A new strategy for the on-column exopeptidase cleavage of poly-histidine tagged proteins

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ABSTRACT

This paper describes a new strategy, which aims to make on-column poly-histidine tag removal more useful in the production of recombinant proteins by improving the yield and efficiency of on-column exopeptidase cleavage. This involves improvement of the on-column cleavage condition by using imidazole concentrations in the range of 100–500 mM in the cleavage buffer. At 300 mM imidazole, maximum on-column cleavage yield (in excess of 99%) was achieved in 3 h of incubation. However, as a result of the increased imidazole concentration, this new strategy of on-column cleavage results in some residual uncleaved poly-histidine tagged proteins (∼0.1%) and the production of cleaved dipeptides, both of which need to be further removed in a subsequent step. A method involving the recirculation of recovered proteins and peptides through the immobilized metal affinity chromatography (IMAC) column (same-column recirculation) was found to be superior to subtractive IMAC for the purpose of contaminant clearance. Recovery of the detagged target proteins was achieved using 10 column volumes of recovery buffer, which had the effect of diluting the imidazole concentration to a suitably low level for contaminant removal by same-column recirculation. This strategy was also applicable at a higher adsorbent loading of 10 mg protein/mL adsorbent with an optimal ratio of 200 mU of DAPase per mg of adsorbed tagged maltose binding protein (MBP), giving a cleavage yield of 99.1% in 3 h. Finally, on-column cleavage conditions including the effect of protease concentration and incubation time on the new strategy have been investigated and comparisons are made for different tag removal strategies.

1. Introduction

With the ever increasing product titer in the cell culture, the technical bottleneck in the manufacturing of biopharmaceuticals has shifted to the downstream purification of therapeutic proteins. As a result, considerable pressure is being exerted to find innovative ways to reduce this cost. Today much of the therapeutic protein production requirement has been achieved by contract manufacturing organizations with multiproduct plants. In such scenarios, a generic and cheap platform technology is a highly sought-after tool to improve cost-effectiveness. Due to its intrinsic nature, immobilized metal ion affinity chromatography (IMAC) can provide a generic approach to primary capture chromatography suitable to meet the requirements mentioned above. In fact, ever since the introduction of poly-histidine fusion tags and IMAC, the technology has gained popularity as a platform technology capable of being incorporated into most existing downstream flowsheets [1,2]. In addition, IMAC adsorbents can be easily regenerated and retain adsorption capacity and robustness following multiple cycles of cleaning and reuse, giving consistent success across all scales from laboratory preparation to manufacturing [3–7].

Nevertheless, extended therapeutic use of recombinant proteins containing poly-histidine fusion tags requires the specific removal of the fusion tag in order for the protein product to achieve the expected immune response in patients. In practice, most poly-histidine tags are followed by a suitable amino acid sequence that facilitates the removal of the tag by enzymes, particularly endopeptidases. Nonetheless, commercially available endopeptidases such as thrombin, tobacco etch virus protease (TEV), and factor Xa have long suffered problems such as the need for high ratios of enzyme to tagged protein, long incubation times, the generation of incorrect terminal amino acids, and the occurrence of non-specific internal cleavage [3,8]. Alternatively, the extra sequence needed to be recognized by some proteases is not necessary when exopeptidases are used to remove the poly-histidine fusion tag. In fact, achieving effective fusion tag removal is the greatest hurdle in the wider use of the poly-histidine tag in protein production. IMAC technologies coupled with the use DAPase, a recombinant exopeptidase used to remove the tag sequence, aims to overcome this obstacle. DAPase is a recombinant rat dipeptidyl aminopeptidase I (DPPI) obtained by heterologous expression in insect cells that is part of the TAGZyme system; it also contains a poly-histidine tag, which is linked to

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its carboxyl terminus and cannot be self-cleaved as DAPase is an aminopeptidase that only cleaves from the amino terminus [9–11]. The use of DAPase can avoid incomplete and unspecific cleavage in tag removal processes, resulting in higher process efficiency and yield [3–5,10–14]. A second, subsequent IMAC step has been used at the end of the tag removal process to remove process contaminants generated from the cleavage reactions as well as the DAPase itself [3]. As a result, the use of DAPase satisfies the validations that require the use of recombinant reagents with no animal-derived reagents, highly specific cleavage, and total clearance of all contaminants from the target protein preparation.

The enzymatic removal of poly-histidine fusion tags whilst the target protein remains bound to the IMAC column, otherwise known as on-column detagging, offers benefits when compared to the batch mode of enzymatic cleavage process in terms of a reduction in the overall number of steps, better control of the cleavage conditions, and mild conditions for product recovery. Nevertheless, the removal of fusion tags whilst the target protein is adsorbed to an adsorbent has only been performed previously with endopeptidases [15–18] and only recently has on-column exopeptidase cleavage been proven feasible [19]. In the presence of a sufficient concentration of the imidazole (an efficient competitor for the IMAC adsorption sites), it was possible to combine the benefits of using the exopeptidase system with the on-column cleavage method.

However on-column tag removal by an exopeptidase suffered from problems such as the slow on-off binding rates of tagged target proteins and tagged enzymes [19]. Consequently, new strategies that aim to enable the on-column process to achieve higher yields at higher efficiencies are proposed here. This study describes the development of a procedure to achieve an on-column digestion method that allows most of the benefits of the combination of the use of DAPase with on-column tag removal to be realized. Higher efficiencies in column-based detagging enable the processing of more labile proteins that are unstable in the otherwise prolonged incubation period needed for cleavage of the tags. Furthermore, it is essential that the processes achieve very high cleavage yields, particularly when used for multimeric proteins. For the latter, product recovery can be significantly lower than that for monomeric proteins as the cleavage yield has an increased influence on the overall recovery. This is because the presence in a multimeric protein, that is otherwise completely detagged, of a single subunit that is only partially cleaved, can immobilize the entire protein on the IMAC column during the subsequent contaminant clearance step [14]. Finally, the rates of on-column cleavage are compared to that of batch cleavage to better understand the cleavage mechanism and the advantages and drawbacks of applying different detagging methods by DAPase are discussed.

2. Materials and methods

Disodium hydrogen phosphate, sodium chloride, tris(hydroxmethyl)aminomethane, EDTA, imidazole, 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[TM] FF Crude, HisTrap[TM] HP, Tricorn[TM] Mono S[TM] 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 room temperature and all buffers were made up with deionized (DI) water, filtered by 0.22-μm filters, and degassed before use unless otherwise noted.

The hexa-histidine tagged cleavage enzyme DAPase and two Escherichia coli strains producing hexa-histidine tagged maltose binding protein (MBP) namely, HT6-MBP and HT15-MBP were kindly provided by Unizyme Laboratories (Horsholm, Denmark). Both strains of MBP are N-terminus hexa-histidine tagged proteins encoded by an Escherichia coli clone pTrcHis/TOP10. HT6 has six consecutive histidine residues whereas HT15 has a glutamine residue inserted between each of the six histidine residues. The host strain was Escherichia 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.

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. 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 $4000 \times g$, 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 resedimented 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, 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 $14,000 \times g$ for 30 min at 4 °C.

2.3. IMAC purification

A 5 mL HisTrap[TM] FF Crude column, coupled to an Akta[TM] Explorer automated chromatography controller, was equilibrated with 10 column volumes of the IMAC equilibration buffer (100 mM NaH_2PO_4, 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 wash buffer (100 mM NaH_2PO_4, 20 mM imidazole, 0.5 M NaCl, pH 7.5). When the UV absorbance at 280 nm had fallen to the zero baseline, the adsorbed proteins were eluted via a step change 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 elution were collected during elution.

2.4. Dialysis

5 mM EDTA was added to the protein samples to chelate 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 of 12–14 kDa. The Visking dialysis tube (regenerated cellulose supplied dry) used was cut to the appropriate length according to the amount of sample required and the pre-use cleaning steps for the membrane were followed according to the supplier's washing instructions. The tube was then dialyzed overnight against the cleavage buffer.

2.5. FPLC analysis by Mono S^{TM} 5/50 GL cationic exchanger

The progress of cleavage of poly-histidine tag from protein can be monitored quantitatively using cationic exchange chromatography [10]. A column of Tricorn^{TM} Mono S^{TM} 5/50 GL (1 mL) was coupled to an Akta^{TM} 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 [20]. 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^{TM} HP column, coupled to an Akta^{TM} Explorer automated chromatography controller, was equilibrated with 10 column volumes of the recovery buffer (20 mM NaH$_2$PO$_4$; 150 mM NaCl, pH 7.0) at 1 mL/min. The purified protein sample was then loaded onto the column at 1 mL/min. Whilst the proteins remained adsorbed to the resin, 1 mL of exopeptidase solution containing varying amounts of total enzyme activity and imidazole concentration 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 (100 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.5) 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. Same-column recirculation

In this procedure, at the end of the on-column incubation with enzyme, protein recovery was completed by washing the IMAC column coupled to an Akta^{TM} Explorer automated chromatography controller with 10 column-volumes of the recovery buffer at 1 mL/min. The collected recovery fraction was then mixed with a vortex mixer to achieve homogeneity before being reloaded to the same IMAC column for removal of the partially and non-cleaved
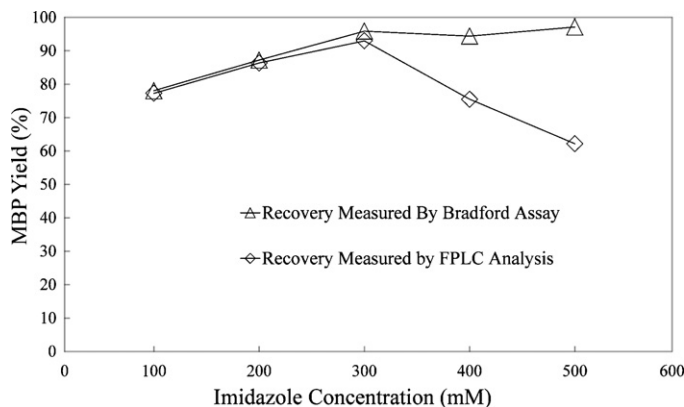

Fig. 1. Optimization of imidazole concentration for on-column exopeptidase cleavage, 0.5 mg of protein loading/mL adsorbent, 23 °C, 200 mU of DAPase, 24-h incubation.

proteins at a flowrate of 1 mL/min. The final recovery step was followed by elution with the IMAC elution buffer to elute the tagged contaminants still adsorbed to the column. The column effluents from the pre- and post-recirculation steps as well as the elution phase were collected for further protein analyses. The total protein concentration was measured by the Bradford assay.

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 [21]. The concentration of DAPase in terms of U/mL was determined in this activity assay where 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. 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 value of ΔA_{405}/min calculated at the end of the measurements.

3. Results and discussion

3.1. The effect of increasing imidazole concentration

In an attempt to improve the yield of the original on-column exopeptidase cleavage protocol [19], it is necessary to increase the imidazole concentration of the cleavage buffer. This can minimise the strength of the binding of the tagged target protein and DAPase to the IMAC column, which allows greater molecular mobility to occur. The effect of imidazole concentration in the range of 100–500 mM on the MBP yield of the on-column recovery fractions was examined. As shown in Fig. 1, the product yield in the recovery fractions measured by the Bradford assay increased with an increase in imidazole concentration with very high yields of 95.9% and 97.1% at imidazole concentrations of 300 mM and 500 mM respectively. Nevertheless, it was suspected that under the conditions tested, the protein concentration measured in the recovery fractions by the Bradford assay might include a mixture of cleaved and partially or uncleaved products because of the higher concentration of imidazole in the column at the time of recovery which would prevent species with residual tags from binding to the column. Indeed as shown in Fig. 1, the FPLC analysis showed varying amounts of partially cleaved products in the recovery fractions,

with recovery fractions for 100–300 mM imidazole containing close to 100% native fully detagged MBP whereas the recovery fraction for 500 mM imidazole contained only 64.0% native detagged MBP.

The result of the FPLC analysis shows that the yield of native detagged MBP does not always increase with an increase in imidazole concentration. In fact, there exists an optimum imidazole concentration of 300 mM as seen in Fig. 1. The yield of the on-column cleavage reaction at this optimum imidazole concentration of 300 mM was 93.0%. According to a study of the variation of binding strength with imidazole concentration performed previously [19], the tagged protein should start to elute from the IMAC column at an imidazole concentration of about 350 mM. Consequently, 300 mM imidazole in the cleavage buffer used for the new on-column cleavage strategy was adequate in minimizing binding of the tagged species to the IMAC column as it is very close to the minimum imidazole concentration needed for tagged protein elution. The decline in native detagged MBP yield observed during on-column detagging performed at imidazole concentrations greater than 300 mM can be explained by the decrease in DAPase activity in the presence of high concentrations of imidazole. Imidazole can affect the cleavage efficiency and yield of DAPase digestion by decreasing enzymatic cleavage rate, probably due to a competitive inhibitory effect on DAPase catalysis as suggested by the supplier of DAPase. Consequently, in a conventional batch cleavage protocol, it is recommended that IMAC elution fractions be buffer exchanged into a buffer containing no imidazole or diluted with the cleavage buffer prior to adding DAPase for tag removal [22].

The inefficiency of the original on-column exopeptidase reaction in not achieving complete tag removal might have been a direct effect of the deactivation of DAPase over the long period of on-column incubation [19]. Consequently, the effect of prolonged incubation of DAPase for 24 h at 37 °C on its enzymatic activity was examined. The results show that the rate of substrate cleavage was indeed reduced after a 24-h incubation at 37 °C. The loss of enzyme activity amounted to about 33% of the original DAPase activity present before the incubation commenced. This shows that increasing the temperature to 37 °C probably enhanced enzymatic activity at the beginning of the detagging reaction, but over time, the enzyme became deactivated. However, the results suggest that the reason why the on-column exopeptidase cleavage yields obtained after incubation at 37 °C for 24 h did not reach 100% cannot be attributed to the lack of DAPase activity in the later stages of the incubation. The remaining 67% of residual DAPase activity would have been sufficient to complete the on-column cleavage reaction assuming there were no limitations on the diffusion of the tagged enzymes to their cleavage sites on the tagged proteins.

3.2. The comparison of different protease systems

Considering that the cleavage efficiency of exopeptidase systems when used in the batch mode is higher than the endopeptidase systems [3], the drop in cleavage efficiency for DAPase when being used in the on-column mode could possibly be due to its intrinsic mechanism of tag removal. In order to verify this, the effect of imidazole concentration on the cleaved protein yields for on-column exopeptidase cleavage by DAPase performed in this work was compared to that for on-column endopeptidase cleavage by TEV protease described in the literature [23]. Since both enzymes and the target proteins are hexa-histidine tagged, it is possible to compare directly the two enzyme systems based on their intrinsic mechanism of cleavage in an on-column environment. Fig. 2 shows that for both systems, the on-column cleavage yields increased with higher imidazole concentration in the cleavage buffer, although the exopeptidase system required a much higher concentration of imidazole to achieve comparable on-column product yields. In fact, limited or no on-column cleavage occurred at low imidazole
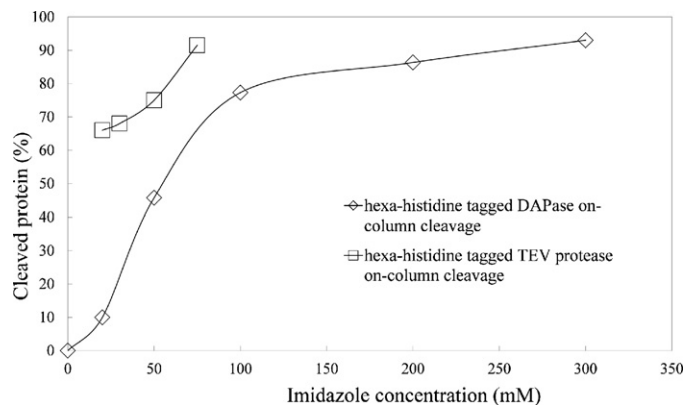

Fig. 2. The effect of imidazole concentration on the percentage of cleaved protein for on-column cleavage processes using different hexa-histidine tagged enzymes, namely the exopeptidase DAPase and the endopeptidase TEV. 1-mL HisTrap™ column, 0.5 mg of target protein loading/mL adsorbent, 23 °C.

concentrations for the exopeptidase system. This is because the presence of imidazole affects also the on-off binding behavior of the tagged target protein, which directly influences the extent of on-column cleavage in the case of exopeptidase tag removal. For endopeptidase tag removal the enzyme does not need to come in-between the N-terminal end of the tag and the adsorbent surface for cleavage to occur, and consequently, relatively high yields of cleaved protein were achieved at imidazole concentrations below 50 mM. The rate of on-column cleavage for the endopeptidase system is much higher than that of the exopeptidase system as the experimental incubation period for the former need only be 4 h to achieve greater than 90% cleavage compared to a 24-h incubation period when DAPase is used.

3.3. Same-column recirculation

The new on-column cleavage strategy developed here involves increasing the imidazole concentration in the cleavage buffer to the optimum concentration of 300 mM determined earlier to be particularly effective in increasing the on-off binding kinetics for both the poly-histidine tagged exoprotease and the poly-histidine tagged target protein, whilst minimizing enzyme inactivation. As shown in Fig. 3(a), the on-column cleavage yield was in excess of 99% in an experiment carried out at 37 °C for 3 h. Note that the elution conductivities of native MBP and HT15-MBP were 48.6 mS/cm and 70.9 mS/cm respectively when separated on the Mono S™ 5/50 GL column. The residual 0.07% of un-cleaved poly-histidine tagged MBP can be attributed to the steric hindrance of the protein molecules in the reaction mixture similar to that observed in batch cleavage experiments as suggested by the supplier of DAPase [22]. As shown, it is possible to not only reduce the on-column incubation time to 3 h, but also increase the on-column exopeptidase cleavage yield achieving in excess of 99% of native cleaved product. Nevertheless, the new on-column cleavage strategy necessitates the addition of a subtractive IMAC purification step [10] in order to remove the process contaminants derived from the on-column exopeptidase cleavage reactions. The use of the same type of column for this purpose as used for the initial IMAC capture in the purification process would be advantageous because the downstream flowsheet would become simpler. The proposed same-column recirculation stage acts as a negative chromatography step in which the product recovery fraction is applied again to the IMAC column in order to capture tagged impurities whilst allowing the native fully detagged protein to flow through the column. As shown in Fig. 3(b) and (c), the same-column recirculation procedure successfully purified the native detagged protein
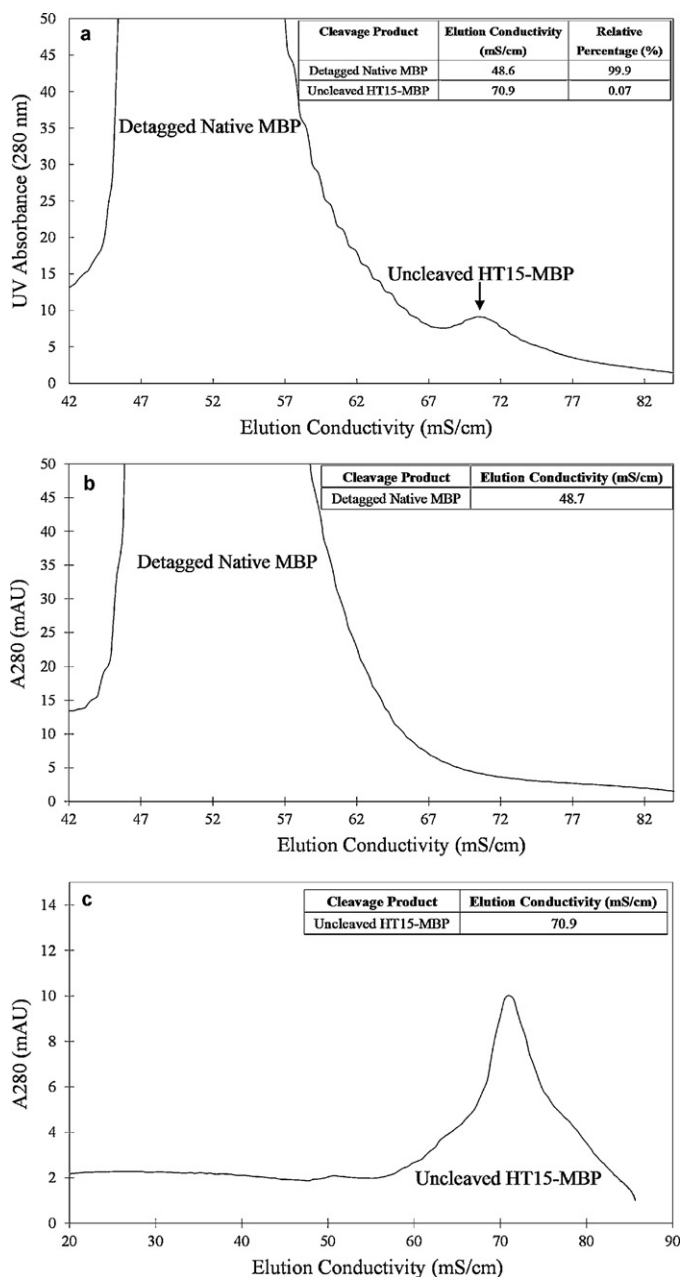
Cleavage Product	Elution Conductivity (mS/cm)	Relative Percentage (%)
Detagged Native MBP	48.6	99.9
Uncleaved HT15-MBP	70.9	0.07

Cleavage Product	Elution Conductivity (mS/cm)
Detagged Native MBP	48.7

Cleavage Product	Elution Conductivity (mS/cm)
Uncleaved HT15-MBP	70.9

Fig. 3. (a) Chromatogram of the recovery fraction analyzed before same-column recirculation; 3-h incubation, 300 mM imidazole, 37 °C, 0.5 mg of protein loading/mL adsorbent. (b) Chromatogram of the recovery fraction analyzed after same-column recirculation. (c) Chromatogram of the elution fraction analyzed after same-column recirculation.

from the residual non-cleaved tagged proteins. Only one passage of the recovery fraction through the column was needed to increase substantially the purity of the native detagged protein. The ability to perform same-column recirculation of the recovery fraction directly for contaminant clearance relies on the fact that with the on-column exopeptidase cleavage protocol, recovery of reaction products can be achieved by irrigating the column with a recovery buffer containing no imidazole. This results in sufficient dilution of the residual imidazole concentration in the recovery fraction such that that the tagged impurities are subsequently retained on the IMAC column when the recovery fraction is applied to it. An additional buffer-exchange step to eliminate the imidazole and a re-equilibration step to condition the second IMAC column as are needed in the conventional subtractive purification protocol
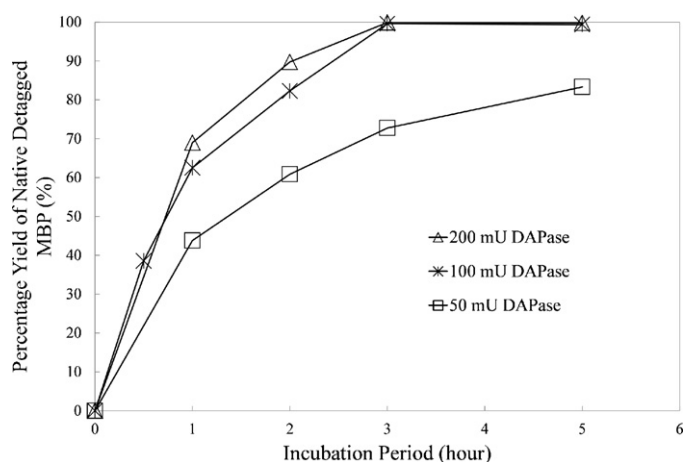

Fig. 4. The effect of the amount of DAPase activity used on the time course of the yield of native detagged MBP for the new strategy of on-column exopeptidase cleavage. 0.5 mg of protein loading/mL of adsorbent was digested with 50–200 mU of DAPase enzyme at 37 °C.

become redundant here. Indeed the success of this recirculation method depends only on the dilution factor of the recovery fraction necessary to allow the re-binding of the tagged contaminants during the recirculation as well as the binding capacity of the IMAC column for tagged contaminants.

3.4. The comparison of different poly-histidine tag cleavage strategies

The optimal conditions for cleavage were found from a series of experiments involving on-column exopeptidase cleavage using DAPase. The effects of the protease to tagged target protein ratio and incubation time adopted on the performance of the new on-column cleavage strategy were investigated. The percentage yield of native detagged MBP was determined for cleavage reactions conducted at 37 °C. As shown in Fig. 4, for a series of experiments conducted at a protein loading of 0.5 mg/mL, an incubation period of 3 h using 100 mU of DAPase provided the optimum on-column cleavage conditions. The optimal ratio of 200 mU of DAPase per mg of adsorbed tagged MBP was also applicable at a higher protein loading of 10 mg protein/mL adsorbent where an on-column cleav-
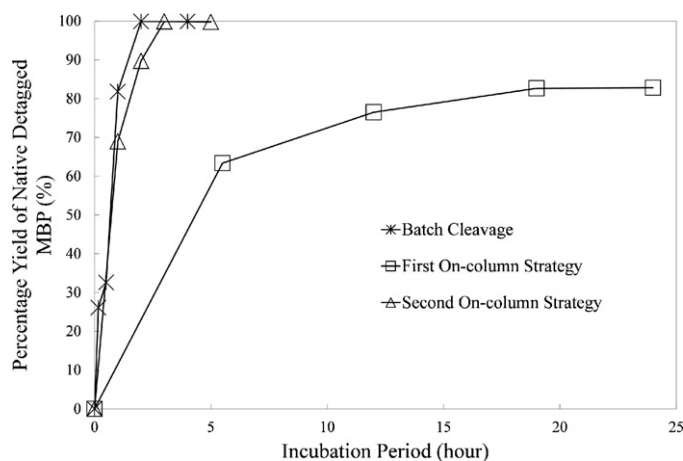

Fig. 5. Comparison of the two on-column cleavage strategies and the conventional batch cleavage method. Batch cleavage performed with 50 mU of DAPase 37 °C, 0.5 mg protein/mL. Original on-column strategy performed with 200 mU of DAPase at 37 °C, 0.5 mg protein/mL of adsorbent [19]. New on-column strategy performed with 200 mU of DAPase at 37 °C, 0.5 mg protein/mL of adsorbent.
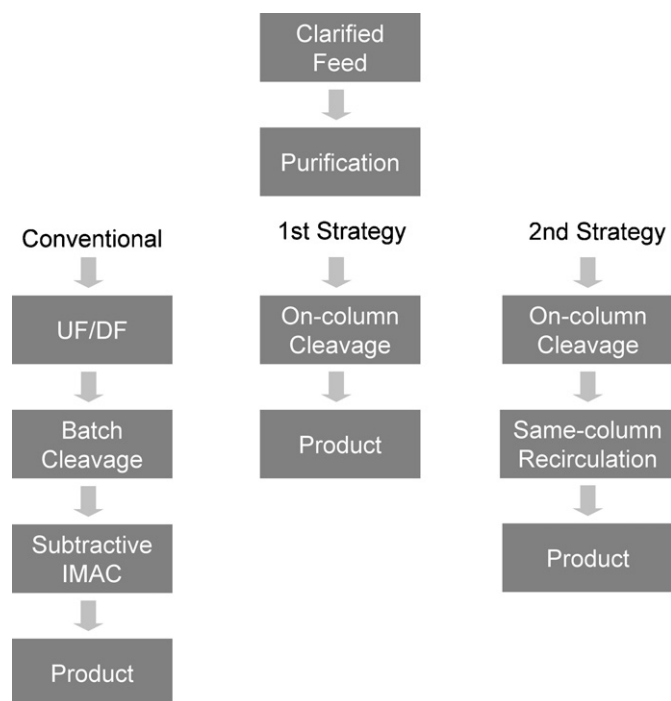
Fig. 6. Comparison of the two on-column cleavage strategies and the conventional batch cleavage method.

age yield of 99.1% of native detagged MBP was achieved after 3 h incubation at 37 °C.

As shown in Fig. 5, an analysis can be performed to compare the detagging yields for the two on-column detagging strategies and the conventional batch cleavage method. The results show that for the new on-column strategy, the apparent cleavage kinetics and mechanism are more similar to those in the batch mode and both can achieve very high yields of native detagged MBP as opposed to the original on-column strategy where incomplete cleavage occurred [19]. Similarly, the batch method and the new on-column strategy are both efficient and offer much higher productivities than the original on-column strategy. Comparing the two on-column strategies to the batch mode of cleavage in terms of the amount of protein being processed per volume, the amount of protein loading in the on-column strategies is limited by the binding capacity (~10 mg of poly-histidine tagged MBP per mL of HisTrap adsorbent) and the volume of IMAC adsorbent used. In addition, the amount of DAPase/mg of tagged target protein required is four times higher for both on-column strategies in comparison to that used in the batch method.

However as shown in Fig. 6, the number of processing steps is significantly reduced with the column-based cleavage strategies compared to the conventional batch method which needs additional recovery and wash steps. Consequently, the overall recovery of product may also be improved with the on-column strategies as a result of the process being intensified. Any additional purification stages for tag removal add to the costs of adopting IMAC chromatography strategies. When using the conventional way of eluting products from IMAC columns, the process requires the dilution or diafiltration of the elution fractions obtained using either low pH or buffers containing histidine and imidazole prior to batch cleavage. In the original on-column strategy, the elimination of steps such as buffer exchange can save time and costs in the setup, sanitization, pre and post-use testing, storage, and processing associated with these extra steps. With the new strategy, the success of the

same-column recirculation step to achieve the clearance of tagged contaminants introduces minimal additional cost and effort to the on-column exopeptidase detagging strategy. This eliminates the need for any pre-use and post-use cleaning and equilibration steps involved in adding the second, separate subtractive IMAC column. Finally, both on-column detagging strategies involve the use of only one unit operation, which is highly beneficial to the downstream process economics. With only one chromatographic unit operation, this method of producing highly pure native target proteins can replace multistep chromatographic methodologies and improve process efficiency. This purification approach can furthermore be readily applied to high throughput methodologies, enabling the purification, cleavage and recovery of recombinant proteins in a completely automated manner.

4. Conclusions

A significant advantage of using the on-column detagging strategy described in this work is that the tagged process contaminants can be segregated from the detagged native products during same-column recirculation. This is beneficial since the number of steps, which would otherwise be required to remove these contaminants, can be reduced. In addition, minimizing the number of handling steps also reduces the chance of protein degradation. The other benefit of the new method is that there is no need to perform a buffer exchange step in a separate unit operation compared to the batch cleavage method. Finally, the comparison between the new on-column cleavage strategy and the batch mode of cleavage revealed that the two probably have a similar cleavage mechanism. Considering that the on-column cleavage yield and efficiency are comparable to that of the batch reactions, it is suggested that this new method presents a number of advantages compared to the conventional batch method.

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