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A novel strategy for the purification of a recombinant protein using ceramic fluorapatite-binding peptides as affinity tags

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ABSTRACT

In recent years, affinity fusion-tag systems have become a popular technique for the purification of recombinant proteins from crude extracts. However, several drawbacks including the high expense and low stability of ligands, their leakage during operation, and difficulties in immobilization, make it important to further develop the method. The present work is concerned with the utilization of a ceramic fluorapatite (CFT)-based chromatographic matrix to overcome these drawbacks. A heptapeptide library exhibiting a range of properties have been synthesized and subjected to ceramic fluorapatite (CFT) chromatography to characterize their retention behavior as a function of pH and composition of the binding buffer. The specific binding and elution behavior demonstrates the possible application of CFT-binding peptides as tags for enhancing the selective recovery of proteins by CFT chromatography. To materialize this strategy, a phage-derived CFT-specific sequence KPRSVSG (Tag1) with/without a consecutive hexalysine sequence, KKKKKKKPRSVSG (Tag2), were fused at the C-terminus of an enhanced green fluorescent protein (eGFP). The resulting gene constructs H-eGFP, H-eGFP-Tag1 and H-eGFP-Tag2 were expressed in *Escherichia coli* strain BL-21, and the clarified cell lysate was applied to the CFT column equilibrated with binding buffer (20–50 mM sodium phosphate, pH 6–8.4). Sodium phosphate (500 mM) or 1 M NaCl in the respective binding buffer was used to elute the fused proteins, and the chromatographic fractions were analyzed by gel electrophoresis. Both the yield and purity were over 90%, demonstrating the potential application of the present strategy.

1. Introduction

Recombinant protein-based drugs are already significant components of the therapeutic arsenal. They contribute greatly to the rapid growth of biopharmaceuticals in the field of medicine [1–4]. However, the purification of recombinant proteins from crude extracts, that represents the largest portion of the entire cost of bioprocessing still remains as a major bottleneck in biopharmaceutical industries [5,6]. As a consequence, a raise in the product price, and a decrease in the pace of its development, limit the accessibility of those products to the patients. In this context, affinity fusion-tag methods arise as popular tools for the purification of recombinant proteins [7–9]. These methods entail a generic strategy for the single-step purification of the fused proteins from a complex biological mixture [10,11].

The small peptide tags share some distinct beneficial features, for example they have minimal effects on the structure and bioactivity of proteins [12], they act simply in terms of purification [13], and they can be removed specifically to produce the native protein [14]. Moreover, various proteins can be purified using a common method [15]. Immobilized metal ions [16], proteins [17], antibodies [18], and complementary peptides [19] are the most commonly used ligands in this type of chromatography. However, some limiting factors such as the difficulties in ligand immobilization [20]; the possibility of metal ion leakage [12]; the low stability of protein or antibody ligands [14]; and the possible contamination of the final product [21], show the necessity for further development of this method.

Calcium phosphate based ceramic apatites have been widely used as leading adsorbent of biomolecules [22–24]. Apatite (AP) chromatographic matrix itself shows functionalities toward

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biomolecules [25–27], so there is no need of ligand immobilization. Moreover, the components of AP materials are neither antigenic nor cytotoxic [28]. Therefore, it is expected that utilization of ceramic fluorapatite (CFT) stationary phase in affinity-tag systems may overcome some of the traditional drawbacks as mentioned earlier. A model approach is presented here to verify the applicability of CFT-binding peptides (CBPs) as tags to enhance the selective recovery of proteins by CFT chromatography.

Retention of biomolecules on AP surfaces is mainly dominated by phosphoryl cation exchange and calcium ion regulated interactions [25,29]. However, previous studies also reported that retention of some positively charged biomolecules cannot be explained by a simple cation exchange mechanism [30,31]. Interestingly, some AP materials retain negatively charged biomolecules despite their net negative surface charge; the interaction mechanisms are less clear albeit [29]. The complexity in interactions eventually makes the AP surface selective for specific biomolecules. In previous work, we have identified CFT-specific heptamer peptides from phage display selection [32]. The peptide F5-4, KPRSVSG, was the most frequent among the phage-derived CBPs, and it was observed that peptide F5-4 can be eluted specifically from the CFT column. The specific retention behavior of the CBPs illuminates the possibility for their use as tags in CFT chromatography. The objective of the present work was to verify this strategy.

The well-known green fluorescent protein (GFP) with a typical rigid beta barrel structure, first isolated from the bioluminescent jellyfish *Aequorea victoria*, exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range [33,34]. Due to this particular property, GFP has been widely used to monitor promoter activity, the localization and motility of proteins, and the interactions between biomolecules *in vivo or in vitro* [35–37]. In downstream processing, GFP is often utilized to investigate the efficiency of tags [11,38,39]. Here, an enhanced mutant of GFP (eGFP) [40], a 241-amino acid protein with improved fluorescent properties was used to investigate the efficiency of CBPs as tags. In this work, CBPs were fused genetically at the C-terminus of the eGFP and the resulting gene constructs were then expressed in *Escherichia coli* (*E. coli*). Afterwards, the clarified cell lysates were applied to the CFT column and the retention behavior of the fused proteins was determined under different chromatographic conditions. The chromatographic fractions were subjected to gel electrophoresis, and the performance of the current strategy was evaluated by analyzing the gel images.

2. Materials and methods

2.1. Materials

Ceramic fluorapatite (40 μm particle size, 600–800 Å pore size) was purchased from BioRad Laboratories (Hercules, California, USA). Preloaded Wang resin, Fmoc (9-fluorenylmethoxycarbonyl)-amino acid derivatives, N-methylpyrrolidone (NMP), piperidine, 1-hydroxybenzotriazole (HOBt), N,N-diisopropylethylamine (DIEA), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), dimethylformamide (DMF), trifluoroacetic acid (TFA), and 1,2-ethanedithiol (EDT) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). *E. coli* strain BL21 was obtained from Life Technologies (New York, USA). The gene corresponding to enhanced green fluorescent protein (eGFP), containing the peptide sequence HHHHHH (H_6) on the N-terminus and with or without the CFT-specific peptide sequences on the C-terminus, was synthesized and cloned in the pUC18 by GenScript (Piscataway, USA). The sequence and molecular weight (MW) of the resulting constructs are shown in Table 1. It has been demonstrated that GFP containing a poly-histidine (H_6) tag does not bind to AP materials [39], therefore, a H_6-tag was fused to eGFP constructs here to monitor their expression by immobilized-metal affinity chromatography (IMAC). Reagents and instruments used for the gel electrophoresis were obtained from GE Healthcare (New Jersey, USA). All other chemicals and reagents were analytical grade.

Table 1
Sequence and molecular weight (MW) of the enhanced green fluorescent protein (eGFP) and the eGFP constructs.

Construct	Sequence	MW (kDa)
eGFP	MSRVSKGEELFTGVVPILVELDGDVNGHKFSVSGE GEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGV QCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDD GNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGH KLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDG SVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKD PNEKRDHMVLLEFVTAAGITLGMDELYK	27.2
H-eGFP	MHHHHHH-*eGFP*	28
H-eGFP-Tag1	MHHHHHH-*eGFP*-KPRSVSG	28.75
H-eGFP-Tag2	MHHHHHH-*eGFP*-KKKKKKKPRSVSG	29.5

2.2. Peptide synthesis

Peptide synthesis was carried out on an automated peptide synthesizer (ABI-433A, Applied Biosystems, California, USA) using the standard solid phase peptide synthesis (SPPS) method [41]. Fmoc protecting group was removed from the N-terminal of the preloaded resin bound amino acid by 20% piperidine in NMP, and the C-terminal of another Fmoc-amino acid was activated using HOBt/HBTU/DIEA (1:1:2) in DMF. Afterwards, the activated amino acid was allowed to react with the deprotected N-terminal of the resin bound amino acid. A cleavage cocktail (82.5% TFA, 5% phenol, 5% water, 5% thioanisol and 2.5% EDT) was used to separate the desired peptide from the resin as well as to remove the acid-labile side chain protecting groups.

2.3. Mass spectrometry

The molecular masses of the synthesized peptides were checked by matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (Autoflex II, Bruker Daltonics, Bremen, Germany). The MALDI-ToF analysis was performed in positive-ion mode using a HCCA (α-cyano-4-hydroxycinnamic acid) matrix prespotted AnchorChip target (Bruker Daltonics, Bremen, Germany). Peptide sample was dissolved in 1% TFA at a concentration of 1 mg/mL, diluted 10 times, and 2 μL of peptide solution was applied on the spot. Afterwards, the sample plate was injected into the MALDI-ToF instrument. At the beginning of the analysis, calibration was run with standard peptide sample, and the acquired calibration data was applied to the measurements of real samples for the mass correction.

2.4. Characterization of the retention of the peptides in CFT chromatography

The chromatographic experiments were performed on BioCAD® 700E (Applied Biosystems, Foster City, USA) equipped with a fraction collector (FC 203B, Gilson, Middleton, USA) and with a UV detector set at 214 nm. A Tricorn column (5/50, GE Healthcare, Munich, Germany) was packed with 20% slurry (w/v) of the CFT beads in degassed 200 mM sodium phosphate ($Na_2HPO_4 + NaH_2PO_4$), pH 9.2. The experiments were carried out at pH 7.4. Here 5–20 mM sodium phosphate (Na-phosphate) and

1% TBS (50 mM Tris–HCl pH 7.5, 150 mM NaCl) were used as the binding buffers (buffer A), and the experiments were run at a flow rate of 1 mL/min. The CFT column was equilibrated properly with buffer A, then 500 μL of the peptide sample (2 mg/mL) was injected, the column was washed with buffer A (5 mL). Afterwards, a gradient elution was performed with 20 mL of 600 mM Na-phosphate (buffer B).

2.5. Production of the fusion protein

The eGFP gene constructs H-eGFP, H-eGFP-Tag1, and H-eGFP-Tag2 were expressed in *E. coli* strain BL-21. Single colonies of transformed *E. coli* harboring the eGFP gene construct were picked from ampicillin plates and inoculated into 10 mL of media containing ampicillin (0.1 mg/mL). The overnight incubation was carried out on a shaker at 37 °C. The overnight culture was inoculated into a 1 L media containing ampicillin (0.1 mg/mL). The culture was grown to OD_{600} 0.9–1.0 in a shaker at 37 °C, and eGFP expression was induced with the addition of IPTG to a final concentration of 0.25 mM. After overnight growth at 28 °C, the cells were harvested by centrifugation at 5000 × *g* and resuspended in 20 mL of lysis buffer (50 mM Na-phosphate, pH 7.4). The suspension was lysed through pulsed sonication using a Branson Sonifier 150 (Branson Ultrasonics Corporation, Connecticut, USA) on ice for 5 min. The lysate was centrifuged at 10,000 × *g* for 20 min at 4 °C, and the remaining cellular debris was discarded.

2.6. Fluorescense microscopy

The eGFP constructs H-eGFP, H-eGFP-Tag1, and H-eGFP-Tag2 expressing cells were monitored using a fluorescence microscope (Axiovert 200, Carl Zeiss, New York, USA) equipped with filter 8 (GFP range). The induced cell culture samples were centrifuged at 5000 × *g* for 3 min, and the pellets were washed with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Afterwards, the pellets were suspended gently in PBS buffer. The suspension was transferred to a glass slide and subjected to analysis under the microscope. The images were captured at a magnification of 100 by Axiocam MRc (Carl Zeiss, New York, USA).

2.7. Evaluation of protein expression

The expression of designed constructs was examined by analyzing the cell lysate supernatant *via* IMAC. An aliquot of the supernatant was loaded onto a metal chelate spin column (Nunc ProPur IMAC spin columns, Thermo Scientific, IL, USA). The column was pre-equilibrated with five column volumes of lysis buffer containing 10 mM imidazole. After washing with the same buffer containing 30 mM imidazole, elution was performed with 250 mM imidazole. The crude extracts and eluate fractions were analyzed with 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie brilliant blue staining was used to detect the protein bands in the gel.

2.8. Purification of the fused proteins by CFT column chromatography

Purification of the fused proteins was carried out on AKTAprime plus (GE Healthcare). A 20% slurry (w/v) of CFT beads in a degassed packing buffer (200 mM Na-phosphate, pH 9.2) was used to pack a Tricorn column (5/50, GE Healthcare). Na-phosphate and NaCl gradient elutions were performed with increasing salt concentrations. For the Na-phosphate elution, the binding buffer (buffer A) was 20–50 mM Na-phosphate and the elution buffer (buffer B) was a 500 mM Na-phosphate. For the NaCl elution, buffer A was 20 to 50 mM Na-phosphate and buffer B was 1 M NaCl in 20 mM Na-phosphate. At the beginning of each experiment, the CFT column was equilibrated properly with buffer A. The cell lysate was diluted four times with 10 mM Na-phosphate, pH 7.4, and 5 mL of the diluted cell lysate (sample) was loaded onto the column. Afterwards, the column was washed with buffer A (10 mL), a gradient elution was performed with 0 to 100% buffer B (20 mL), and the column was washed with 100% buffer B. The experiments were run at a flow rate of 1 mL/min. After each run, the column was regenerated with 500 mM Na-phosphate. The crude extracts, flow-through, and elution fractions were analyzed by 12.5% SDS-PAGE. Protein bands were visualized by silver staining.

Table 2
Influence of the composition of the binding buffer on the retention of the peptides in CFT column chromatography.

Peptide ID (sequence)[a]	5 mM NaP	10 mM NaP	20 mM NaP	TBS (1%)
N_0 (HLPPWTQ)	– – –	– – –	– – –	– – –
E_1 (LHSELPT)	– – –	– – –	– – –	+++
D_1 (DHPAVAQ)	– – –	– – –	– – –	+++
E_2 (GSESEGG)	Negligible	– – –	– – –	+++
D_4 (DDDDGGG)	+++	– – –	– – –	+++
E_6 (EEEEEEG)	+++	Negligible	– – –	+++
K_1 (KTTGLTV)	– – –	– – –	– – –	+++
K_2 (SKTKNNL)	+++	+++	+++	+++
K_4 (KKKKGGG)	+++	+++	+++	+++
K_6 (KKKKKKG)	+++	+++	+++	+++
R_1 (SQMHLRY)	– – –	– – –	– – –	+++
K_1R_1 (KPRSVSG)	+++	+++	+++	+++
R_2 (QRHTRPN)	+++	+++	+++	+++
R_4 (RRRRGGG)	+++	+++	+++	+++
N_{DK} (SSPDKSF)	– – –	– – –	– – –	+++
N_{EK} (KKTPEEG)	+++	– – –	– – –	+++

[a] Amino acids are presented as single letter in the sequences.
NaP represents sodium phosphate buffer.
(+++) represents retained and (– – –) represents non-retained.

3. Results

3.1. Peptide synthesis and analysis

In our previous work, CBPs were identified from a randomized heptamer phage display peptide library. Peptides with diversified physico-chemical properties have been found in the selected phage-derived sequences. Therefore, it is difficult to predict their retention mechanism in CFT chromatography. However, peptides that will be used as tags should have specific retention behavior. For this reason, an investigation was performed to understand the underlying facts behind the retention of peptides on CFT (Table 2). Peptides exhibiting a range of properties, such as, acidic amino acid rich peptides E_1 (LHSELPT), D_1 (DHPAVAQ), E_2 (GSESEGG), D_4 (DDDDGGG), E_4 (EEEEGGG) and E_6(EEEEEEG), basic amino acid rich peptide K_1 (KTTGLTV), K_2 (SKTKNNL), R_1 (SQMHLRY), R_2 (QRHTRPN), R_4 (RRRRGGG), K_4 (KKKKGGG) and K_6 (KKKKKKG), and neutral peptide N_0 (HLPPWTQ), N_{DK} (SSPDKSF) and N_{EK} (KKTPEEG) were synthesized and subjected to CFT chromatography under different conditions. Here, the sequence ID that starts with 'F' was selected from phage display selection.

3.2. Characterization of the retention of the peptides in CFT chromatography

Retention of the peptides on CFT was verified with 5, 10 and 20 mM Na-phosphate binding buffer at pH 7.4. Table 2 represents the effects of the composition of the mobile phase on peptide binding to CFT in dynamic condition. Neutral peptide N_0 and the positively charged peptides (K_1 and R_1) having a net charge of +1 were not retained in any composition of Na-phosphate

Table 3
Influence of net charge and isoelectric point on the retention of peptides on CFT.

Peptide ID (sequence)	Isoelectric point	Net charge	Sodium phosphate concentration (mM) at the point of elution at pH 7.4	
			TBS (1%)[a]	20 mM NaP[a]
E_1 (LHSELPT)	5.1	−1	170	–
E_4 (EEEEGGG)	3	−4	185	–
D_4 (DDDDGGG)	2.75	−4	190	–
E_6 (EEEEEEG)	2.9	−6	195	–
K_1R_1 (KPRSVSG)	11.5	+2	240	220
E_2K_4 (EEKKKKG)	10.2	+2	260	235
K_4 (KKKKGGG)	11	+4	355	340
R_4 (RRRRGGG)	12.9	+4	290	275
K_6 (KKKKKKG)	11.2	+6	465	445
N_{EK} (KKTPEEG)	7.05	0	180	–

[a] Binding buffer at pH 7.4.
NaP represents sodium phosphate.

binding buffer. However, the column with 5 mM Na-phosphate buffer retained the neutral peptide NEK. The positively charged peptides having a net charge of +2 or more were retained in all applied compositions of the binding buffer. Interestingly, none of the acidic peptides were retained by the column when the binding buffer was 10 or 20 mM Na-phosphate. Therefore, 1% of TBS buffer was considered as binding buffer for observing the effect of different types of binding buffers on retention. In this case, all peptides but N_0 were retained by the column.

A gradual elution was performed with 600 mM Na-phosphate, pH 7.4. The peptides E_1, D_4, E_4, E_6, N_{DK}, N_{EK}, K_1R_1, R_4, K_4 and K_6 were eluted with approximately 170, 190, 185, 195, 175, 180, 240, 275, 355 and 465 mM Na-phosphate, respectively, when the binding buffer was 1% TBS. The effects of the net charge and isoelectric point (IEP) of the peptides on retention are shown in Table 3. The composition of the buffer at the point of elution of neutral peptide N_{DK} or N_{EK} was very similar to that of the acidic peptides. Therefore, it is suggested that the retention of N_{DK} or N_{EK} was mainly dominated by the interactions between glutamic acid (E) and calcium ions (Ca^{+2}) on the CFT surface. This clearly shows that Ca^{+2} (C-site) regulated interactions do not depend on the net charge of peptides, whereas phosphate (P-site) regulated interactions on the CFT surface do depend on the net charge of peptides [42]. Some interesting features were observed regarding the effect of different types of acidic or basic amino acid residues, the retention times (RTs) of the lysine (K) containing peptides were longer than that of the arginine (R) containing peptides, whereas the RTs of acidic amino acid (D and E) containing negatively charged or neutral peptides were similar. However, it was not possible to correlate the retention behavior and IEP values of the peptides [29].

A comparison between salt concentrations required to elute the charged peptides from the CFT column is shown in Fig. 1. This feature of binding and elution definitely indicates some of the benefits regarding the use of positively charged peptides as tags for the purification of recombinant proteins by CFT chromatography [9]. It can be assumed that the composition of the mobile phase can be selected to restrict the retention of negative charged proteins as has been demonstrated with negatively charged peptides, and the proteins fused with positively charged peptides could be eluted specifically from the CFT column. This hypothesis shows the possible application of the most frequent phage-derived CFT-binding peptide F-4 (KPRSVSG) as tag.

3.3. Production of the fusion proteins

The eGFP constructs (Table 1) were expressed in *E. coli*, and the protein expressing cells were monitored by fluorescence microscopy after 8 h of IPTG induction. The presence of the designed

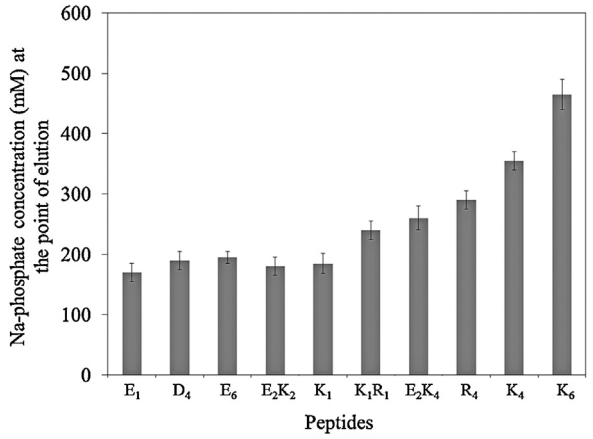

Fig. 1. Comparison between the compositions of the mobile phase required for the elution of the peptides. Buffer A was 1% of TBS and buffer B was 600 mM Na-phosphate. A gradient elution was performed with 0–100% buffer B (20 mL), and the experiments were run at pH 7.4 with a flow rate of 1 mL/min.

constructs in the supernatant was investigated with immobilized metal ion affinity chromatography (IMAC). The crude lysate and the elution fractions from the IMAC spin column were subjected to the SDS-PAGE analysis. The protein bands of H-eGFP, H-eGFP-Tag1 and H-eGFP-Tag2 corresponded to their approximate theoretical molecular weight (MW) of 28, 28.75 and 29.5 respectively. Fig. 2 represents the SDS-PAGE image of the crude extracts and the respective eluate fraction from IMAC.

3.4. Purification of H-eGFP-Tag1 by CFT chromatography

The retention of the designed constructs on the CFT surface was investigated through chromatographic experiments. A Tricorn column (5/50) was packed with CFT beads, and the experiments were run at a flow rate of 1 mL/min. Here, 20 mM Na-phosphate was used as the binding buffer (buffer A), and the experiments were performed at a pH ranging from 6 to 8.4. A four-fold diluted cell lysate (5 mL) was passed through the column, and the elution was performed with 1 M NaCl in the respective binding buffer. Construct H-eGFP-Tag1 was eluted with approximately 550 mM NaCl in 20 mM Na-phosphate at pH 6.5. The crude extract, flow through and eluate fractions were analyzed with SDS-PAGE, and this analysis shows that the construct was separated by the CFT column with high purity and yield. Fig. 3(A) represents the chromatographic profile and Fig. 3(B) represents the corresponding SDS-PAGE analysis for the purification of H-eGFP-Tag1 at pH 6.5. A sharp protein band corresponds to the approximate MW (28.75) of H-eGFP-Tag1

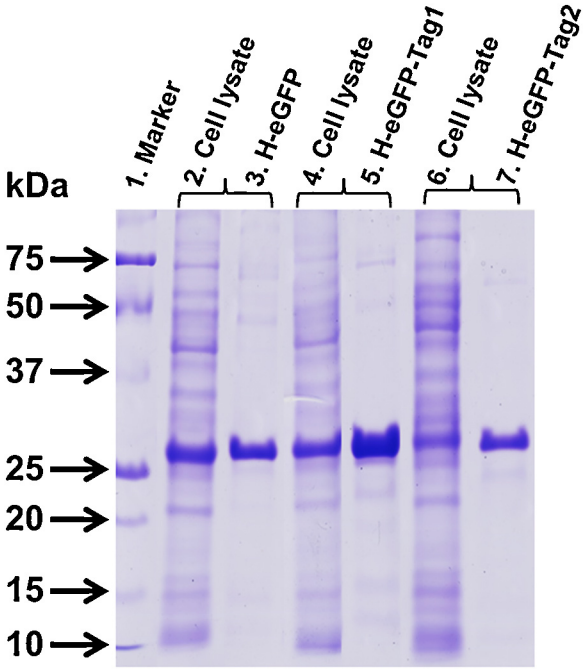

Fig. 2. SDS-PAGE analysis (Coomassie brilliant blue staining) of the cell lysate and the corresponding eluate fractions obtained from the IMAC spin column. Lanes: 1, molecular weight markers; 2, lysate of *E. coli* cells expressing H-eGFP; 3, eluate fraction containing H-eGFP; 4, lysate of the H-eGFP-Tag1 expressing *E. coli* cells; 5, eluate fraction containing H-eGFP-Tag1; 6, lysate of the H-eGFP-Tag2 expressing *E. coli* cells; 7, eluate fraction containing H-eGFP-Tag2.

is found in cell lysate (lane 2) and eluted fractions (lane 4). Whereas a very tiny band of H-eGFP-Tag1 is found in the flow through fraction (lane 3), and apparently no other significant protein bands are visible in lane 4 expect the band for H-eGFP-Tag1. This clearly indicates the high efficiency of the present approach that can separate a target protein with a purity of over 90%.

Table 4 represents the effect of pH on the retention of the constructs. The retention of H-eGFP-Tag1 decreased significantly when the experiments were performed at a pH beyond 7, and apparently, there was no retention at pH 7.4. In Section 3.2, it was observed that the electrostatic interactions play a key role behind the affinity of Tag1 toward CFT. Therefore, the pH dependent retention might

Table 4
Effect of pH on the retention of the constructs.

Binding buffer	pH	H-eGFP	H-eGFP-Tag1	H-eGFP-Tag2
20 mM sodium phosphate	6	– – –	+++	+++
	6.5	– – –	+++	+++
	7	– – –	+++	+++
	7.4	– – –	Negligible	+++
	8.4	– – –	– – –	+++

(+++) represents retained and (– – –) represents non-retained.

be due to an alteration of the net surface charge of CFT and H-eGFP-Tag1 [29]. It is certain that the positive charge density of the construct is lower than that of the original peptide. The lower surface charge density of H-eGFP-Tag1 might reduce the strength of the interactions between CFT and the construct at higher pH conditions. Therefore, the construct was washed out through the column instead of being adsorbed. This indicates that the successful implementation of this technique requires the optimization of chromatographic conditions.

3.5. Purification of H-eGFP-Tag2 by CFT chromatography

The retention behavior of the peptides shows that peptides composed of lysine residue have higher RTs than other peptides. Therefore, six lysine residues (K_6) were fused together with Tag1, KKKKKKKPRSVSG (Tag2), for conducting the purification at elevated pH. In this case, 20 or 50 mM Na-phosphate was used as the binding buffer (buffer A), a four-fold diluted cell lysate (5 mL) was passed through the column, and the elution was performed with 500 mM Na-phosphate or 1 M NaCl in the respective binding buffer. Fig. 4 represents the chromatographic profile for the purification of the H-eGFP-Tag2 at pH 7.4. Two major peaks were found in the chromatograms during elution, especially in the case of Na-phosphate elution. However, there was a sufficient distance between two peaks, indicating a difference in retention properties between the proteins corresponding to the first and second peaks. The presence of the construct H-eGFP-Tag2 in the second peak was confirmed through SDS-PAGE analysis, which is represented in Fig. 5. The construct was eluted from the CFT column with approximately 430 mM Na-phosphate or 850 mM NaCl at pH 7.4 when buffer A was 20 mM Na-phosphate. Approximately 420 mM Na-phosphate or 800 mM NaCl was required to elute the construct when buffer A was 50 mM Na-phosphate.

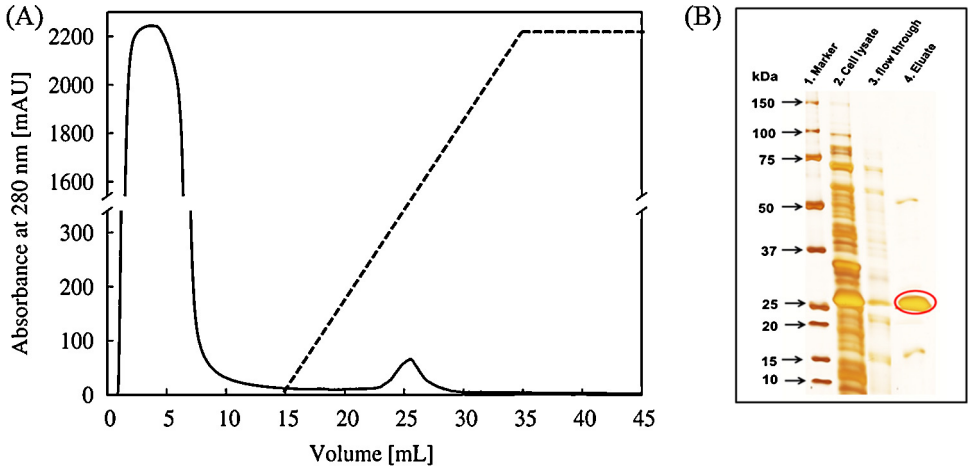

Fig. 3. (A) Chromatographic profile and (B) the corresponding SDS-PAGE results for the purification of construct H-eGFP-Tag1 by the CFT column at pH 6.5. A Tricorn column (5/50) was packed with the CFT beads, and the experiments were run at a flow rate of 1 mL/min. The binding buffer (buffer A) was 20 mM Na-phosphate and the elution buffer (buffer B) was 1 M NaCl in 20 mM Na-phosphate. After sample (5 mL) loading, the column was washed with buffer A (10 mL), and a gradient elution was performed with buffer B (20 mL).

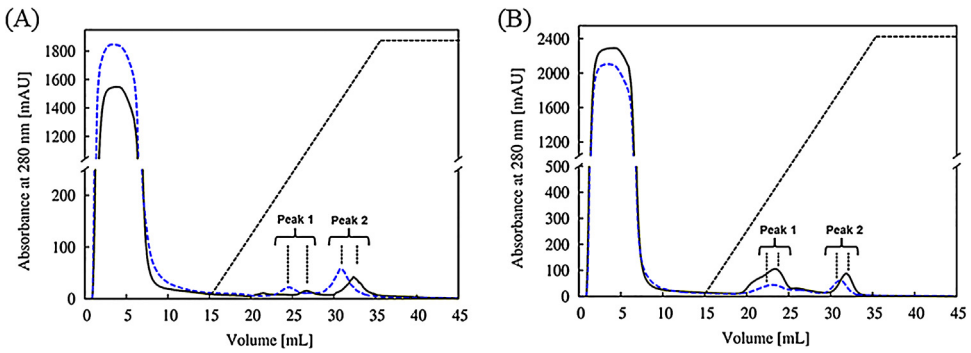

Fig. 4. Chromatograms obtained for the purification of H-eGFP-Tag2 at pH 7.4 when the elution buffer was (A) 1 M NaCl or (B) 0.5 M Na-phosphate in the respective binding buffers. (—) 20 and (—) 50 mM Na-phosphate were used as the binding buffers (buffer A). A Tricorn column (5/50) was packed with CFT beads, and the experiments were run at a flow rate of 1 mL/min. After sample loading (5 mL), the column was washed with buffer A (10 mL), and a gradient elution was performed with increasing concentration of NaCl or Na-phosphate (20 mL). After each run, the column was washed with elution buffer (10 mL).

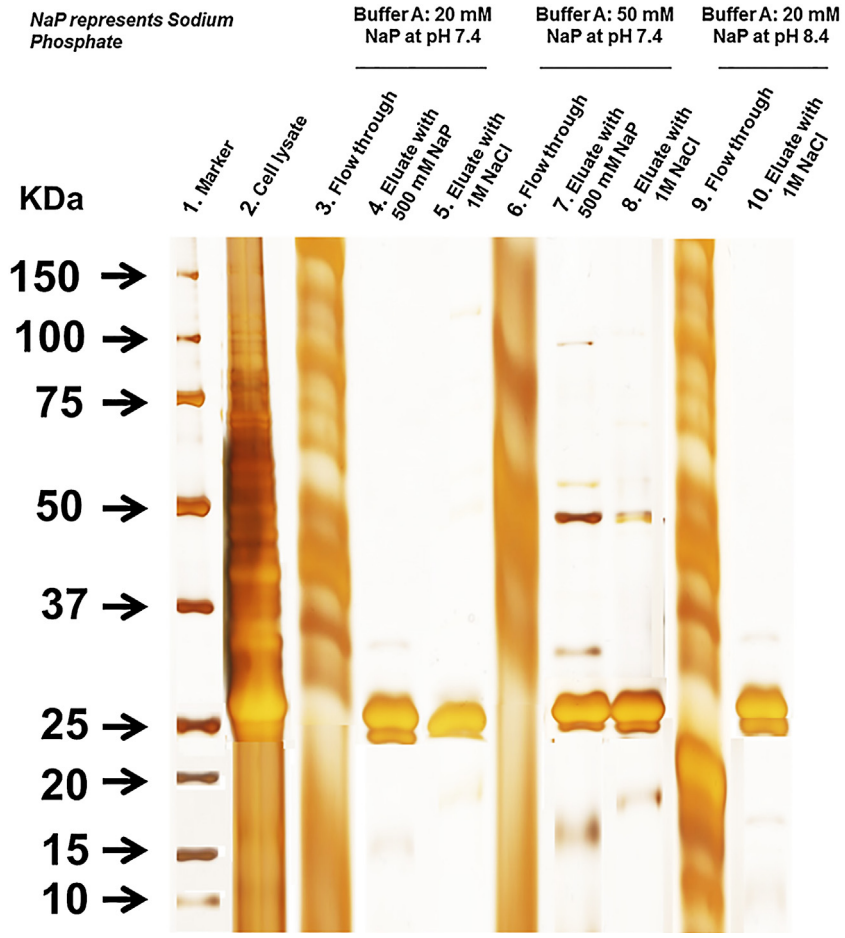

Fig. 5. SDS-PAGE analysis (silver staining) of the eluate fractions obtained from the CFT column for the purification of H-eGFP-Tag2 at pH 7.4 (Lane 1–8) and at pH 8.4 (Lane 9 and 10). Lanes: 1, molecular weight markers; 2, lysate of the H-eGFP-Tag2 expressing *E. coli* cells; 3, column flow through when the binding buffer (buffer A) was 20 mM Na-phosphate at pH 7.4; 4 and 5, eluate fractions when buffer B was 500 mM Na-phosphate or 1 M NaCl in the respective buffer A; 6, column flow through when buffer A was 50 mM Na-phosphate at pH 7.4; 7 and 8, eluate fraction when buffer A was 50 mM Na-phosphate at pH 7.4, and buffer B was 500 mM Na-phosphate or 1 M NaCl in the respective buffer A; 9, column flow through when the buffer A was 20 mM Na-phosphate at pH 8.4; 10, eluate fraction at pH 8.4 when buffer B was 1 M NaCl in respective buffer A.

These results show that increasing increments of Na-phosphate concentration from 20 to 50 mM in the binding buffer (buffer A) did not have much influence on the retention of H-eGFP-Tag2. However, SDS-PAGE analysis (Fig. 5) of the eluted fractions showed that a higher purity was obtained when buffer A was 20 mM Na-phosphate. It can be assumed that a slightly higher salt content in buffer A might have reduced the intra- or intermolecular interactions of some proteins [43,44]. Therefore, the functional groups on the surface of those proteins became available for interaction with the CFT, and thus the RTs of some of those proteins were close to the RT of the H-eGFP-Tag2.

In order to further broaden the investigation regarding interaction mechanisms; the chromatographic experiments were performed at higher pH conditions. Fig. 6 represents the

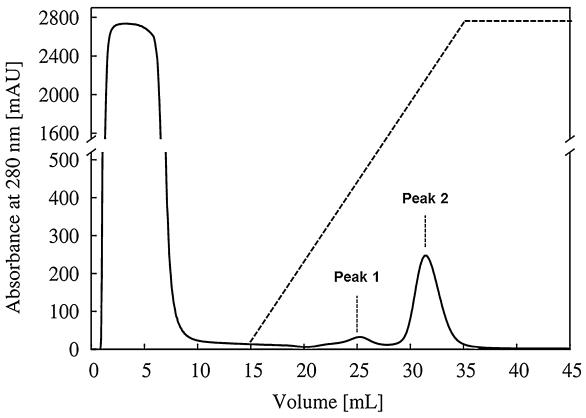

Fig. 6. Chromatograms obtained for the purification of H-eGFP-Tag2 at pH 8.4. In this case, the clarified lysate (non-diluted) was directly applied to the CFT column. A Tricorn column (5/50) was packed with the CFT beads, and the experiments were run at a flow rate of 1 mL/min. The binding buffer (buffer A) was 20 mM Na-phosphate and the elution buffer was 1 M NaCl in 20 mM Na-phosphate.

chromatogram when the experiments were run at pH 8.4. In this experiment, cell lysate (non-diluted) was directly subjected to the column. The construct H-eGFP-Tag2 was eluted with approximately 825 mM NaCl in 20 mM Na-phosphate. The elution fraction was subjected to SDS-PAGE analysis (Fig. 5), which confirmed a high degree of purification. Interestingly, there was no significant difference between the salt concentrations required to elute the construct at pH 7.4 and 8.4. Moreover, at both pH conditions, the SDS-PAGE analysis shows that both the purity and yield were above 90% when buffer A was 20 mM Na-phosphate. For the determination of yield more precisely, the ratio of the construct in flow-through fraction to the corresponding eluted fraction was measured by a fluorescent spectrophotometer (F-2500, Hitachi, Japan), which indicated an overall yield of 90–94%.

4. Discussion

Due to the complex physico-chemical properties of proteins and the possibility of denaturation, their purification is a challenging task [45]. The structure and biological activities of proteins can be altered at any step of the purification process, however an efficient method needs to yield high activity and recovery of the isolated proteins [46]. In this context, small peptide affinity-tag systems are capable of reaching the objective of maximizing purity and yield without effecting the tertiary structure and biological activity of most of the proteins [19,47]. The present work is focused on further development of this technique using ligand-less CFT stationary phase in this type of chromatography to overcome some of the traditional drawbacks such as leakage and inactivation of ligands, high expense, possible contamination of the final product by residual ligand coupling reagents, and the subsequent environmental burden [48,49]. As a first step to materialize the concepts proposed above, peptides with the ability to bind to CFT were discovered in our previous work [32]. Yet, the peptides that will be used as tags should have specific retention behavior [11,50]. Therefore, a peptide library with diversified physico-chemical properties has been synthesized here and subjected to CFT chromatography. The effects of net charge, isoelectric point and type of amino acid content of the peptides on their binding to CFT were evaluated. Overall, the retention behavior of the peptides indicates that the positively charged peptides having net charge +2 or more can be specifically eluted from the CFT column, which is important when considering them for use as tags for the purification of recombinant proteins. Moreover, the retention time of the consecutive lysine containing peptide was longer than other peptides. Therefore, a phage derived positively charged peptide F5-4, KPRSVSG, with or without a hexalysine sequence, KKKKKKKPRSVSG, were fused at the C-terminus of the eGFP.

The intense green color of the eluted fraction from the CFT column implies that both Tag1 and Tag2 sustained their CFA-binding ability without affecting the fluorescent properties of the fused protein. The retention of the construct H-eGFP-Tag1 on CFT decreased significantly when the experiments were run at pH above 7. However, the native Tag1 was retained by the CFT column at pH 7.4. On the other hand, at pH 6.5, interactions between H-eGFP-Tag1 and CFT surface were stronger than the interactions between the native Tag1 and the CFT surface [32]. This clearly indicates the partial positive charge contribution of the N-terminal histidine residues H_6 to the net charge of the construct at pH lower than 7.

Two major peaks were found in the chromatograms obtained for the construct H-eGFP-Tag2. The second peak corresponded to the construct; however the retention properties of the proteins of the first peak are not clear. Nevertheless, the relative area of the first peak decreased with the increase of Na-phosphate content or pH of the binding buffer. In the section 3.2, it was observed that 20 mM Na-phosphate, pH 7.4 was able to prevent interactions between CFT and six glutamic or aspartic acid containing peptides or the peptides bearing a net positive charge of +1. Therefore, it can be assumed that proteins corresponding to the first peak contain surface exposed positively charged or highly negatively charged binding sites or phosphate groups. In this case, the positively charged binding sites should bear a net charge of +2 or +3. However, the positively charged proteins or binding sites might contain acidic amino acids that can interact with the C-site (Ca^{+2}) of CFT. Therefore, the interactions between those proteins and CFT are expected to be of a mixed mode, and thus compared to NaCl elution, the first peak area was higher with the Na-phosphate elution at pH 7.4, as the phosphate ion is a much stronger eluent than the chloride ion to break the bonds between proteins and the C-site of CFT. It has been reported that the interactions between proteins and the C-site of apatite (AP) materials increase with the increment of pH of the mobile phase, while the interactions between proteins and the P-site (PO_4^{3-}) of AP materials decrease [29,31]. Therefore, it can be hypothesized that the biomolecules corresponding to the first peak are a mixture of positively and negatively charged proteins. The lower area of the first peak at pH 8.4 can be explained by the fact that the NaCl in the elution buffer was not able to elute the proteins that were strongly associated with the C-site of CFT under elevated pH conditions. On the other hand, at pH 6.5, the C-site interactions decreased and some of those proteins that interacted with the P-site of CFT might elute with construct H-eGFP-Tag1, so there were no separate peaks. This assumption can also be supported by a protein band near 50 kDa found in the SDS-PAGE analysis of the eluted fractions during the purification of H-eGFP-Tag1 (Fig. 3B).

In this work, an IMAC spin column was used to ensure the expression of the desired constructs. Still, it might be possible to compare the efficiency between IMAC and the present strategy to some degree. The SDS-PAGE analysis reports for the eluted fractions obtained from these two columns (Figs. 2 and 5) show that the efficiency of both the purification strategies is quite similar. However, as discussed, the limitations of the traditional IMAC systems [12], suggest that CFT chromatography could be better alternative to the traditional IMAC techniques. Nevertheless, further study is required for a detailed comparison between these two techniques.

5. Conclusions

To the best of our knowledge, this is the first study reporting CFT-binding peptides (CBPs) as tags for the purification of recombinant proteins. The eGFP gene constructs with or without CBPs, H-eGFP,

H-eGFP-Tag1 and H-eGFP-Tag2, were expressed successfully in *E. coli* strain BL-21. Expression of the constructs was confirmed by SDS-PAGE analysis followed by IMAC. Chromatographic experiments were performed to examine the efficiency of the CFT column for the purification of the fused proteins under dynamic conditions. The construct without a CFT-binding peptide, H-eGFP, was not retained by the CFT column under any of the applied experimental conditions. It is known that eGFP is a hydrophobic protein [51], and that the CFT surface possesses a high degree of hydrophilicity [52]. Therefore, the retention of H-eGFP on a CFT surface was not expected. In the case of constructs H-eGFP-Tag1 and H-eGFP-Tag2, the color of the eluate fractions primarily indicated the retention, while SDS-PAGE analysis of the chromatographic fractions showed that the column was able to separate the fused proteins H-eGFP-Tag1 and H-eGFP-Tag2 directly from the crude extracts with high purity and yield. It is significant that both the constructs, H-eGFP-Tag1 and H-eGFP-Tag2, were bound to CFT directly from the crude extract, demonstrating that the fused peptide tags sustained their CFT-binding ability without influencing the fluorescent properties of their fused partner. Overall, both the tags were able to enhance the adsorption and selective elution of the fusion protein. The chromatographic experiments showed that the design of an optimum tag and process optimization are both important when applying this technique for the separation of various proteins from biological mixtures. It can be concluded here that the use of a CFT matrix in a fusion-tag system can exist as an alternative approach for the purification of recombinant proteins, which could overcome many of the traditional drawbacks.

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