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Adsorptive detagging of poly-histidine tagged protein using hexa-histidine tagged exopeptidase

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ABSTRACT

The ubiquitous use of poly-histidine fusion tags has made the purification of the recombinant target proteins much simpler, although the presence of residual fusion tags can generate immunogenic products or products with changed biological activities. This work presents a generic method of removing poly-histidine fusion tags from recombinant proteins through the use of a hexa-histidine tagged exopeptidase (DAPase) when both tagged species are adsorbed to the immobilized metal affinity chromatography (IMAC) adsorbent. Adsorptive detagging was performed in the presence of 50 mM imidazole in order to allow the cleavage reaction by the hexa-histidine tagged DAPase to occur. The progress of batch and adsorptive detagging by DAPase of maltose binding protein (MBP) tagged with two variants of hexa-histidine fusion tag was successfully monitored using cationic exchange chromatography. A single-step, column-based detagging strategy was then optimized to maximize the recovery of native MBP. The kinetics of batch and on-column digestion for both HT6 and HT15 fusion tags were investigated. The process involved the sequential removal of dipeptides during the digestion of full-length fusion protein down to its fully detagged native form. During the course of tag digestion, 4 and 7 different intermediates were detected for HT6 and HT15 tagged MBP respectively. The characteristics of on-column cleavage of poly-histidine fusion tags by DAPase as a function of incubation temperature and amount of protease activity used were examined. It was found that the influence of fusion tag design on the batch and column-based detagging yield and efficiency was substantial. In addition, the structural difference of fusion tags affects the binding strength of the fusion protein, which can influence the resulting product purity. Despite being a longer tag, HT15 fusion tag was the preferred sequence for shortening the time needed for on-column detagging. These results can be applied to the wider use of the proposed platform protocol for the on-column cleavage of poly-histidine tagged proteins using exopeptidases.

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1. Introduction

There is a growing demand for protein-based therapeutics and proteins for structural determination. In order to produce these proteins, highly expensive purification steps are required. Consequently, there is a need for a highly flexible, specific and, yet economic purification technology. The technology of using poly-histidine fusion tag in conjunction with immobilized metal affinity chromatography (IMAC) fits these requirements. A poly-histidine tag is a short amino acid chain containing at least six histidine residues that can be attached to the end of virtually any protein to allow its purification by IMAC. The many benefits of IMAC include its high specificity, low cost, mild elution conditions, ease of regeneration and sanitization to common industrial standards for multiple cycles of processing. In fact, the abundance of literature involving the use of IMAC manifests it as one of the most efficient tools for purifying recombinant products from crude feedstocks and can even be used for large-scale production of therapeutics [1–5].

Nevertheless, one major obstacle in making the processing of poly-histidine tagged proteins using IMAC the technology of choice for purifying proteins of industrial interest such as therapeutics is the need to remove the fusion tags post-purification. It is essential to have the tag sequences completely cleaved because the native form of the target protein would be required, in the case of therapeutics, to produce the expected immune response in patients. By far the most prevalent method for the cleavage of fusion tag involves the use of proteases. There are two classes of proteases, namely endopeptidases that cleave internal peptide bonds of a protein and exopeptidases that cleave terminal amino acids of a protein. Most commercially available cleavage proteases are endopeptidases such as thrombin, tobacco etch virus protease (TEV), and factor Xa. These endopeptidases suffer various drawbacks such as the need for high ratios of enzyme to tagged

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protein, long incubation times, the production of incorrect terminal amino acids, and the occurrence of non-specific internal cleavage [1,6].

On the other hand, the use of exopeptidases provides a solution to these problems. Exopeptidase removal of fusion tags is highly specific and the use of exopeptidase cleavage significantly reduces the amount of enzyme required, which is particularly important for operation at industrial scale when the cost of employing high grade proteases becomes prohibitive. Nevertheless, when using enzymes in downstream processing of therapeutic proteins, the purity and the origin of the cleavage enzymes are important. Most commercially cleavage enzymes are derived from animal sources which carry risks of viral and prion contamination. In order to address such issue, this work involves the use of a recombinant rat dipeptidyl aminopeptidase I (DPPI) called DAPase obtained by heterologous expression in insect cells that is part of the TAGZyme system [7–9]. Recent studies have demonstrated that the TAGZyme system is the preferred method of detagging due to its high specificity and efficiency [1–3,8–12]. DAPase (also known as cathepsin C) (EC 3.4.14.1) is a lysosomal cysteine peptidase belonging to the papain family. When a poly-histidine fusion tag is located at the N-terminal end of the target protein, DAPase can be used to remove the tag after the protein has been purified. The recombinant DAPase is itself engineered to have a poly-histidine tag fused to its C-terminal for the ease of its removal after the completion of the batch enzymatic cleavage reaction. In fact, most commercially available proteases are also tagged for the ease of their removal; for instance, TEV from Invitrogen is poly-histidine tagged while factor Xa from Roche Applied Science is fused with biotin in order for the separation of the cleavage enzymes from the target proteins to be accomplished by adsorption to immobilized metal ions and immobilized streptavidin, respectively.

Alternatively, it is possible to perform the cleavage of fusion tags from the target protein while it remains bound to the IMAC column during the primary recovery process; this is known as adsorptive detagging. The development of such on-column tag digestion methodology reduces the overall number of processing steps needed as the incubation of target protein in the cleavage enzyme solution is performed directly following the purification step in the same unit operation. In addition, product recovery at the end of the cleavage reaction can be achieved without the use of imidazole, by washing the column with a wider range of buffer systems. This is in contrast to the competitive elution using imidazole required for tagged target proteins in the traditional method, which is sometimes detrimental to the integrity of the protein products [13]. Moreover, adsorptive detagging allows simpler protein processing, for instance, an extensive buffer-exchange step can be avoided as the conditions required for on-column cleavage can be changed simply by washing the column with the desired cleavage buffer. Nevertheless, the success of the adsorptive detagging protocol requires careful optimization of the on-column conditions to ensure that good cleavage performance can be maintained. Similar to conventional batch cleavage methods, system parameters such as time for cleavage, ratio of protease to substrate, buffer pH and ionic strength, and incubation temperature should all be taken into consideration. On-column cleavage has only seen use with certain endopeptidase systems [13–16]. In fact, it has been generally accepted that the inherent nature of exopeptidase cleavage does not work with the adsorptive detagging method. Recently, the first successful demonstration of on-column exopeptidase cleavage has been described [17]. It was found that in the presence of 50 mM imidazole in the cleavage buffer, on-column tag removal by an exopeptidase such as DAPase could be achieved. It was hypothesized that the introduction of imidazole induced a dynamic binding situation for the poly-histidine tagged species which allowed (1) the diffusion of the exopeptidase to its substrate and (2) on-off binding characteristics of the poly-histidine tagged target protein that enable the tagged exopeptidase to diffuse between the surface of the IMAC adsorbent and the fusion tag on the target protein and thus allowing the exopeptidase to bind to the N-terminal end of the fusion tag and to commence the cleavage reaction. Such an approach has made it possible to combine the many benefits of using an exopeptidase system with those of employing an adsorptive detagging method. This paper describes a novel application of an industrial-relevant hexa-histidine tagged exopeptidase system on poly-histidine fusion tags and IMAC, both of which have a long history of proven performance [18,19]. This can provide a solution to the challenges in protein purification include reduction of manufacturing cost, development of specific, flexible processes for both product purity and diversity, and integration of upstream and downstream processes. Issues related to the implementation and improvement of the proposed platform technology for wider use in protein purification are discussed.

2. Materials and methods

Disodium hydrogen phosphate, sodium chloride, tris(hydroxmethyl)aminomethane, EDTA, imidazole (minimum 99% pure), nickel chloride, cysteamine–HCl, ampicillin, Luria Broth (LB) media, LB agar, Gly-Phe-*p*-nitroanilide, *N*,*N*-dimethylformamide, isopropyl β,D-thogalactopyranoside (IPTG), sodium hydroxide, β-mercaptoethanol, hydrochloric acid, acetic acid, glycerol, Bradford reagent, magnesium chloride, citric acid were purchased from Sigma–Aldrich (Poole, UK). BugBuster® Master Mix was purchased from Novagen (Nottingham, UK). HisTrap™ FF Crude, HisTrap™ HP, Tricorn™ Mono S™ 5/50 GL columns were purchase from GE Life Sciences (Amersham, UK). Dialysis membranes were purchased from Medicell International Ltd. (London, UK). All experiments were performed at 23 °C and all buffers were made up with deionized (DI) water, filtered by 0.22-μm filters, and degassed before use unless otherwise noted.

Two *Escherichia coli* strains producing hexa-histidine tagged MBPs were kindly provided by John Pedersen of Unizyme Laboratories (Horsholm, Denmark). Both strains of hexa-histidine tagged MBP are N-terminus hexa-histidine tagged proteins encoded by an *E. coli* clone pTrcHis/TOP10. The host strain was *E. coli* TOP10 (Invitrogen, Paisley, UK) cloned with an expression plasmid pTrcHisA, B, or C (Invitrogen) with ColE1 origin, ampicillin resistance gene laclq repressor gene and the Trc promoter. MBP exists in solution under physiological conditions as a monomer with a molecular weight of 43 kDa. The hexa-histidine tagged cleavage enzyme DAPase was also kindly provided by John Pedersen.

2.1. Fermentation

The procedures for fermentation were adapted from the protocols provided by Unizyme Laboratories (Horsholm, Denmark). Single colonies of hexa-histidine tagged MBP were picked from the clones provided and streaked onto fresh LB agar plates and incubated overnight or until the colonies had grown to approximately 1 mm in diameter. Single colonies were picked from the LB agar plates on which hexa-histidine tagged MBP had been grown and inoculated in 500 ml of LB media supplemented with 100 mg/ml ampicillin. All fermentations producing MBP were grown aerobically in an orbital incubator at 36 °C and agitated at 200 rpm. When the optical density measurement at 600 nm reached 0.6–0.9, samples were taken and mixed with glycerol for cell banking at −85 °C. For all strains producing MBP, expression was induced by adding IPTG to a final concentration of 300 μM and incubated for an additional 4 h. Cells were harvested by centrifugation at 4000 × *g*, at

4 °C for 15 min. The supernatant was then discarded and DI water added to the pellets to wash away any residual media. The cells were then re-sedimented under the same conditions. The supernatant was again discarded and the centrifuge tubes were weighed to measure the pellet weight. The cell pellets were then stored at −20 °C until used in cell disruption.

2.2. Cell disruption

The cell disruption procedure was primarily adapted from the BugBuster® Master Mix User's Manual. Frozen cell pastes were thawed on iced water and BugBuster® Master Mix was added to the cell suspension at a ratio of 5 ml of the reagent per gram of wet cell paste. The cells were lysed by mixing the suspension for 30 min. The mixture was then suspended in the feedstock preparation buffer (100 mM NaH_2PO_4, 10 mM β-mercaptoethanol, 40 mM imidazole, 4 mM $MgCl_2$, 0.5 M NaCl, pH 7.5) in the ratio of 5 volumes of the feedstock preparation buffer per gram of pre-weighed cell paste and then mixed for 15 min. It was then centrifuged at 14,000 × *g* for 30 min at 4 °C.

2.3. IMAC purification

The IMAC purification procedure was primarily adapted from the HisTrap™ FF Crude Chromatography User's Manual provided by GE Healthcare. A 5 ml HisTrap™ FF Crude column, coupled to an Akta™ Explorer automated chromatography controller, was equilibrated with 10 column volumes of the IMAC equilibration buffer (100 mM NaH_2PO_4, 20 mM imidazole, 0.5 M NaCl, pH 7.5) at 5 ml/min. The *E. coli* homogenate was then loaded onto the column at 1 ml/min. At the end of protein loading, loosely bound proteins were washed from the bed using the IMAC equilibration buffer. When the UV absorbance at 280 nm had fallen to the zero baseline, the adsorbed proteins were eluted via step elution from the IMAC equilibration buffer to the IMAC elution buffer (100 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.5). Fractions of the column eluate were collected during elution.

2.4. Dialysis

Imidazole used during IMAC purification inhibits the activity of the cleavage enzymes, therefore samples must be exchanged into the appropriate cleavage buffer prior to enzyme cleavage. 5 mM EDTA was added to the protein samples to inactivate any residual nickel ions that may have been leached from the adsorbent during the IMAC process. The samples were dialyzed by being placed in a semi-permeable dialysis tube with an appropriate molecular weight cut-off (MWCO) of 12–14 kDa. The tube was then dialyzed overnight against the cleavage buffer.

2.5. Mono S™ 5/50 GL analysis

A column of Tricorn™ Mono S™ 5/50 GL (1 ml) was coupled to an Akta™ FPLC automated chromatography controller. The binding buffer used was 20 mM citric acid/NaOH pH 4.0. 50 μl of the tag digestion sample was added to 950 μl of the binding buffer and applied to the column through the sample loop at a flow rate of 1 ml/min. Elution was performed by a salt gradient elution over 5 column volumes using 20 mM citric acid, 1 M NaCl, pH 4.0 as the final buffer.

2.6. Batch poly-histidine tag cleavage

Bradford assays were performed on the dialyzed sample to determine the protein concentration. The protein concentrations in each sample were adjusted to an appropriate test concentration before adding the cleavage enzyme. For DAPase, an equal volume of 20 mM cysteamine–HCl was added to activate the enzyme before use as recommended in the TAGZyme System handbook.

2.7. IMAC on-column poly-histidine tag cleavage

A 1-ml HisTrap™ HP column, coupled to an Akta™ Explorer automated chromatography controller, was equilibrated with 10 column volumes of the recovery buffer (20 mM NaH_2PO_4; 150 mM NaCl, pH 7.0) at 1 ml/min. The purified protein sample was then loaded onto the column at 1 ml/min. A wash was performed after sample loading using the cleavage buffer. When the chromatogram showed a baseline of zero UV absorbance at 280 nm, the post-load wash was terminated. While the proteins remained adsorbed to the resin, 1 ml of exopeptidase solution containing varying amounts of total enzyme activity was fed into the column. The column was then incubated for a defined period of time to allow on-column poly-histidine tag removal to occur. At the end of the incubation time, the column was washed with the recovery buffer to recover the cleaved proteins and this process was followed by elution with the IMAC elution buffer to recover any non-cleaved proteins still adsorbed to the column. The column effluents from loading, washing, and elution phases were collected for further protein analyses. The total protein concentration was measured by the Bradford assay [20].

2.8. Binding strength of poly-histidine tagged proteins

The method used to evaluate the binding strength of HT6 and HT15 was adopted from GE Healthcare Application Note [21] in order to compare the result with that described in the literature. An 1-ml HisTrap™ HP column was washed and equilibrated with binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl) and wash buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole) respectively. A flowrate of 1 ml/min was used throughout and a 0–100% linear gradient from the washing buffer to the elution buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole) in 10 column volumes was used.

2.9. Enzymatic assay of DAPase

DAPase activity can be determined spectrophotometrically by measuring the initial rate of hydrolysis of the chromogenic substrate Gly-Phe-*p*-nitroanilide (Gly-Phe coupled to para-nitroanilide) according to Planta and Gruber [22], who concluded that the splitting of Gly-Phe-*p*-nitroanilide is a rapid, sensitive, and convenient method for the estimation of cathepsin C in their work. In an attempt to develop a standard operating procedure for the chromogenic DAPase activity assay, a method for determination of the concentration of active DAPase in terms of units/ml in DAPase enzyme stock solutions was employed in accordance with that recommended by the enzyme supplier. One unit is defined as the amount of enzyme required to convert 1 μmol of substrate per minute under the described conditions. If the concentration of DAPase in the test sample is below or above the limits set for this protocol, the dilution factor (40×) can be varied by addition of smaller or larger volumes of the DAPase assay buffer. The cysteamine in the DAPase assay buffer activated the DAPase and further diluted (40× dilution) the test sample with the substrate solution. This final mixture was then transferred to the cuvette in the spectrophotometer for measurements at 405 nm. The reading of adsorption for each 5-s interval was recorded for 60 s and the ΔA_{405}/min calculated at the end of the measurements.

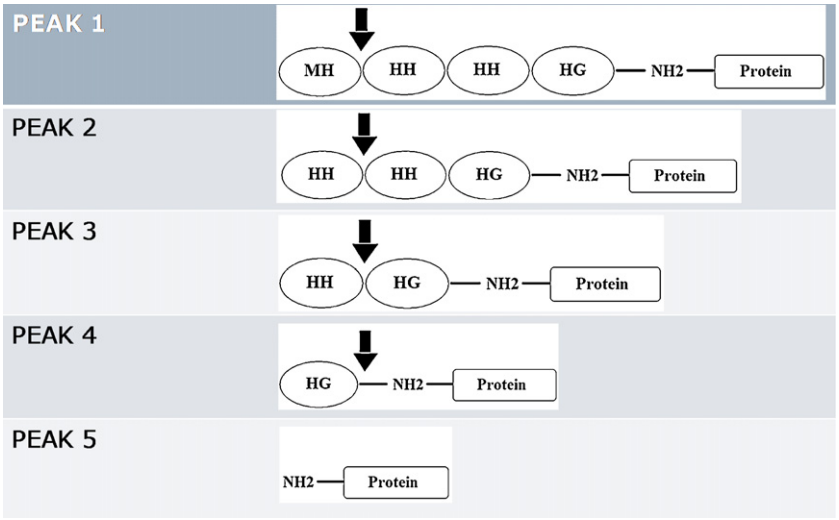

Fig. 1. Theoretical structure prediction of the progress of dipeptide removal of a HT6 fusion tag by DAPase. The numbering of each peak for each intermediate species corresponds to the peak numbers on the FPLC chromatogram shown in Fig. 2.

3. Results and discussion

3.1. Progress of poly-histidine tag cleavage

Poly-histidine tagged MBP was chosen as the model protein, which was obtained in a pure form from crude cell extracts using packed bed IMAC purification [17]. The pooled elution fractions from the IMAC purification contained high concentrations and homogeneity of MBP as analysed by SDS-PAGE. Prior to either the batch or on-column exopeptidase cleavage experiments, dialysis was performed against the cleavage buffer. This step was needed to remove the imidazole from the elution fractions and any residual nickel ions that may have leached from the IMAC column during product elution. EDTA was also added to chelate any residual nickel ions that might cause unwanted metal-catalysed oxidation of the protein. In order to monitor the progress of poly-histidine tag removal, it is essential to find reliable analytical methods that can be used to detect the presence of poly-histidine tags of various lengths. Cation-exchange chromatography was chosen as the method of analysis to confirm the stepwise release of dipeptides containing positively charged histidine residues [8]. A Tricorn™ Mono S™ 5/50 GL column was used in this work to develop a high-resolution FPLC analysis. The aim of using the Mono S™ cation exchanger with the FPLC system was to quantify and accurately monitor the stepwise cleavage of HT6-MBP to MBP, which was helpful in the subsequent development of the new process.

Fig. 1 shows the structure prediction of a HT6 fusion tag, which is made up of four dipeptides. As shown, the cleavage of a HT6 fusion tag by DAPase would result in the sequential loss of 4 positive charges due to the removal of the dipeptide MH, the two HH, and the HG dipeptides. This was confirmed by the chromatogram shown in Fig. 2. There were four products that eluted earlier than HT6-MBP generated sequentially during the reaction. The chromatograms of native detagged MBP and uncleaved HT6-MBP proteins gave retention conductivities at 49.0 mS/cm and 79.4 mS/cm respectively when separated on the Mono S™ 5/50 GL column. The 3 species of intermediates during the digestion process have retention conductivities of 52.4 mS/cm, 63.8 mS/cm, and 74.9 mS/cm. Note that to generate a native MBP control standard, prolonged batch digestions of hexa-histidine tagged MBP with DAPase were performed. At the end of the batch digestions, N-terminal sequencing was performed on the assumed native MBP sample to confirm the amino acid sequence that was present. A N-terminal sequence of KIEEG was detected, which matched the expected sequence of the N-terminal of native MBP. This native MBP sample was then analysed by a Mono S™ column, and the elution of the native

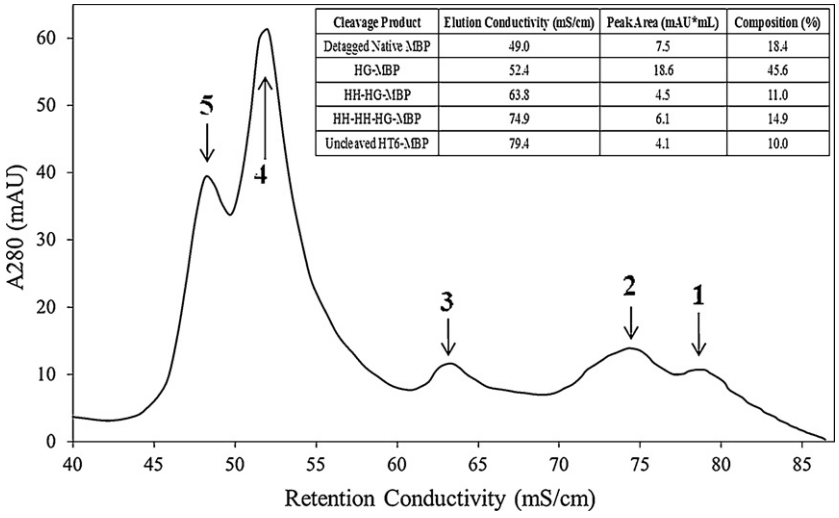

Fig. 2. Chromatogram showing the generation of different species of intermediates during the progress of poly-histidine tag removal from HT6-MBP by DAPase digestion.

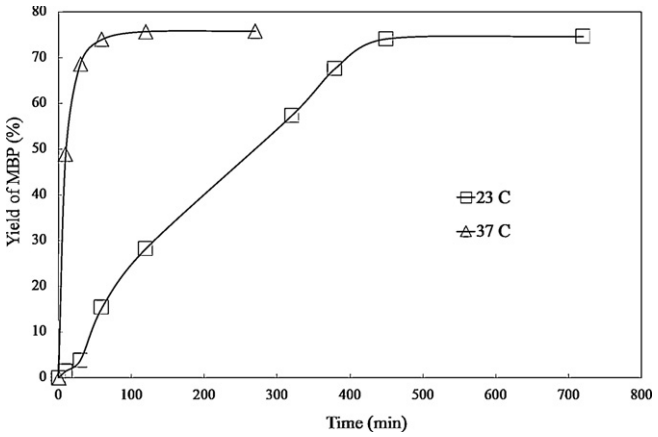

Fig. 3. The effect of incubation temperature of 23 °C and 37 °C on the time course of the yield of MBP liberated HT6-MBP. 5 mg/ml HT6-MBP was digested with 300 mU of DAPase enzyme in the batch mode. Yield of MBP was calculated using the area of the MBP peak on the FPLC chromatogram as described previously.

MBP peak occurred at exactly the same conductivity as peak 5 in Fig. 2. The result of the N-terminal sequencing of the cleaved protein further confirmed the precision of fusion tag cleavage by exopeptidase DAPase. The results also showed that the Mono S™ 5/50 GL column can be used to provide sufficient resolution to separate all the intermediate cleavage products so that chromatograms can be analysed to estimate the amounts of the various histidine tagged proteins and the results obtained correlated to the theoretical cleavage mechanism. Hypothetical structure predictions of intermediate species during conversion of HT6-MBP to MBP using DAPase digestion can be drawn to agree with each of the peaks obtained from the FPLC chromatograms.

In order to maximize the recovery of native detagged MBP, further experiments were performed to identify the optimal cleavage conditions by varying the operating conditions such as the operation temperature and initial enzyme concentration on the yield of HT6-MBP conversion to MBP. Quantitative analyses to determine the yields of conversion were performed based on the chromatograms produced by the UNICORN software provided with the AKTA FPLC. The yield of a selected protein was calculated according to the following equation:

$$\text{Yield}\,(\%) = \frac{\text{Area of a selected peak}}{\text{Sum of areas of all peaks}} \times 100\%$$

3.2. Effect of important system parameters

The effect of temperature on the batch cleavage of HT6-MBP was analysed by digesting a 5 mg/ml solution of HT6-MBP with 300 mU DAPase. Fig. 3 shows the yield of HT6-MBP conversion to MBP with time when performed at 23 °C and 37 °C in the batch mode. The activity of the DAPase enzyme was found to be stable at both temperatures. The conversion was more efficient when performed at 37 °C when compared to results obtained at 23 °C. After 1 h of digestion, the yield of MBP produced in the reaction incubated at 37 °C was 74%. The yield was more than 50% lower when incubation occurred at 23 °C. The reaction was found to achieve 75% conversion of HT6-MBP to MBP after 2 h incubation time at 37 °C. In contrast, 7.5 h at 23 °C was needed in order to achieve a similar yield of MBP. This finding shows that the DAPase digestion of HT6-MBP in the batch mode is strongly dependent on temperature.

The effect of temperature on the on-column exopeptidase cleavage of HT6-MBP was analysed by digesting 0.5 mg of HT6-MBP with 200 mU of DAPase. Fig. 4 shows the yield of HT6-MBP conversion to MBP at various stages of the on-column exopeptidase cleavage reaction at 23 °C, 37 °C, and 45 °C. The on-column cleavage reactions were found to occur at all three temperatures, with the on-column cleavage reaction being most efficient at 37 °C. After 24 h of incubation, the yield of MBP obtained at 37 °C was 77%. The yield was about 32% lower when the on-column cleavage reaction was carried out at 23 °C, with a recovery of only 45%. In an attempt to further increase both the product yield and the efficiency of the on-column cleavage by DAPase, an incubation temperature at 45 °C was also examined. As shown in Fig. 4, increasing the temperature to 45 °C seemed to have a detrimental effect on the product yield. Comparing the on-column cleavage reactions at 37 and 45 °C, raising the temperature up to 45 °C proved to be unfavourable for the process.

The results show that the on-column cleavage reaction is also strongly dependent on temperature. The trend for the on-column cleavage results seem to reflect that observed with the batch digestion experiments. This observation is in accordance with information provided by other suppliers of commercially available endopeptidases, which are usually reported to have a maximum temperature of operation at 37 °C when used in the soluble form. In order to verify the effect of temperature on the activity of DAPase itself, enzymatic assays were performed at the batch operation temperatures. Samples were activated and assayed in reaction buffer complemented with chromogenic pNA-labeled peptide substrate as described under Section 2. The activity for the sample held at 23 °C was found to have a relative activity of 58% compared to that at 37 °C (100%). Consequently, the trends observed in the on-column experiments are most likely due to the variation in enzymatic activity of DAPase in response to the change in temperature, in a similar manner to that observed in the temperature-variation experiments on the batch cleavage of HT6-MBP by DAPase shown in Fig. 3. In fact, the same temperature dependence for the hydrolysis of HT6 tag and the chromogenic substrate used in the enzymatic assays was observed for the hydrolysis of another fusion tag, HT15, described in later sections. Lastly, the diffusivity of DAPase probably increased with a rise in temperature to a certain extent, which might also have helped to enhance the yield of the on-column exopeptidase cleavage reactions.

The effect of the amount of added DAPase on the yield of detagged products from HT6-MBP was investigated by varying the amount of DAPase used in the on-column exopeptidase cleavage experiments over a wide range from 0 mU to 1000 mU. Adsorptive detagging of 0.5 mg of HT6-MBP was performed at the optimum temperature of 37 °C. As shown in Fig. 5, most of the MBP was recovered during the product recovery step. The recovery fraction contained only native detagged MBP as confirmed by the FPLC analysis, indicating successful removal of the hexa-histidine fusion tag from the originally tagged proteins. Nevertheless, not all of

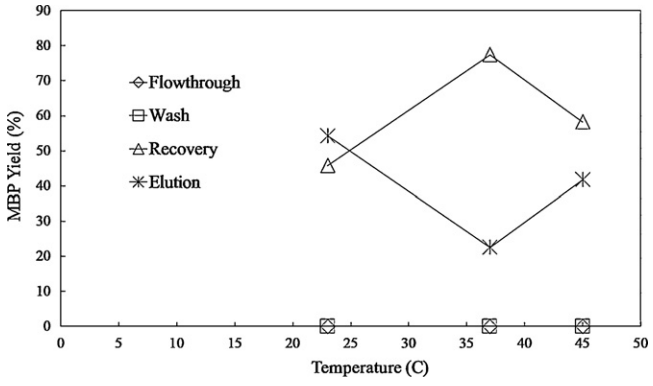

Fig. 4. The effect of temperature on the on-column exopeptidase cleavage reaction yield; on-column cleavage buffer contained 50 mM imidazole, 0.5 mg HT6-MBP loading, 200 mU of DAPase, 24-h incubation.

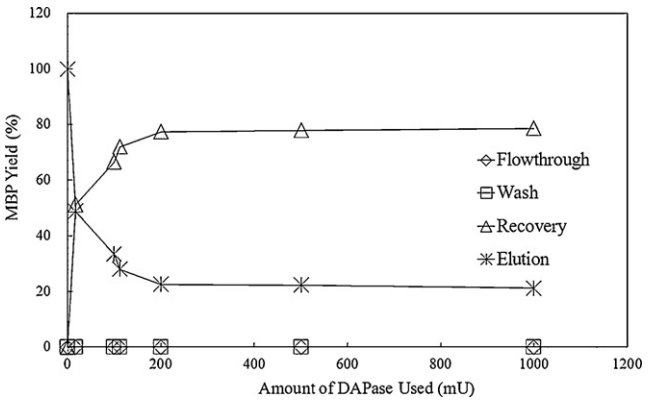

Fig. 5. The effect of the amount of DAPase activity used on the on-column cleavage reaction yield; on-column cleavage buffer contained 50 mM imidazole, 0.5 mg HT6-MBP loading, 37 °C, 24-h incubation.

the adsorbed HT6-MBP was detagged after the on-column incubation as shown by varying amounts of various tagged proteins being found subsequently in the elution fractions eluted by the IMAC Elution buffer containing 0.5 M of imidazole. This could be due to limitations in the rate of diffusion of DAPase limiting its access to the cleavage sites of protein molecules that are adsorbed within the adsorbent matrix in a way that obscures the cleavage sites. In contrary to expectations, however, a higher cleavage yield was not observed with an increase in DAPase activity from 200 mU to 1000 mU. After 24 h of incubation, the percentage yields of MBP did not increase significantly in experiments where the enzyme concentration was greater than 200 mU; the on-column cleavage reactions achieved yields of 78% and 79% for additions of 200 mU and 1000 mU of DAPase respectively.

The results suggest that the amount of exopeptidase was not controlling the rate of the on-column cleavage reactions when present above an enzyme activity of 200 mU. This corresponds to the earlier deduction that as all cleavage reactions appeared to reach equilibrium after 24 h of incubation, there existed a maximum percentage of poly-histidine tagged MBP available for reaction. It is possible that at a DAPase activity of 200 mU, the available sites on the tagged protein for enzyme catalysis were fully occupied by enzyme molecules. Increasing the amount of enzyme beyond 200 mU could do nothing to overcome the steric hindrance experienced by the remaining tagged proteins that were probably in an unfavourable configuration for enzymatic cleavage.

3.3. The effects of different poly-histidine tag design

The particular amino acid sequence of a poly-histidine fusion tag as a result of the nucleotide sequence designed to encode it can have a great impact on the overall performance of the resulting construct during expression, post-translational processing, and tag removal [8]. The detagging characteristics of HT6 and HT15 constructs were compared in order to provide some underlying principles for designing poly-histidine tags. HT6 and HT15 are both poly-histidine tags containing six histidines, where HT6 has an N-terminal Met residue followed by six His residues and a Gly residue (sequence of HT6: MHHHHHHG) and HT15 has other amino acid residues inserted between its six histidine residues (sequence of HT15: MKHQHQHQHQHQ). The HT6 fusion tag that has been used so far in this work represents the commonly used hexa-histidine tag sequence described in the literature. Both constructs have a natural stop point in the tag sequence frame for DAPase enzyme. In addition, both constructs have an even number of amino acid residues in the tag, which means the complete removal of the fusion tags can be performed by DAPase digestion alone.

As before, a Tricorn™ Mono S™ 5/50 GL column was used to develop a high-resolution FPLC analysis to follow the progress of DAPase digestion of HT15-MBP to MBP. As shown in Fig. 6, it was assumed that the DAPase cleavage of HT15 tagged protein would result in the sequential loss of 7 positive charges due to the removal of each of the 7 dipeptides. Consequently, 8 species would be separated during the progress of the stepwise digestion over time. The chromatograms in Fig. 7(a)–(c) were taken from a batch cleavage experiment in which 0.8 ml of 2 mg/ml of HT15-MBP was digested with 500 mU of DAPase and incubated at 37 °C for 0 min, 10 min or 60 min respectively. Indeed, the cleavage of HT15-MBP by DAPase resulted in the sequential loss of 7 positive charges due to the removal of the dipeptide MK and the six HQ dipeptides as confirmed by the chromatograms shown in Fig. 7(a)–(c). There were seven products that eluted earlier than HT15-MBP which were generated sequentially during the reaction. The 6 species of intermediates detected during a typical DAPase digestion process have retention conductivities of 53.1 mS/cm, 57.5 mS/cm, 59.5 mS/cm, 61.4 mS/cm, 64.6 mS/cm, and 68.0 mS/cm. The retention conductivities of native MBP and HT15-MBP were 49.1 mS/cm and 70.3 mS/cm respectively when separated on the Mono S™ 5/50 GL column. Note that the native detagged MBP products produced from both variants of poly-histidine tagged MBP saw the elution of the detagged target protein at a conductivity of 49 mS/cm, while the uncleaved form of HT6-MBP had a higher elution conductivity than its HT15 counterpart (79 mS/cm and 70 mS/cm respectively). This further confirmed the validity of this method of analysis and also showed that the difference in binding strength for the two different tag variants to the cationic exchange resin used. The results showed that the tag cleavage of HT15-MBP to MBP was indeed a time dependent reaction and DAPase provided the expected precise tag cleavage. This was in agreement with results obtained with a different N-terminal poly-histidine tag fused to different recombinant proteins but in which the same DAPase TAGZyme system was used [12].

It is important to take into consideration the rate of tag removal when designing poly-histidine tags. In order to compare the cleavage efficiency for HT6 and HT15, batch digestions were carried out at 37 °C with 50 mU of DAPase. As shown in Fig. 8, the yields of native MBP over 240 min of incubation time were plotted for HT6-MBP and HT15-MBP. The results show that HT15-MBP out-performed HT6-MBP in terms of cleavage efficiency, and when HT15-MBP was employed nearly complete tag removal was achieved after only 2 h of batch digestion. Based on these results, although the HT15 tag is longer and thus requires the cleavage of more dipeptides, it appears that this construct shows a superior cleavage efficiency and higher yield of detagged protein compared to the use of the HT6 tag. This is in accord with previous observations in which the tag cleavage rate was found to be independent of tag length [12]. Likewise, in studies of the stepwise release of dipeptides by DAPase it was found that the constitute dipeptides of HT6 and HT15, namely Met-His, His-His, His-Gly, Met-Lys, and His-Gln, were all excellent substrates for the enzyme DAPase [8]. During the conversion of HT6-MBP and HT15-MBP to MBP, the reactions have been shown previously to involve the sequential loss of four (Met-His, 2 × His-His, and His-Gly) and seven dipeptides (Met-Lys and 6 × His-Gln) respectively. Consequently, based on these numbers of dipeptides that need to be removed, the results in this work further suggest that the rate of cleavage of the types of dipeptides present in a HT15 tag is higher than those present in a HT6 tag. Perhaps the positioning of certain dipeptides in the HT6 tag make it relatively slower to cleave when compared with other longer tags. It has been shown that despite the fact that Gly-His is generally thought of as being an excellent DAPase substrate, the incorporation of Gly-His as the penultimate C-terminal dipeptide in tags fused to hTNFa resulted in a markedly decreased cleavage rate [8].

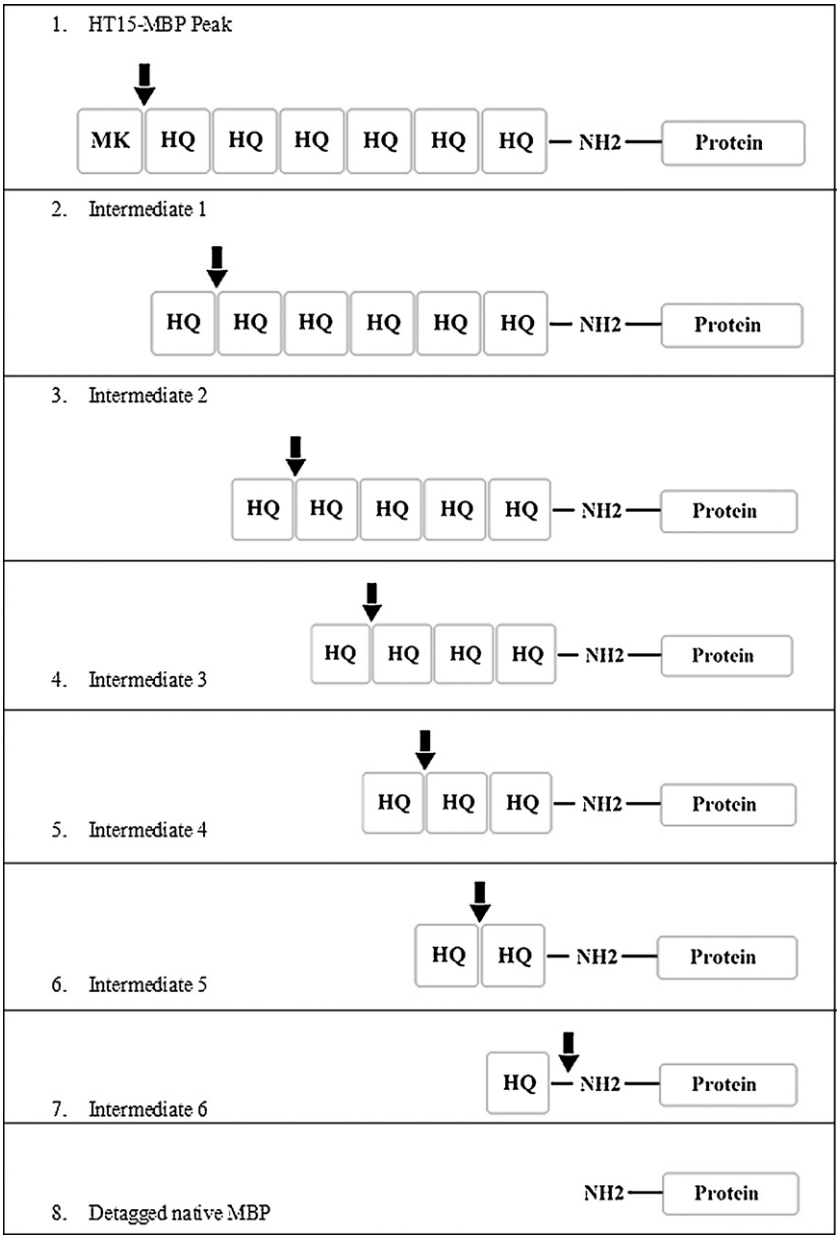

Fig. 6. Theoretical structure prediction of the progress of dipeptide removal of a HT15 fusion tag by DAPase. The numbering of each peak for each intermediate species corresponds to the peak numbers on the FPLC chromatogram shown in Fig. 7.

3.4. *Effect of HT15 fusion tag on adsorptive detagging by DAPase*

The binding strength of poly-histidine tagged proteins to IMAC adsorbents has an indirect impact on the purification performance of the fusion protein. This is due to the fact that the higher the binding strength, the higher the imidazole concentration that can be added the binding and wash buffers as it is desirable to maintain as high an imidazole concentration as possible in the binding and wash buffers in order to prevent non-specific binding and thereby increase product purity. In order to determine the relative binding strengths of HT6 and HT15 tagged proteins, the percentages of the elution buffer needed in the gradient elution to elute HT6-MBP and HT15-MBP were determined as illustrated in the chromatograms in Fig. 9(a) and (b). The higher the percentage of elution buffer needed to achieve elution indicated the stronger the binding strength of the poly-histidine fusion tag. In previous studies which have also used MBP as a model protein under similar experimental conditions, it has been found that whether the poly-histidine fusion tag is located on the N-terminal or C-terminal of the protein does indeed affect the strength with which the fusion protein binds to the IMAC adsorbent [21]. As shown in Fig. 9(a) and (b), the amount of imidazole needed in the elution buffer to elute the MBP tagged with HT6 and HT15 fusion tags were 364 mM and 299 mM respectively. This shows that HT15 has a lower binding strength to IMAC than HT6 despite both tags contain 6 histidine residues. As mentioned earlier, HT15 has a lower elution conductivity (70 mS/cm) than HT6 (79 mS/cm) when separated by the Mono S^{TM} cationic exchanger, suggesting that HT15 is less positive than HT6. Both results can be attributed to the insertion of the glutamine (Q) residues, which have a neutral charge, in the HT15 sequence between the positively charged histidine residues. The inclusion of the glutamine residues in the hexa-histidine tag decreases the effect of the histidine residues in terms of both the metal-binding ability and the overall positive charge as shown by the relative binding strength of HT15 to nickel ions being lower and the earlier elution of HT15 in the cationic exchange separation. As a result, during the purification

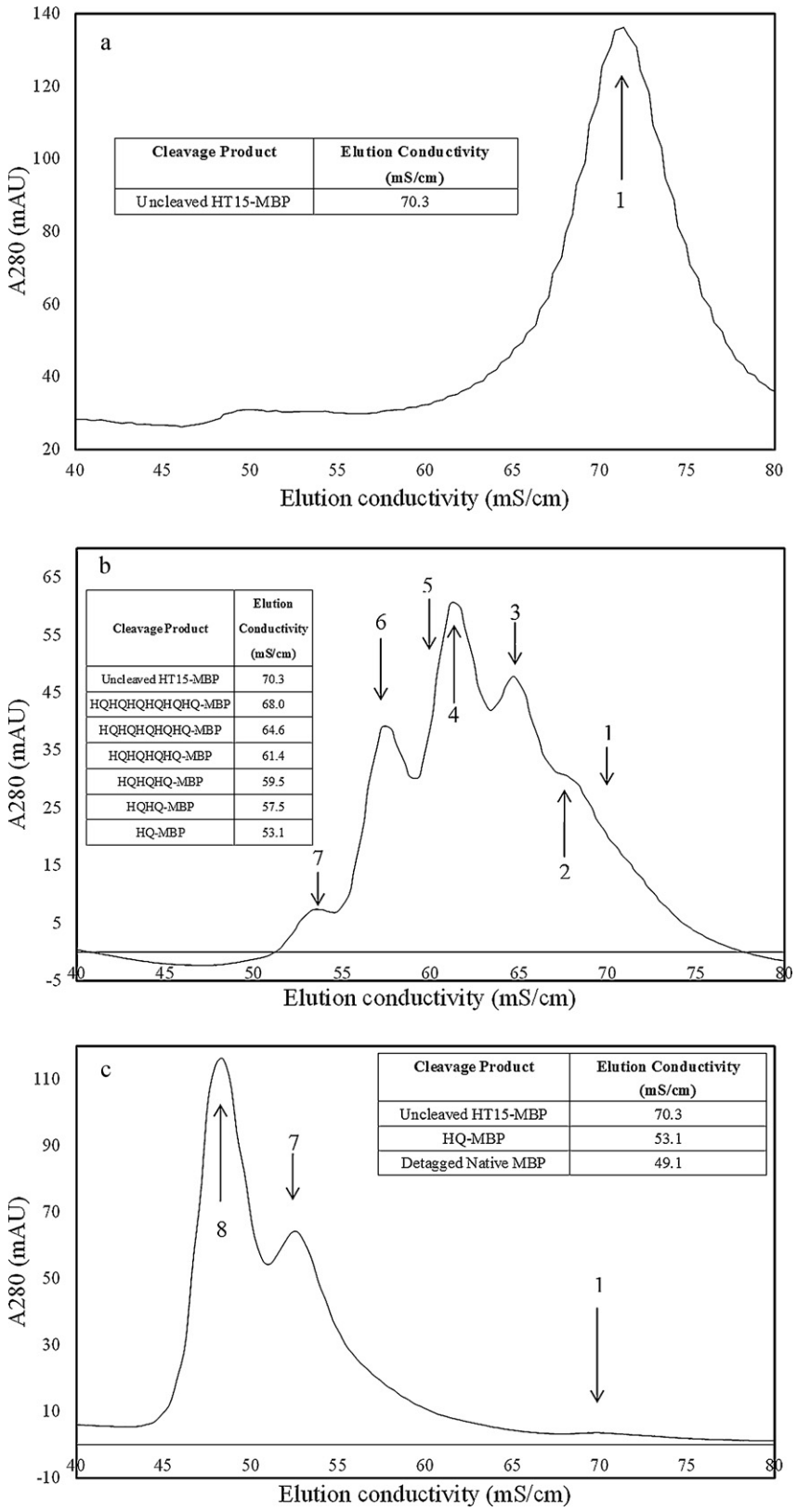

Fig. 7. Chromatograms showing the generation of different species of intermediates during the progress of poly-histidine tag removal from HT15-MBP by DAPase digestion. (a) Chromatogram recorded at the start, time 0 min of batch digestion. (b) Chromatogram recorded after 10 min of batch digestion and (c) chromatogram recorded after 60 min of batch digestion.

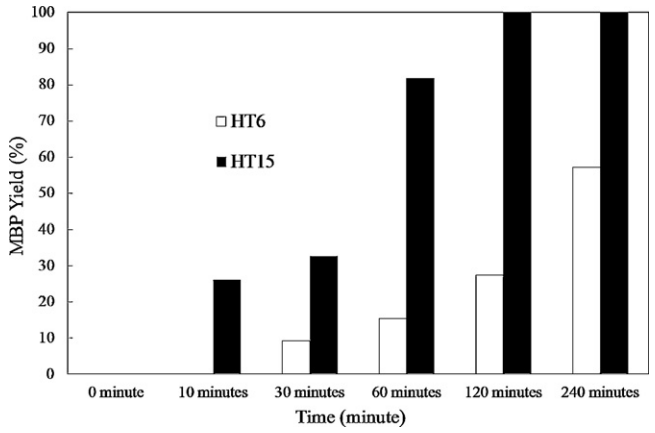

Fig. 8. The effect of tag design on the time course of the yield of MBP. HT6-MBP and HT15-MBP were digested with 50 mU of DAPase enzyme in the batch mode at 37 °C. Yield of MBP was calculated using the area of the MBP peak on the FPLC chromatograms.

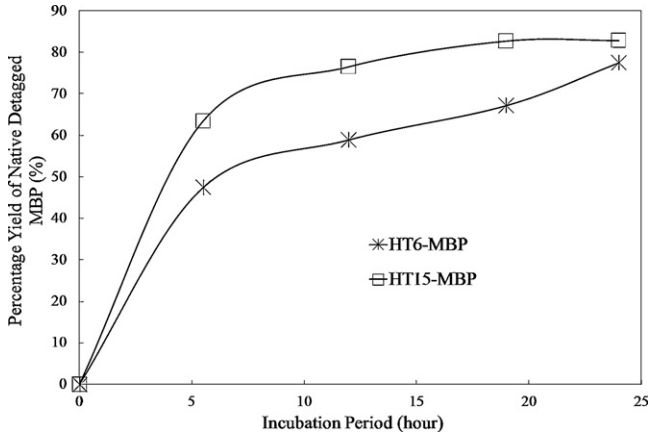

Fig. 10. The effect of incubation time on the percentage yield of native detagged MBP for on-column exopeptidase cleavage experiments using HT6-MBP and HT15-MBP; on-column cleavage buffer contained 50 mM imidazole, 0.5 mg of protein loading, 200 mU of DAPase, 37 °C.

of HT6 tagged fusion proteins, a higher imidazole concentration can be used in the binding and wash buffers to prevent non-specific binding, which might result in higher product purity. It follows then that in terms of purification performance, HT6 can be a preferred fusion tag than HT15.

The effect of incubation time on the percentage yield of native detagged MBP for the on-column exopeptidase cleavage of HT6-MBP and HT15-MBP was then investigated. Fig. 10 shows that the on-column cleavage reaction was more efficient when the HT15 fusion tag was used, as the yield of native detagged MBP was higher for the on-column removal of the HT15 tag throughout the range of test incubation time. On-column cleavage of HT15-MBP by DAPase reached 77% (93% of the equilibrium yield) after 12 h; a comparable yield of 77%, was achieved for the on-column cleavage experiment using HT6-MBP only after 24 h of incubation. It was previously found that by increasing the period of the on-column cleavage reaction under the same conditions up to 50 or 97 h for HT6-MBP did not increase the yield of detagged MBP compared to that achieved with 24-h incubation [17]. These results suggested that an incubation period of 24 h was sufficient for the on-column cleavage experiments to reach equilibrium for HT6-MBP. The results of the on-column cleavage experiments are in accordance with those observed during batch mode of cleavage, in which a HT15 tag was also found to be more efficiently removed than an HT6 tag. Based on these results, an incubation period of 12 h should be used for the on-column cleavage of HT15-MBP by DAPase in future experiments as this seems to be the optimum time. Furthermore, the use of an HT15 fusion tag should be chosen preferentially over a HT6 fusion tag for adsorptive detagging by DAPase as it effectively shortens the on-column incubation time required to achieve a comparable yield to the equilibrium value of native detagged MBP.

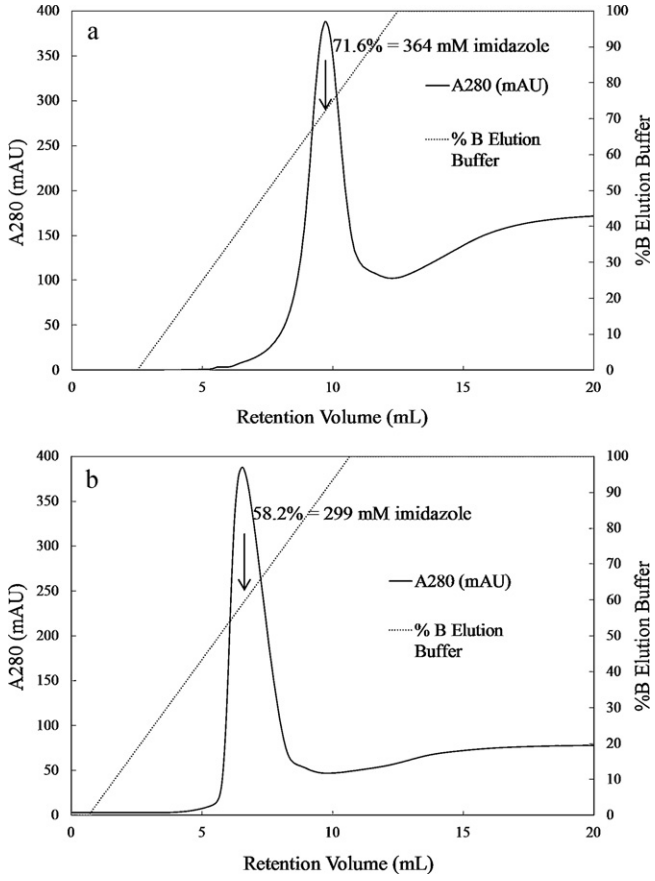

Fig. 9. (a) The elution profile of HT6-MBP and (b) the elution profile of HT15-MBP. The higher percentage elution buffer (B) needed for elution the stronger binding of the fusion tag.

4. Further discussion

Therapeutic application of recombinant fusion tagged proteins requires the protein to meet stringent requirements of structural integrity and purity. Furthermore, large-scale manufacturing of these recombinant proteins would need the factors affecting production cost, namely purification, fusion tag removal, and contaminant clearance, be well characterized. Emphasis of this work is given to the development of the column-based detagging process in combination with an exopeptidase system and strategies for increasing the process yield. The proposed method allows protein product with authentic, native N-terminal to be recovered. In addition, the column-based exopeptidase detagging technique described in this work provides an alternative to conventional batch enzymatic digestion in an attempt to reduce processing steps and improve overall operation economics. For process scale operations, the on-column exopeptidase cleavage method is preferred to the batch strategy because the isolation of the native detagged proteins can be achieved in a single chromatographic step. Even with an exopeptidase such as DAPase, digestion can occur on the column as long as adequate degree of dynamic binding is maintained and the accessibility to the cleavage site is not blocked or hindered.

This can allow the purification, on-column exopeptidase cleavage, and product recovery to be carried out in one-column step, effectively reducing the number of processing steps and minimizing the loss of protein during the multiple change-overs necessary in the conventional batch method.

Nevertheless, in comparison to another case of on-column cleavage but with an endopeptidase [14], longer incubation periods and higher temperature were required to achieve the same degree of product yield in the exopeptidase cleavage experiments described here. 200 U of AcTEV Protease, a hexa-histidine tagged endopeptidase, was used to cleave the tagged target protein adsorbed on-column with an incubation period of 8 h [14]. Considering that both works involved the use of hexa-histidine tagged enzymes and that the earlier results of batch digestion in the literature showed that exopeptidase cleavage by DAPase was indeed more efficient than endopeptidase cleavage in the batch mode [9], it seemed that in terms of the two obstacles to on-column exopeptidase cleavage identified earlier, limited molecular diffusion of the tagged proteases to their targets was the easier barrier to overcome. It follows that it is more difficult to achieve frequent exposure of the N-terminus of the tagged MBP to the exopeptidase in order to enhance the rate of peptide cleavage and shorten the period of on-column incubation required. As a result, the rate limiting step of the on-column exopeptidase cleavage can be identified to be the frequency of the reversible, on-off binding of the tagged target protein to the IMAC adsorbent, a property that can be enhanced by optimising the fusion tag sequence or by increasing the imidazole concentration. Experiments comparing the cleavage yield and efficiency for the on-column exopeptidase cleavage of different tag variants by DAPase in Section 3.4 can help elucidate the rate limiting factor to a certain extent. Since the HT15 fusion tag was found to have a lower binding strength to the IMAC adsorbent than the HT6 fusion tag, it is expected that the cleavage yield and efficiency for its on-column exopeptidase cleavage will be higher than that of HT6 under the same conditions due to the less strongly bound HT15-tagged protein being more frequently desorbed. Although the fact that the HT15 fusion tag contains dipeptides that are more readily removed should also be taken into account, the results of this experiment partially corroborate the identification of the aforementioned rate-limiting step. Future attempts to further improve the productivity of the adsorptive detagging method can involve increasing the frequency of desorption of the tagged target protein by further optimising the fusion tag design or the on-column cleavage conditions, both of which aiming to improve the dynamics of binding to the adsorbent and the intrinsic kinetics of the cleavage reaction.

5. Conclusions

When more purification steps than are really needed and process inefficiencies occur in a downstream purification process, an alternative approach is to use poly-histidine fusion tags. This might sidestep the design of the support matrix, affinity ligand or coupling chemistry as well as the need of studying the biomolecule properties to be purified. In fact, the design of affinity fusion tags can enable the maximization of both upstream product expression level and downstream productivity and recovery as a single entity. The results demonstrate that successful batch and adsorptive detagging of MBP constructed with two different poly-histidine fusion tags using DAPase can be achieved. The ability of poly-histidine tagged DAPase to digest its tagged protein substrates appeared to be significantly affected by the presence of the latter's binding interactions with the affinity matrix when compared to the batch mode of digestion. When cationic exchange chromatography was used to monitor the progress of poly-histidine tag removal, species with different elution conductivities were isolated, which were all in agreement with the structure prediction of their corresponding N-terminal sequences. Temperatures that were effective for batch digestion of poly-histidine tagged MBP also achieved high yields during the adsorptive detagging experiments. The results also suggest that the amount of enzyme present was not controlling the rate of the adsorptive detagging reactions above an enzyme activity of 200 mU used per milliliter of adsorbent. The influence of fusion tag design was found to be substantial on the batch digestion yield and efficiency. The chosen tag design was used effectively in shortening the on-column incubation time required to achieve the equilibrium yield of native detagged MBP. Finally, the difference in the composition of fusion tags affects the binding strength of the fusion protein, which can have an impact on the purification performance. The results presented in this paper can improve the commercial adoption of IMAC technology for the production of recombinant proteins as well as the synchronization of upstream and downstream bioprocess design to facilitate the development of a productive platform process.

References

[1] J. Arnau, C. Lauritzen, G.E. Petersen, J. Pedersen, Protein Expr. Purif. 48 (2006) 1.
[2] H. Block, J. Kubicek, J. Labahn, U. Roth, F. Schäfer, Protein Expr. Purif. 57 (2008) 244.
[3] V. Gaberc-Porekar, V. Menart, Chem. Eng. Technol. 28 (2005) 1306.
[4] Q. Liu, J. Lin, M. Liu, X. Tao, D. Wei, X. Ma, S. Yang, Protein Expr. Purif. 54 (2007) 212.
[5] L. Tan, D. Kim, I. Yoo, W. Choe, Chem. Eng. Sci. 62 (2007) 5809.
[6] R.J. Jenny, K.G. Mann, R.L. Lundblad, Protein Expr. Purif. 31 (2003) 1.
[7] C. Lauritzen, J. Pedersen, M.T. Madsen, J. Justesen, P.M. Martensen, S.W. Dahl, Protein Expr. Purif. 14 (1998) 434.
[8] J. Pedersen, C. Lauritzen, M.T. Madsen, S.W. Dahl, Protein Expr. Purif. 15 (1999) 389.
[9] J. Arnau, C. Lauritzen, J. Pedersen, Nat. Protoc. 5 (2006) 2326.
[10] N. Abdullah, H.A. Chase, Biotechnol. Bioeng. 92 (2005) 501.
[11] M. Kenig, S. Peternel, V. Gaberc-Porekar, V. Menart, J. Chromatogr. A 1101 (2006) 293.
[12] F. Schafer, A. Shafer, K. Steinert, J. Biomol. Tech. 13 (2002) 158.
[13] M.H. Hefti, C.J. Van Vugt-Van der Toorn, R. Dixon, J. Vervoort, Anal. Biochem. 295 (2001) 180.
[14] R. Bhikhabhai, A. Sjöberg, L. Hedkvist, M. Galin, P. Liljedahl, T. Frigård, N. Pettersson, M. Nilsson, J.A. Sigrell-Simon, C. Markeland-Johansson, J. Chromatogr. A 1080 (2005) 83.
[15] C. Dian, S. Eshaghi, T. Urbig, S. McSweeney, A. Heijbel, G. Salbert, D. Birse, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 769 (2002) 133.
[16] J. Nilsson, S. Ståhl, J. Lundeberg, M. Uhlén, P.A. Nygren, Protein Expr. Purif. 11 (1997) 1.
[17] W.K. Kuo, H.A. Chase, Biotechnol. Progr. 26 (2010) 142.
[18] E. Hochuli, H. Bannwarth, R. Dobeli, R. Gentz, D. Stuber, Nat. Biotechnol. 6 (1988) 1321.
[19] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[20] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[21] Amersham Biosciences, Application Note 18-1145-18 (2002) 1.
[22] R.J. Planta, M. Gruber, Anal. Biochem. 5 (1963) 360.