7] causes a similar block in processing by LepB. Further studies have introduced basic amino acids (lysine and arginine) at the N-terminus of the mature protein for OmpA [11] and PhoA [7, 12], and observed decreases in the efficiency of signal peptide processing. Hence it is clear that there are signals on the mature side of the protein that are important for efficient processing by SPase I.

Previously we reported that zero aromatic amino acids were found at P2' in a group of 143 verified type I signal peptidase cleavage sites in *Escherichia coli* [13]. In

accordance with our previous findings, none of the verified type I SPase data-set contained either a proline at P1' or a arginine at P2' [13]. To this date, no other studies have investigated the direct effects of aromatic amino acids at this position *in vitro*. In this study, we used site directed mutagenesis of maltose binding protein (MBP) as a model system of secretion as well as biophysical analysis to assess the impact of introducing aromatic amino acids at P2'.

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2. Materials and Methods

2.1 Molecular cloning techniques

Cloning was carried out in *E. coli* DH5 α (*F*- (*80dlacZ* Δ *M15*) (*lacZYA-argF*) *U169 hsdR17* (*r-m+*) *recA1 endA1 relA1 deoR*). Protein expression was carried out in *E. coli* TL225 (*F*- [*araD139*]_{B/r} Δ (*argF-lac*)169 λ *zce-727::Tn10 flhD5301* Δ (*fruK-yeiR*)725(*fruA25*) *relA1 rpsL150(strR) maltI101*(Const) Δ *malE444 deoC1*). Ampicillin was used at 100 μ g/ml. All PCR reactions were carried out using KOD (Novagen). Ligations using T4 DNA ligase were performed according to manufacturer's instructions (New England Biolabs). DNA sequencing was done using the Big Dye Terminator method (Griffith University DNA Sequencing Facility).

2.2 Constructing *malE* without the *lacZ* α on pMALp2e

To make the wild-type *malE* gene without the *lacZ* α present, we amplified *malE*, using pMALp2e as template, with the primers *malE* and *malE_R* (Table S1). The reverse primer adds the last four amino acids of wild-type MalE not originally present (encoding the sequencing RITK) before adding a stop codon. This deletes the linker region and *lacZ* α present before on the original plasmid. The 1210 bp PCR product was digested with NdeI and PstI, and ligated into the pMALp2e vector digested with the same enzymes. The ligation was transformed into DH5 α and putative clones with the wild-type *malE* gene (without the *lacZ* α region) were confirmed by sequencing.

2.3 Construction of aromatic amino acid mutants at P2' in MBP

To make the aromatic mutants at P2', the *malE* construct without the *lacZ* α (made above) was used as a template for inverse PCR to introduce the base-changes at P2'. Briefly, a common forward primer (MBP_invF) was paired with the appropriate mutant primer (Table S1) in a PCR reaction. PCR products generated were treated with T4 PNK (NEB, M0201S) before being ligated with T4 DNA ligase and transformed into DH5 α . Putative clones were confirmed by DNA sequencing (Griffith University DNA Sequencing Facility) for the presence of the correct aromatic amino acid at P2'. The plasmids containing the correct sequences were then transformed into TL225, a *malE* deficient strain.

2.4 Generating the anti-preMBP antibody

The protocol followed was as described in Jen, Djoko, Bent, Day, McEwan and Jennings [14]. Five mice were inoculated with 40 μg / mouse of a peptide ($^1\text{MKIKTGARILALTTM}^{15}$), which comprises the first 15 amino acids of the MBP signal peptide. The peptide was synthesised and conjugated with keyhole limpet hemocyanin (KLH) by Mimotopes (Clayton, VIC, Australia). The first inoculation of each mouse contained the peptide solubilised in 200 μl Freund's Complete Adjuvant / mouse. Subsequent boosts with 20 μg peptide / mouse at days 35 and 49 was solubilised in Freund's Incomplete Adjuvant (200 μl / mouse). The sera was tested by ELISA against non-KLH linked peptide to confirm the presence of anti-preMBP antibodies.

2.5 Maltose binding protein purification

Maltose binding protein (MBP) mutants were purified using amylose column buffer as per manufacturer's instructions (New England Biolabs). Briefly, a starter culture of 4 ml LB-ampicillin was grown overnight then added to 400 ml LB-ampicillin. This culture was then grown for 3 h at 37°C with shaking, before being pelleted by centrifugation and stored at -20°C overnight. Pellets were resuspended in column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) containing one tablet of EDTA-free protease inhibitor cocktail (Merck, 11836170001) and 200 μg DNase. Cells were lysed by sonication, lysates were then centrifuged at 10,000 g for 10 min and passed through a 0.45 μm filter. The remaining supernatant was passed by gravity flow over a 2 ml amylose column and washed with column buffer. MBP was eluted in 1 ml fractions with 10 mM maltose in column buffer. Pure protein fractions were pooled together and concentrated using a 10 kDa cut off spin column (Merck).

2.6 SDS-PAGE analysis

For western blot analysis, whole cell lysates or purified proteins were subjected to SDS-PAGE and transferred onto PVDF membrane. The membranes were immunoblotted with either anti-MBP (NEB, E8032S) or anti-preMBP (developed in this study, see materials and methods), then detected using anti-mouse IgG (Sigma, A3562). Sypro Ruby stain (Thermo Fisher Scientific, S12000) was performed as per manufacturer's instructions, after SDS-PAGE of pure MBP protein samples.

2.7 *N-terminal Sequencing of MBP variants*

Approximately 3 μg of all purified protein samples (MBP-wt and aromatic mutants) were run on a 4-12% SDS PAGE gel and transferred onto PVDF. The protein bands were stained with coomassie (0.1 % (w/v) in 50% methanol), and visualised by destaining in 50% methanol. The band corresponding to mature MBP was cut-out and sent to Monash Biomedical Proteomics Facility for sequencing of the first five residues by Edman degradation.

2.8 *Surface plasmon resonance competition assay*

Competitor assays were performed using the Pall Pioneer FE. LepB Δ 2-76 (MyBioSource.com, MBS1116891) was immobilised onto flow cell 3 of a COOH5 Biosensor chip and flow cell 2 the blank immobilised surface using amine coupling using EDC-NHS reactions. Briefly, EDC-NHS mix was flowed at 10 μL per minute for 10 minutes across flow cell 2 and 3. LepB Δ 2-76 was then flowed across flow cell one at 5 μL per minute for 20 minutes in sodium acetate pH 4.5 at a concentration of 25 $\mu\text{g}/\text{mL}$. Ethanolamine was then flowed at 10 μL per minute for 10 minutes to block any remaining active NHS. OneStep assay with regeneration were run of both wild-type signal peptide, the aromatic amino acid P2' peptides, and the proline P1' peptide to determine the affinity and kinetics. Competition assays were performed using NextStep injections in the OneStep assay builder in the following buffer: 50 mM Tris-HCl, 1% Triton X-100, pH 8.1. Aromatic amino acid P2' peptides, and the proline P1' peptide, were used as the first injection (A) and wild-type signal peptide as the second injection (B) with PBS used as negative controls. The competition injection was run for 60 seconds with the A starting at 10 μM at time zero and reducing across the injection time with the B component increasing across the injection reaching 10 μM at 60 seconds. Data was collected using the Pioneer Software package and analysed using Qdat analysis software.

3. Results

3.1 *Effect of aromatic amino acids at P2' in maltose binding protein*

To determine whether the presence of aromatic amino acids at P2' had an effect on secretion, we first modified *malE::lacZ* gene present on pMALp2e vector that encodes MBP:LacZ α . The region encoding LacZ α was deleted and the last three amino acids of wild-type MBP (see materials and methods) was added. This enabled us to study the effects of aromatic amino acids at P2' on secretion with a wild-type version of MBP, without any confounding effects due to the presence of LacZ α at the C-terminal end.

Our previous study examined amino acid biases in the mature region of 143 verified signal peptidase I processed signal peptides in *E. coli* and reported that none of the exported proteins had an aromatic amino acid at position P2' [13]. To determine the biological effect aromatic amino acids at P2' have on the secretion of a secretory protein, MBP was mutated from the wild-type residue, isoleucine, to contain each of the aromatic amino acids (tryptophan, tyrosine, and phenylalanine) at P2'. MBP is a well characterised exported protein and has been used as a model system to study secretion [15-17] and signal peptide processing [9]. After confirming the presence of the amino acid substitutions by DNA sequencing, we first analysed whether secretion was impaired by performing western blots on whole cell lysates. To avoid the presence of endogenous MBP, we conducted these experiments in the *malE* deficient strain, TL225. Initial western blot studies with whole cell lysates using anti-MBP was only able to detect mature MBP, but did not detect the presence of a precursor protein band (unprocessed signal peptide + mature protein) (Figure 1A). Hence, any potential impairment in secretion by the presence of aromatics at P2' would need more sensitive methods to detect unprocessed signal peptide (preMBP). To that end, we decided to generate our own antisera specific to the MBP signal peptide.

3.1.1 *Generating anti-preMBP antisera*

To generate the anti-preMBP antisera, mice were immunized with a peptide containing the first 15 amino acids of the MBP signal peptide (see materials and methods). The resulting antisera enabled detection of unprocessed signal peptide from

maltose binding protein. Testing whole cell lysates of the MBP variants in strain TL225, we were unable to clearly detect preMBP in these lysates (Figure 1B).

3.1.2 *Higher amounts of unprocessed precursor (preMBP) in aromatic P2' mutants of maltose binding protein*

To increase the amount of MBP loaded onto a gel, all MBP aromatic variants and MBP-wt were purified under the same conditions (see materials and methods). To detect whether there was an increase in the amount of unprocessed preMBP in these samples, equal amounts of purified material were run on SDS-PAGE and analysed using Sypro Ruby stain (Figure 2B) as well as by western analysis using anti-MBP (Figure 2A) and anti-preMBP (Figure 2C). We were able to detect the presence of precursor more clearly with the Sypro Ruby stain and with our derived anti-preMBP antibody (Figure 2B, C). The MBP_I28F phenylalanine mutant clearly accumulated the most precursor MBP relative to MBP-wt, followed by the MBP_I28Y tyrosine mutant. The MBP_I28W mutant did not accumulate any noticeable amount of preMBP. Bands were also observed below the mature protein with the anti-preMBP (Figure 2C) and Sypro Ruby stain (Figure 2B). These lower molecular weight bands correlated with samples that contained more precursor MBP. These results clearly show that the presence of phenylalanine and tyrosine at P2', but not tryptophan, increase to a small extent the amount of preMBP in the sample.

3.1.3 *Processing by SPase I*

A previous study by Fikes *et al* looking at residues important in MBP signal peptide processing found that alternative processing sites can be used when the P1 alanine (A26) was mutated to various residues, including tyrosine and phenylalanine [18]. This alternative site at A24/L25 [18]. To see if changing the P2' isoleucine to aromatic amino acids causes the same effect, we verified the signal peptide processing site by N-terminal sequencing. We observed no change in the processing site by SPase, as all mature proteins started at K27 (Figure S1 and Table S2). So the presence of a tyrosine or phenylalanine at P2' increases the amount of preMBP, but do not change the SPase I processing site.

3.2 *Surface plasmon resonance (SPR) competition assay*

To see if the increase in preMBP detected in the purified samples was due to slower processing by signal peptidase, LepB, we designed peptides around signal peptide cleavage site (from P6 – P6', see Table 1). The LepB used in this study was the $\Delta 2-76$ deletion version. LepB $\Delta 2-76$ is soluble and lacks both N-terminal transmembrane domains [19]. It has been well characterised and used in many studies to analyse LepB processing signal peptides [20]. A peptide containing a proline at the P1' position was included, as it has previously been shown it binds but is not cleaved by LepB [9].

For the SPR assay, LepB $\Delta 2-76$ was attached to the chip via amine coupling and the peptides flowed over the chip. Initial screening showed that the MBP-wt (wild-type) peptide had a similar on rate to the MBP_W P2', MBP_Y P2', MBP_F P2' peptides (ones with aromatic amino acids at P2'), and to MBP_P P1' (with a proline at P1'), but had a significantly faster off rate (Table 2). These on and off rates result in higher affinity binding by the peptides with aromatic amino acids at P2' (MBP_W, Y, F P2') compared to MBP-wt peptide. To determine if a peptide with an aromatic amino acid at the P2' position could affect the binding of the wild-type peptide, competition assays were performed, the results of which are shown in Figure 3. Pre-injection of the phenylalanine P2' peptide (MBP_F P2') completely inhibited the binding of the MBP-wt peptide, with no response above base line of the MBP_F P2' peptide control observed. Pre-injection with the tyrosine P2' peptide (MBP_Y P2') resulted in a significant reduction in MBP-wt peptide binding, with a 92.3% reduction in response. While the MBP_W P2' (tryptophan P2' peptide) reduced the MBP-wt peptide binding by 80.4%. The MBP_P P1' (proline P1' peptide) showed the highest affinity and slowest off rate with 99.1% blocking of MBP wild-type observed (Figure 3).

4.0 Discussion

A vital step in the secretion process is the removal of the N-terminal signal peptide by signal peptidase. The amino acids surrounding the cleavage site have been shown to be important in the processing of exported proteins by LepB. These sequence features include the Ala-X-Ala rule [4], with a preference for small uncharged residues at positions -1 and -3 relative to the signal peptide cleavage site, and a net negative charge in the early mature protein region [6, 7], especially at P2' [13, 21]. We previously reported a bias against aromatic amino acids at the second position after the cleavage site (P2') of exported proteins processed by the type 1 signal peptidase in *E. coli*, LepB [13]. There have been no studies testing the impact on secretion of aromatic amino acids at this position to date. Determination of the effect of aromatic amino acids at this position will help to increase the understanding of signal peptidase I substrate specificity, and thereby improve the accuracy of signal peptide prediction *in silico*.

To determine if the presence of an aromatic amino acid at P2' would lead to a change in the processing rate of LepB *in vivo*, MBP was mutated to contain each of the aromatic amino acids at this position. These MBP variants were purified after growth in rich media and the amount of precursor (unprocessed signal peptide + mature protein) and mature MBP was detected using various protein resolving techniques (Figure 2. A-C). There was no difference in the amount of mature MBP, however, there was an increase in the amount of precursor protein in both the tyrosine and phenylalanine samples. These data indicate defective secretion of MBP containing a tyrosine or phenylalanine at P2', but not for tryptophan. Each aromatic amino acid leads to a different degree of accumulation of unprocessed precursor protein, with phenylalanine showing the greatest affect. Unexpected low molecular weight bands were also detected by Sypro Ruby stain, as well as the anti-preMBP antibody. These bands are presumably C-terminally degraded precursor protein, as they retain an intact signal peptide. These bands are also more obvious in the samples with greater accumulation of precursor protein, suggesting that the accumulated precursor is being degraded by the cell.

We hypothesised that the accumulation of unprocessed precursor protein was due to inefficient cleavage or release by LepB. To compare the ability of LepB to bind and

release each signal peptide, synthetic peptides were made that comprise the six amino acids either side of the cleavage site of precursor MBP, with either an aromatic amino acid at P2' or proline at P1'. The ability for LepB to recognise and bind all tested peptides is comparable to the wild-type as shown by the similar calculated on rates in SPR, demonstrating that this is not the step in the secretion pathway affected by the P2' aromatic amino acids, or proline at P1'. However, the off rate is significantly faster (100-fold) for the wild-type peptide (Table 2), with the P2' aromatic amino acid peptides all having a relatively similar off rate, which in turn leads to a higher affinity binding. The P1' proline peptide, however, had an even slower off rate than the P2' aromatic peptides, which confirms why MBP expressing a proline at P1' is reported to not be cleaved *in vivo* [9]. During secretion, the role of LepB is to bind, process and release proteins, preferably at a fast rate. Therefore, a high affinity is not necessarily a guarantee for a good signal peptide, as the sooner the protein is processed and released, the sooner the next peptide can bind to be processed.

The P2' aromatic amino acid peptides are capable of blocking the wild-type peptide from binding to LepB, as shown using the SPR NextStep competition assay (Figure 3). This indicates that the presence of an aromatic amino acid at the P2' position, or proline at the P1' position, leads to a “stickier” type of binding to LepB, which in turn would slow down the amount of protein being able to be processed over time and therefore lead to a greater amount of unprocessed protein. These data suggest that the processing/release step of the pre-protein interaction with LepB is hindered by P2' aromatic amino acids, leading to the partial accumulation of precursor protein. The presence of a proline at P1' also affects secretion at this step.

Modelling LepB binding to a precursor protein indicates there are several sites where LepB will interact with both the signal peptide and the N-terminus of the mature protein [22, 23]. At the predicted subsite where the P2' residue of the precursor protein would interact with LepB contains a phenylalanine residue at position 208. In studying interactions between aromatic residues, Anjana *et al* reported that phenylalanine residues can self-associate, while the most number of interactions occur between tyrosine and phenylalanine residues [24]. There were less reported interactions between phenylalanine and tryptophan. If the aromatic amino acid at P2' does interact with the phenylalanine at position 208, it could slow processing by LepB

and lead to the “stickier” binding phenotype of the P2’ aromatic signal peptides to LepB we observed by SPR.

In this study, we provide the first experimental evidence as to why we do not observe aromatic amino acids at position P2’ in exported proteins. We have shown that the presence of aromatic amino acids at P2’ leads to inefficient processing/release by LepB, and confirmed that the presence of a proline at P1’ leads to the same inefficiency. Each aromatic amino acid affects the processing ability of LepB to different extents, with phenylalanine being the most detrimental. Whilst not deleterious to the overall export of proteins via the Sec system, any amino acid that slows down processing of the signal peptide by LepB would likely have been selected against over the billions of years of *E. coli* evolution. Hence, the effect we observe with aromatic amino acids at P2’ likely explains why these are not present in *E. coli* verified exported proteins [13]. The P2’ position of Gram-negative exported proteins appears to play an important role in secretion efficiency, as seen by the bias for negative amino acids, and against positive charged and aromatic amino acids. These data and previous bioinformatic studies suggest signal peptide prediction algorithms should include this information in predicting signal peptide cleavage sites in the future.

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Table 1 Peptides used in SPR. Red box indicates residue change from MBP-wt peptide

Peptide	Sequence
MBP-wt	SASALAKIEEGK
MBP_W P2'	SASALAKWEEGK
MBP_Y P2'	SASALAKYEEGK
MBP_F P2'	SASALAKFEEGK
MBP_P P1'	SASALAPIEEGK

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Table 2 Kinetics of the interaction between signal peptides

Peptide	ka (M ⁻¹ s ⁻¹ - on rate)	kd (s ⁻¹ - off rate)	KD (M - affinity)	Response (RU) of WT after pre-injection of -	% inhibition
MBP-wt	1.5±0.2e4	0.40±0.02	27±4 μM	591.6 ± 19.1	-
MBP_W P2'	1.78±0.03e4	7.29±0.05e-3	409±7 nM	116.2 ± 4.39 (<i>p</i> =1.9x10 ⁻⁶)	80.4%
MBP_Y P2'	2.62±0.06e4	5.01±0.05e-3	191±5 nM	45.7 ± 2.49 (<i>p</i> =1.0x10 ⁻⁶)	92.3%
MBP_F P2'	2.13±0.04e4	7.28±0.05e-3	341±7 nM	-14.1 ± 1.15 (<i>p</i> =6.7x10 ⁻⁷)	100%
MBP_P P1'	1.86±0.04e4	2.48±0.02e-4	13.33±2.1 nM	5.42 ± 0.82 (<i>p</i> =7.6x10 ⁻⁷)	99.1%

Figure 1. A. Detection of MBP_I28 variants using anti-MBP from TL225 whole cell lysates. Equal amounts of cells were loaded onto a 4-12% Bis-Tris gel and MBP was detected with both anti-MBP (A) and anti-preMBP (B).

Figure 2. Analysis of MBP mutants of purified MBP. Approximately 2.8 μ g of pure MBP was loaded onto a 4-12% Bis-Tris gel and the protein detected with either anti-MBP western blot (A), with Sypro Ruby stain (B) or anti-preMBP Western (C). Equal amounts of mature MBP present between the P2' aromatic amino acids and wild-type species can be seen in (A), while an increase in the pre-MBP in the phenylalanine and tyrosine samples can be seen in (B) and (C). All bands of the precursor and mature MBP protein are detected, highlighting the correlation of more precursor protein with less mature protein.

Figure 3. Percentage of MBP-wt wild-type peptide bound to LepB after pre-injection of each of the MBP peptides

Conflicts of Interest:

This work was supported by NHMRC (Australia) Program Grant 1071659 to MPJ, and a CJ Martin Biomedical Fellowship 569913 awarded to YMZ.

There are no patents, or payments from outside sources relevant to this work that need to be disclosed.

There are no interactions from outside sources for this study that need to be disclosed.

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Highlights

- *Aromatic amino acids at P2' slow signal peptide processing by signal peptidase*
- *Leads to an accumulation of unprocessed precursor material*
- *Experimentally explains why there are no aromatic amino acids observed at P2'*

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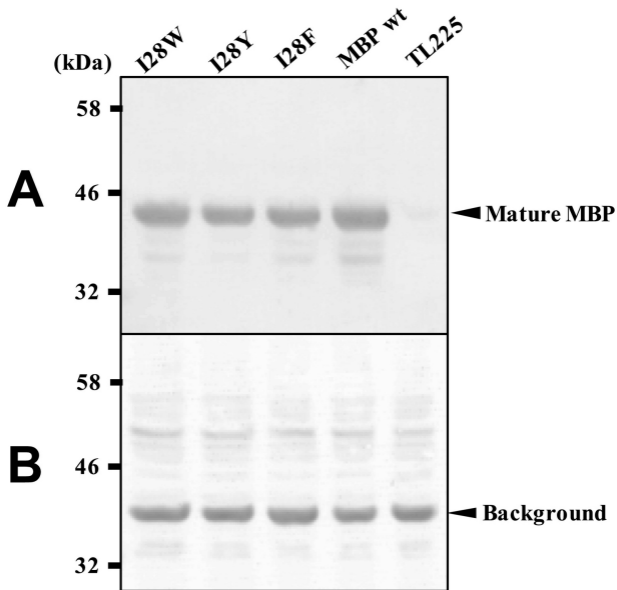


Figure 1

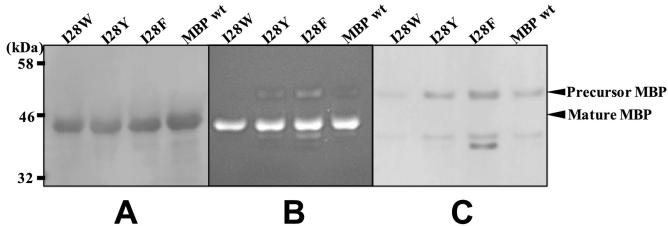


Figure 2

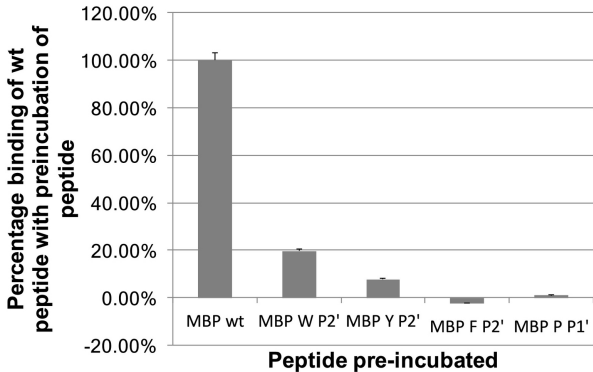


Figure 3