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Strategy for highly selective ion-exchange capture using a charge-polarizing fusion partner

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Abstract

To achieve efficient recovery of recombinantly produced target proteins using cation-exchange chromatography, a novel basic protein domain is used as a purification handle. The proteolytic instability usually encountered for basic peptide tags is avoided by the use of a highly constrained α-helical domain based on staphylococcal protein A into which positively charged amino acids have been introduced. Here we show that this domain, consisting of 58 amino acids with a calculated isoelectric point (p*I*) of 10.5, can be used to efficiently capture different fused target proteins, such as a bacterial DNA polymerase (Klenow fragment), a viral protease (3C) and a fungal lipase (Cutinase). In contrast to standard cation-exchange chromatography, efficient capture can be achieved also at a pH value higher than the p*I* of the fusion protein, demonstrated here by Z_{basic}-Klenow polymerase (p*I*≈5.8) and ZZ-Cutinase-Z_{basic} (p*I*≈7.2) both purified at a pH of 7.5. These results show that the Z_{basic} domain is able to confer a regional concentration of positive charge on the fusion protein even at a relatively high pH. Hence, the data suggest that this domain could be used for highly efficient and selective capture of target proteins at conditions where most host-cell proteins do not bind to the chromatographic resin. The obtained purity after this one-step procedure suggests that the strategy could be an alternative to standard affinity chromatography. Methods for site-specific proteolysis of the fusion proteins to release native target proteins are also discussed.

Keywords: Ion-exchange chromatography; Z_{basic} protein domain; Proteins

1. Introduction

Strategies leading to efficient recovery of recombinant proteins are of importance to facilitate the often time consuming steps of protein purification. One of the main issues is to increase the selectivity of the purification steps in order to reduce the number of unit operations. By making small changes in the target protein, the behavior in the down-stream process can be improved. This has for example been utilized by Abrahmsén et al. who purified a subtilisin variant with an active site Cys-mutation on thiol-Sepharose [1]. However, in many cases it is of importance that the target protein is kept in its native form. In these cases a protein fusion strategy can be employed, where the target protein is expressed with a peptide or protein domain that allows for efficient down-stream processing [2]. Several protein fusion

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strategies involving a purification handle with a specific ligand binding functionality have been developed [3]. These include affinity purification systems such as protein A fusion proteins for IgG column chromatography [4] and immobilized metal affinity chromatography (IMAC) systems where hexahistidyl-tagged proteins are captured on metal charged chelator resins [5]. These systems are typically highly efficient, but for use in industrial size processes the high cost of most of the resins needs to be considered. An additional disadvantage associated with protein based affinity ligands, is that they normally are sensitive to the high pH, often employed in industrial cleaning-in-place (CIP) protocols [6,7], although a recent report suggests that this can be circumvented by protein engineering [8]. In industrial processes alternative chromatographic methods such as ion-exchange chromatography (IEC) are often preferred. The resins used in IEC are much more adapted to large-scale processes since their performance is reproducible over many cycles; protocols for scaling are available and most importantly; the resins can be sanitized without loss of capacity. IEC has become a frequently used method for protein purification, because it has the potential of giving high resolution separation of the loaded proteins and also high recovery of the target protein. However, the performance in a down-stream process is dependent on both the particular target protein and the complexity of the feed-stream. Therefore, extensive optimization is required to find suitable conditions that give the best performance.

Several successful strategies to improve IEC purification based on fusions to charged tags have been developed [9,10]. Also, an interesting strategy was developed by Niederauer et al. [11] who described the use of a genetically fused poly(Arg) and poly-(Asp) tails to the C-terminus of β-galactosidase to achieve an enhanced enrichment in polyelectrolyte precipitation. However, it was necessary to produce the arginine tailed fusion proteins in protease deficient ($OmpT^-$) cells to avoid degradation of the charged polypeptide extension [2].

In this study, we describe the utilization of a highly charged α-helical protein domain, Z_{basic} [12], as a general purification handle to improve ion-exchange chromatographic purification. Purification parameters were first optimized for the recovery of Z_{basic} fused to a bacterial DNA polymerase (Klenow[1]). Using the same parameters, we were able to verify the generality in the procedure by purifying two other target proteins [a viral protease (3C) and a fungal lipase (Cutinase)] fused to Z_{basic}. All three fusion proteins could successfully be captured and purified to homogeneity by a single unit operation.

2. Experimental

2.1. General

Synthetic oligonucleotides were purchased from Interactiva (Ulm, Germany). Gene constructs were verified using solid-phase DNA sequencing [14]. DNA restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). All ion-exchange chromatographic experiments were performed at room temperature, using an ÄKTA Explorer 100 fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden). Isoelectric points (p*I* values) were calculated at: http://www.expasy.ch/tools/protparam.html.

2.2. Strains

Escherichia coli strain RRIΔM15 [15] was used as a host for all gene construction experiments and expression of ZZ-Cutinase-Z_{basic} fusion protein. *E. coli* K-12 strain W3110 [16] was used for protein production for all constructs including Klenow polymerase. *E. coli* strain Bl21(DE3) (Novagen, Madison, WI, USA) was used as a host for production of Z_{basic}-3C fusion protein.

2.3. Construction of expression vectors

All recombinant DNA technology followed standard procedures [17].

2.3.1. Z_{basic}-Klenow polymerase

The gene encoding Z_{basic} was polymerase chain reaction (PCR) amplified from pTrp-Z_{basic2} [12]

[1]The Klenow variant used in this paper lacks 3′→5′ exonuclease activity [13].

using the primers SOHO-2 (5′-GAATGCGCAAC-ACGATGAAGC-3′) and SOHO-3 (5′-TGCTGA-ATTCGAGCTTTTCGGCGC-3′). This introduced an upstream *Fsp*I restriction endonuclease site and a downstream *Eco*RI restriction endonuclease site of the gene encoding Z_{basic}. The PCR product was ligated to pGEM-5zf(+) (Promega, Madison, WI, USA) according to the manufacturer's recommendations. After gene verification the pGEM-construct was restricted with *Fsp*I and *Eco*RI and the gene encoding Z_{basic} was isolated. Thereafter a ligation with pRIT-45 [18] digested with the same enzymes was performed. The resulting construct was labeled pRIT-45-Zb. The original pRIT-45 encodes Z_{wt} followed by Klenow polymerase under control of the trp-promoter.

2.3.2. Z_{basic}-3C

Construction of the vector encoding the Z_{basic}-3C fusion protein was performed by PCR amplification of Z_{basic} gene from pKN1-Z_{basic2} [12]. The PCR product was digested with *Bam*HI and *Eco*RI and ligated with pET24a(+) that had been digested with the same enzymes yielding pET24a(+)-Zb. The gene encoding 3C was recovered from the plasmid pUC19-3C [19] by digestion with *Eco*RI and *Hind*-III. The gene was subsequently ligated with pET24a(+)-Zb, digested with the same enzymes. The resulting plasmid was labeled pT7Zb-3C.

2.3.3. ZZ-Cutinase-Z_{basic}

Construction of the production vector for the ZZ-Cutinase-Z_{basic} fusion protein was performed by subcloning the gene encoding Z_{basic} by PCR to the vector pEZZ-Cutinase [20] using the *Sal*I and *Pst*I restriction endonucleases. The DNA sequence of the resulting gene construct was verified and the vector was labeled pEZZ-Cutinase-Zb.

2.4. Production of proteins

2.4.1. Constructs containing Klenow polymerase

A colony containing pRIT-45-Zb or pRIT-45 was grown overnight in a shake flask at 30°C for pRIT-45-Zb or 37°C for pRIT-45 in 1xMJ minimal media [21] supplemented with 5 g/l yeast extract (Merck, Darmstadt, Germany) and 100 mg/l ampicillin. On the following morning, cultivations were inoculated with 5 ml of the overnight culture to 500 ml fresh media in shake flasks. Protein was produced for 20 h at 37°C for Z_{wt}-Klenow fusion protein and for 24 h at 30°C for Z_{basic}-Klenow fusion protein. After protein production, the cultivations were divided into batches containing 220 ml. The batches were harvested by centrifugation at 4000 *g* for 10 min and the resulting pellets were each resuspended in 25 ml water and frozen.

2.4.2. Z_{basic}-3C

An overnight shake flask culture harboring pT7Zb-3C was grown at 30°C in TSB+YE media [30 g/l tryptic soy broth (Lab M, Topley House); 5 g/l yeast extract] supplemented by 50 μg/l kanamycin. On the following morning 500 ml fresh TSB+YE was inoculated with 5 ml of the overnight culture and the cells were grown until A_{600} reached 1.0. Protein production was induced by adding 240 mg/l isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation after 4 h of production and the resulting pellet was resuspended in 25 ml 50 m*M* sodium phosphate, pH 7.5 and frozen.

2.4.3. ZZ-Cutinase-Z_{basic}

E. coli containing pEZZ-cutinase-Zb was grown in a shake flask overnight in TSB +YE media supplemented with 100 mg/l ampicillin at 30°C. On the following morning 500 ml fresh media was inoculated with 5 ml overnight culture and the cells were grown for 24 h. The cells were harvested by centrifugation. The periplasmic protein fraction was released by osmotic shock [22] and frozen until further analysis.

2.5. Influence of pH on purification of Z_{wt}-Klenow polymerase

Cells corresponding to 15 ml culture were thawed on ice and disrupted by sonication. Insoluble material was removed by centrifugation at 15 000 *g* for 15 min at 4°C, followed by filtration through a 0.45-μm filter. In order to adjust the pH, concentrated running buffer was added to the cleared lysate giving a final concentration of: 20 m*M* etanolamin, pH 9.0; 20 m*M* Tris–HCl, pH 8.0; 20 m*M* bis–Tris–propane, pH 7.0; 20 m*M* bis–Tris, pH 6.0 or 20 m*M* piperazine, pH 5.0. The lysate was loaded on a 1-ml Resource Q

(Amersham Pharmacia Biotech) anion-exchange column previously equilibrated with 10 column volumes (CVs) (10 ml) running buffer. After the loading step the column was washed with 10 CVs (10 ml) running buffer. The bound proteins were eluted using a linear gradient of NaCl from 0 to 1 *M* over 20 CVs (20 ml). The flow was 2 ml/min.

2.6. Cation-exchange chromatography of Z_{basic}-Klenow polymerase

Cells corresponding to a 220 ml culture were thawed on ice and sonicated. Insoluble material was removed by centrifugation at 15 000 *g* for 15 min at 4°C, followed by filtration of the cleared lysate through a 0.45-μm filter. The pH was adjusted by adding: sodium phosphate, pH 7.5, to a final concentration of 50 m*M*; bicine, pH 8.5, to a final concentration of 50 m*M* or a universal AIX 5–9.5 buffer, pH 9.5, containing 50 m*M* 1-methyl piperazine, 50 m*M* bis–Tris and 25 m*M* Tris. An XK16 column containing 24 ml S-Sepharose FF (Amersham Pharmacia Biotech) was equilibrated with 2 CVs of the corresponding buffer, after which the cleared cell lysate was loaded at a linear flow-rate of 60 cm/h. After loading, the column was washed at a linear flow-rate of 180 cm/h with 5 CVs of running buffer followed of elution of the bound proteins, also at a linear flow-rate of 180 cm/h, by a 20 CV linear gradient of NaCl from 0 to 1 *M*. The amount of protein purified was measured using the Bradford protein determination method [23].

2.7. Anion-exchange chromatography of Z_{wt}-Klenow polymerase

Cells corresponding to a 220 ml culture were thawed on ice and subsequently sonicated. Insoluble material was removed by centrifugation at 15 000 *g* for 15 min at 4°C and the cleared lysate was passed through a 0.45-μm filter. The pH was adjusted by addition of bis–Tris–propane, to the filtrate to pH 7.0, giving a final volume of 26 ml and concentration of 20 m*M*. The filtrate was loaded at a linear flow-rate of 60 cm/h onto an XK16 column containing 27 ml Q-Sepharose FF resin (Amersham Pharmacia Biotech) that had been equilibrated at pH 7.0 by 2 CVs of running buffer. After loading, washing was performed by 5 CVs of running buffer. The absorbed material was eluted using a 35 CV NaCl gradient from 0 to 0.5 *M*. Washing and elution was done at a linear flow-rate of 150 cm/h. The amount of protein recovered was measured using the Bradford protein determination method [23].

2.8. Enzymatic activity analysis of Z_{basic}-Klenow polymerase

Fractions containing purified protein were analyzed for Klenow polymerase activity by pyrosequencing [24] on an automated pyrosequencer. Single-stranded DNA with annealed sequence primer was added to the pyrosequencing reaction mixture as described [25]. 10 U (~5.6 μg) of commercial Klenow polymerase (Pyrosequencing, Uppsala, Sweden) was added to the reference wells and the same amount in μg of Z_{basic}-Klenow polymerase was used in sample wells. Stepwise elongation of the primer strand upon sequential addition of the deoxynucleoside triphosphates was carried out.

2.9. Isoelectric focusing (IEF) of Klenow fusion proteins

The isoelectric points were determined using a Model 111 mini IEF cell (Bio-Rad Labs., Upplands Vasby, Sweden). The analysis was done according to the supplier's recommendation. All materials, except for the ampholytes, was from Bio-Rad. Ampholine, pH 3.5–10, was supplied by Amersham Pharmacia Biotech.

2.10. Purification and enzymatic analysis of Z_{basic}-3C

Cells containing Z_{basic}-3C were disrupted by sonication and insoluble material was removed by centrifugation (15 000 *g*) followed by filtration through a 0.45-μm filter. The pH of the cleared lysate was adjusted to 7.5 by addition of sodium phosphate buffer to a final concentration of 50 m*M*. The conductivity was adjusted to 20 mS/cm by the addition of NaCl. The cleared lysate was loaded onto the previously used 24-ml S-Sepharose FF cation exchanger that had been equilibrated with running buffer (50 m*M* sodium phosphate, pH 7.5) at a

flow-rate of 60 cm/h. The column was extensively washed after which bound proteins were eluted using a 20 CV linear gradient of NaCl from 0 to 1 *M*.

2.10.1. Enzymatic activity analysis

Fractions containing protein were analyzed for 3C activity by incubation with ABPII-Δtaq as a substrate protein. ABPII-Δtaq contains the 3C-cleavage sequence EALFQ/GP between the ABP and Δtaq domains and its production and purification has been described elsewhere [19]. Z_{basic}-3C was incubated in a 1:1 molar ratio at 25°C for 12 h with the substrate.

2.11. Purification and enzymatic analysis of ZZ-cutinase-Z_{basic}

The periplasmic protein fraction after osmotic shock was filtered through a 0.45-μm filter and sodium phosphate buffer was added to a final concentration of 50 m*M*, pH 7.5. The conductivity was adjusted to 20 mS/cm by the addition of NaCl. Thereafter the cleared lysate was loaded onto the previously used 24-ml S-Sepharose FF that had been equilibrated with running buffer (50 m*M* sodium phosphate, pH 7.5) at a flow-rate of 60 cm/h. The column was extensively washed after which bound proteins were eluted using a 20 CV linear NaCl-gradient from 0 to 1 *M*.

2.11.1. Enzymatic activity analysis

Fractions containing protein were analyzed for cutinase activity by incubation with *para*-nitrophenyl buturate (pNPB) as described earlier [20]. The hydrolysis of pNPB was monitored photometrically at 405 nm.

3. Results

We have earlier reported on the construction and initial characterization of a strongly charged protein domain, denoted Z_{basic} [12]. It was constructed by the introduction of charged amino acids into a constrained scaffold structure, using rational design. The scaffold chosen was one of the three-helix bundle domains that constitute staphylococcal protein A [26]. This domain has earlier been proven to be poorly recognized by *E. coli* endogenous proteases, most likely due to its constrained α-helical nature. In this paper we have evaluated the positively charged Z_{basic} purification handle for use in ion-exchange chromatography purification of fused target proteins.

3.1. Production and purification of Klenow polymerase

In order to analyze the behavior of Z_{basic} fusion proteins in cation-exchange chromatography, a fusion protein consisting of Z_{basic} and the Klenow fragment of an exonuclease deficient (exo^-) variant of *E. coli* DNA-polymerase I (Z_{basic}-Klenow) was produced as an intracellular protein in *E. coli*. Also, to be able to evaluate the influence of the engineered Z_{basic} domain in this fusion protein, a control protein consisting of the parental Z domain fused to Klenow polymerase (Z_{wt}-Klenow) was produced and analyzed in parallel. The solubility and adsorption characteristics of Z_{wt}-Klenow fusion protein were initially investigated by performing an anion-exchange pH-scouting experiment in which cleared cell lysate aliquots were fractionated at different pH. The fusion protein was most efficiently recovered when a running pH of 7 was used. The p*I* of the fusion protein was determined to be 5.4 (Table 1). When

Table 1
Characterization of the Klenow fusion proteins

Fusion protein	M_r (g/mol)	Measured p*I*	Calculated p*I*[a]	Amount (mg/l)[b]	Yield (%)[c]
Z_{wt}-Klenow	76 893	5.4	5.5	60	90
Z_{basic}-Klenow	77 051	5.8	6.2	60	99

[a] p*I* values were calculated at: http://www.expasy.ch/tools/protparam.html.
[b] Amount of fusion protein recovered after ion-exchange chromatography.
[c] Percent recovered of produced material.

performing anion-exchange chromatography at a running pH lower than 7, some of the fusion protein was collected in the flow-through. Using a running buffer with a pH below 6 or above 8.5 also resulted in a partial loss of the target protein, most likely due to precipitation (data not shown). This initial pH scouting experiment led to the conclusion Z_{wt}-Klenow fusion protein is soluble between pH 6 and 8.5 and also that IEC is most efficient when the pH of the running buffers is close to 7. This is in accordance with earlier described purification protocols of native Klenow polymerase [27].

Thereafter we investigated the influence of pH and conductivity, on a cation-exchange chromatographic procedure of Z_{basic}-Klenow fusion protein. The influence of pH on the yield of the fusion protein is shown in Fig. 1A. Purifications performed at pH 7.5 and 8.5 allowed the isolation of high amounts of essentially pure Z_{basic}-Klenow fusion protein. Interestingly, the p*I*-value of the Z_{basic}-Klenow fusion protein was determined to be 5.8 (Table 1), which is several units lower than the pH of the running buffer. Purification at pH 9.5 resulted in lower recovery of the protein, probably due to precipitation, which is consistent with the results obtained for Z_{wt}-Klenow fusion protein at high pH (data not shown). An interesting observation was that for all three pH values investigated the fusion protein was eluted at a conductivity of approximately 40 mS/cm.

The influence of the conductivity in the loading and washing steps was investigated by the addition of NaCl in the buffers and *E. coli* lysate while performing the purification. In order to maintain high activity of the Klenow polymerase a running pH of 7.5 was used. The results obtained (Fig. 1B) showed that the product was quantitatively recovered when using a conductivity of 8 or 20 mS/cm. At 34 mS/cm the recovered amount of product decreased to 80% and at 47 mS/cm no significant binding to the cation-exchange media could be observed. This led to the conclusion that a running pH of 7.5 and a conductivity of 20 mS/cm were conditions, very stringent and also gentle, that could be used for a quantitative recovery of the fusion protein on the cation-exchange resin used. An example of a chromatogram for the purification of the Z_{basic}-Klenow fusion protein employing the optimized parameters is shown in Fig. 2A. The Z_{basic}-Klenow fusion protein

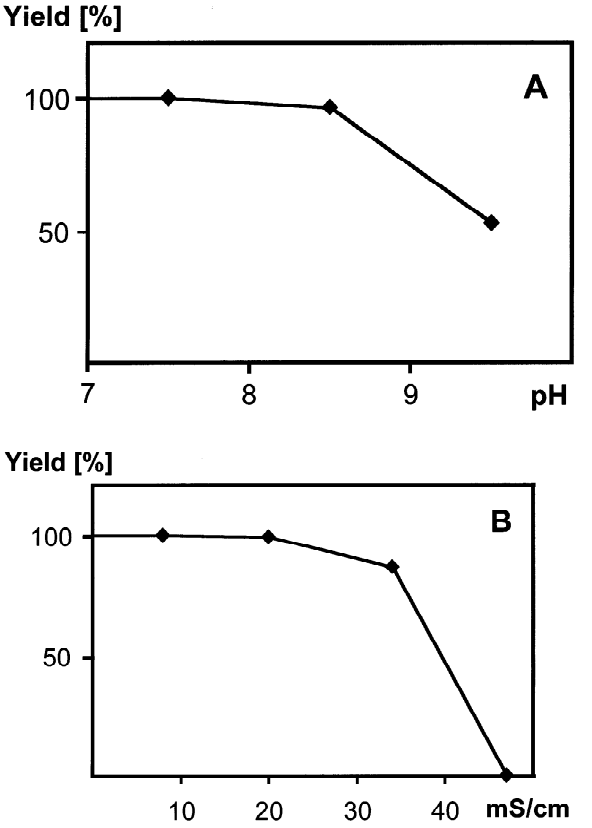

Fig. 1. (A) Influence of pH on the recovery of Z_{basic}-Klenow polymerase. (B) Influence of conductivity during the purification on the amount of Z_{basic}-Klenow polymerase recovered. The conductivity was controlled both in the loading and washing steps by addition of NaCl.

was eluted at a conductivity of about 40 mS/cm and fractions containing the desired product were pooled. The amount purified protein was 60 mg/l cell culture. For comparison the Z_{wt}-Klenow fusion protein was purified on an anion exchanger also at a pH of 7.5 and the resulting chromatogram is shown in Fig. 2B. This fusion protein was found to be eluted at 20 mS/cm and the amount of protein recovered was 60 mg/l cell culture. For both purifications the cleared lysate, flow-through and the pooled fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 3A and B) and agarose gel electrophoresis (data not shown). From a visual examination of the gels (SDS–PAGE for protein and agarose

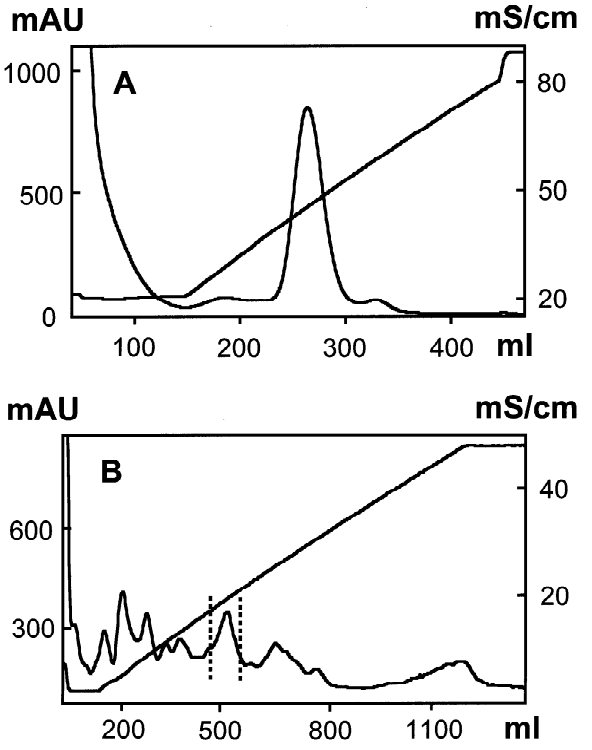

Fig. 2. (A) Cation-exchange purification of Z_{basic}-Klenow polymerase at pH 7.5. Fractions corresponding to the main peak were pooled and analyzed. (B) Anion-exchange purification of Z_{wt}-Klenow polymerase at pH 7.0. Fraction between dashed lines were collected and analyzed.

for DNA) and the chromatograms it could be concluded that Z_{basic}-Klenow fusion protein was >95% pure. For the Z_{wt}-Klenow fusion protein we were also able to achieve a rather high purity of about 80%, due to the very shallow gradient used. However, the pooled material contained higher amounts of contaminating DNA compared to the purified Z_{basic}-Klenow fusion protein. The amount protein lost in the flow-through fractions was also investigated by Western blotting using a protein A specific antibody (Table 1). This showed that about 99% of the Z_{basic}-Klenow fusion protein had been adsorbed to the column and recovered in the pooled fractions. The same analysis for the Z_{wt}-Klenow fusion protein showed that for this protein the recovery was approximately 90%.

The activity of the Z_{basic}-Klenow fusion protein was investigated in DNA sequencing of a model sample using a sequencing-by-synthesis method called pyrosequencing [24,28]. The results obtained from sequencing with Z_{basic}-Klenow polymerase and a commercially available Klenow (exo$^-$) DNA polymerase were virtually identical (Fig. 4).

3.2. Production and purification of other Z_{basic} fusion proteins

In order to evaluate the generality of the Z_{basic}-domain as a purification handle, fusion proteins consisting of Z_{basic} and a viral protease (Z_{basic}-3C, calculated pI=9.7) or a fungal lipase (ZZ-cutinase-Z_{basic}, calculated pI=7.2) were also expressed and purified by cation-exchange chromatography. The Z_{basic}-3C fusion protein was produced intracellularly in *E. coli*. The resulting cleared lysate was adjusted to pH 7.5 and a conductivity of 20 mS/cm corresponding to conditions previously optimized for the purification of the Z_{basic}-Klenow fusion protein. The Z_{basic}-3C fusion protein was found to be eluted at about the same conductivity as earlier shown for the Z_{basic}-Klenow fusion protein. Proteins eluted from the IEC column were pooled and analyzed by SDS–PAGE together with the starting lysate and flow-through fractions (Fig. 5). The pooled fractions were shown to contain a single protein band of the expected molecular size. In addition, the enzyme was shown to display 3C-protease activity.

In the ZZ-cutinase-Z_{basic} fusion protein the Z_{basic} domain was positioned in the C-terminus. The accumulation of the fusion protein was directed to the periplasmic space of the *E. coli* host. After protein production, the periplasmic fraction was released by osmotic shock and subjected to cation-exchange chromatography, again employing the previously optimized parameters of pH 7.5 and a conductivity of 20 mS/cm. This fusion protein was also eluted at a conductivity of about 40 mS/cm. Samples from the applied periplasmic fraction, the eluted proteins and the flow-through were analyzed by SDS–PAGE (Fig. 5). The eluted material contained a single protein of the expected molecular mass. When assaying for hydrolysis of *para*-nitrophenyl buturate the protein displayed cutinase activity.

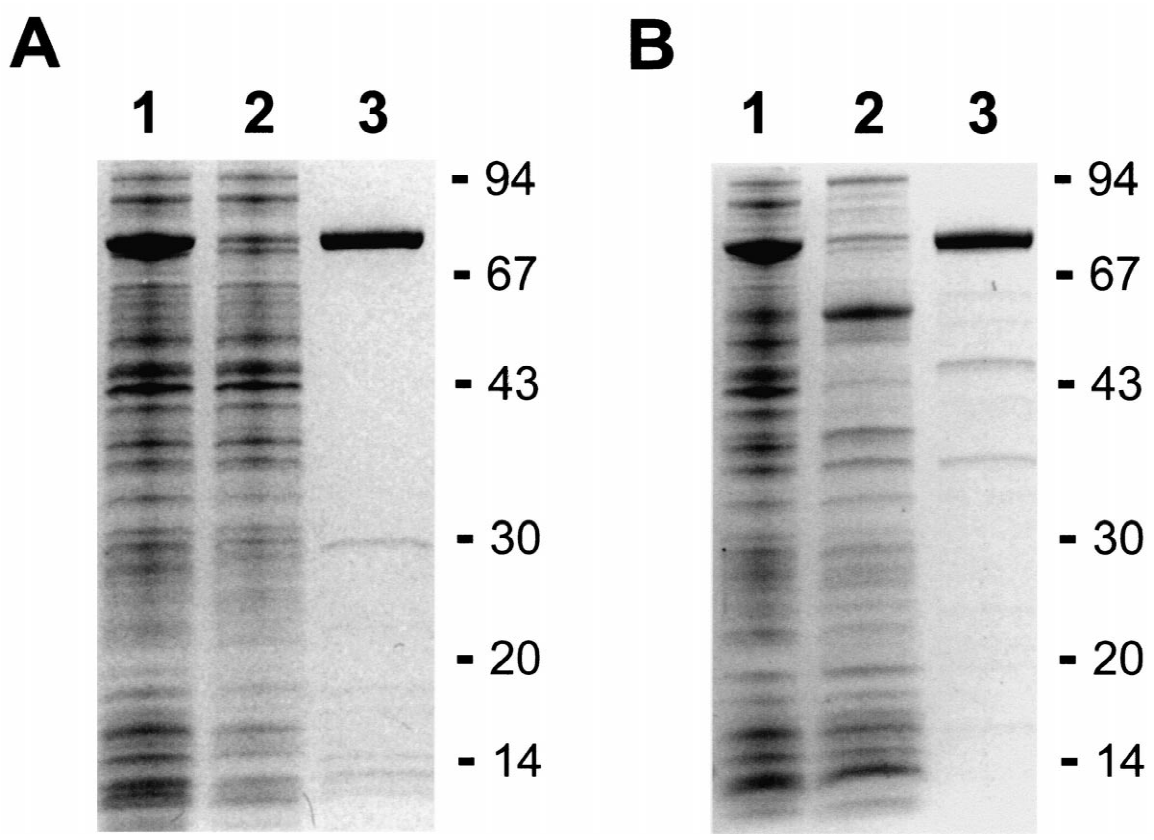

Fig. 3. (A) SDS–PAGE analysis of the purification of Z_{basic}-Klenow polymerase. Lanes: 1=lysate after sonication and clarification; 2=collected flow-through during loading; 3=pooled main peak fractions. Numbers of the right hand side correspond to molecular masses $\times 10^{-3}$. (B) SDS–PAGE analysis of the purification of Z_{wt}-Klenow polymerase. Lanes: 1=clarified lysate after sonication; 2=collected flow-through during loading; 3=pooled peak fractions. Numbers on the right hand side correspond to molecular masses $\times 10^{-3}$.

4. Discussion

In this work we have investigated the highly charged protein domain Z_{basic}, for use as a purification handle in IEC. Three different target proteins were expressed as fusions to Z_{basic} and the chromatographic behavior of the fusion proteins were investigated. To ascertain the properties of Z_{basic} fusion proteins in cation-exchange chromatography and to be able to specify a general set of parameters in the purification procedure, Klenow polymerase was chosen as the first model target protein. Purification of Klenow polymerase by IEC poses a challenge as the polymerase is only soluble at pH values ranging from 6 to 8.5 and has a p*I* value below 6. Therefore, anion-exchange chromatography is required which is not well suited for the purification as it also binds nucleic acids, leading to its co-purification. In order to avoid this, we have used cation-exchange purification of Z_{basic}-Klenow fusion protein. During the optimization of the purification of the Z_{basic}-Klenow fusion protein it was found that a running pH of 7.5 and also a rather high conductivity of 20 mS/cm resulted in an efficient adsorption of the protein to the cation-exchange column and allowed for effective washing without loss of material. This resulted in a highly purified fusion protein in a single-step procedure analogous to what is normally achieved using affinity chromatography. The results obtained during the pH optimization suggest that Z_{basic} fusion proteins have the potential to be purified at even higher pH values (Fig. 1A). However, to maintain a

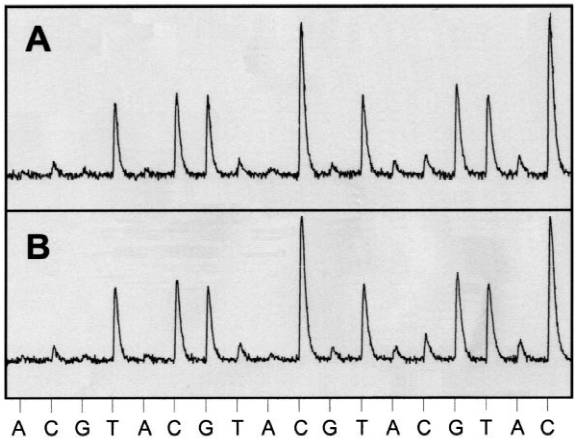

Fig. 4. (A) Pyrogram of the SNP analysis using commercially available Klenow polymerase. (B) Pyrogram of the single nucleotide polymorphism (SNP) analysis using Z_{basic}-Klenow polymerase. The Z_{basic}-Klenow fusion protein gives the same reading length and signal-to-noise ratio as commercially available Klenow polymerase. It is also able to stable read through several bases of the same type such as GG with the same result as the commercially available polymerase.

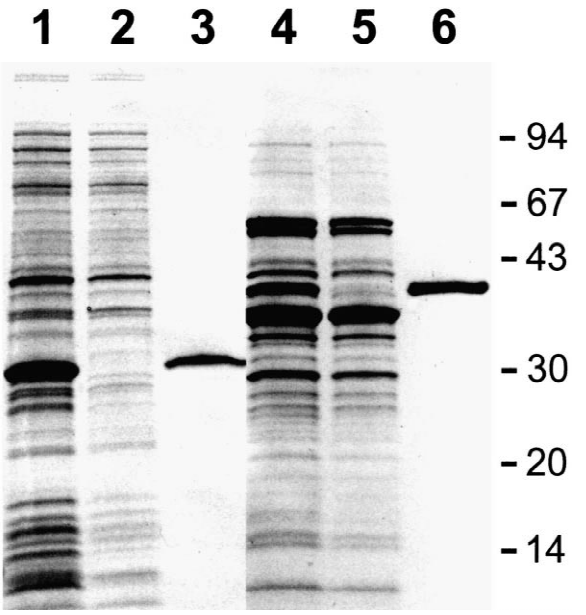

Fig. 5. SDS–PAGE analysis of samples taken during the purification of Z_{basic}-3C and ZZ-cutinase-Z_{basic}. Lanes: 1=cleared lysate after sonication of Z_{basic}-3C; 2=collected flow-through during loading of Z_{basic}-3C; 3=pooled main peak fractions eluted during purification of Z_{basic}-3C; 4=cleared lysate after osmotic shock of ZZ-cutinase-Z_{basic}; 5=collected flow-though during loading of ZZ-cutinase-Z_{basic}; 6=pooled main peak fractions eluted during purification of ZZ-cutinase-Z_{basic}. Numbers on the right hand side correspond to molecular masses $\times 10^{-3}$.

high specific activity it was important not to expose the target proteins to unnecessarily high pH values.

Interestingly, the p*I* value of the complete Z_{basic}-Klenow fusion protein was determined to 5.8, which is significantly lower than the pH used during the cation-exchange purification, a purification procedure normally run at pH values below the isoelectric point. This suggests that the extremely charged Z_{basic} moiety is able to confer a local positive charge concentration in the fusion protein. This observation is further supported by the fact that the fusion protein also can be adsorbed to an anion exchanger at the same pH although in smaller quantities (data not shown).

In parallel, a fusion between the parental Z domain (Z_{wt}) and Klenow polymerase was expressed and purified. This fusion protein was found to be expressed in similar levels as the Z_{basic}-Klenow fusion protein indicating that the Z_{basic} domain does not negatively affect the expression levels despite numerous arginines closely spaced in the primary sequence.

Interestingly, the strongly charged Z_{basic} domain was found not to affect the activity of the fused Klenow polymerase (Fig. 4). This suggests that the Z_{basic} domain does not interact with the fusion partner to any great extent.

This notion is further supported by the successful purification of two other target proteins using the same strategy. The same set of optimized parameters used for purification of Z_{basic}-Klenow fusion protein was also used to purify Z_{basic}-3C and ZZ-cutinase-Z_{basic}. This suggests that it should be possible to device a set of universal parameters with regards to conductivity and pH of the feed-stream for the purification of a wide range of Z_{basic} fused target proteins.

The handle could be expressed both as an N-terminal and C-terminal fusion to the target protein. Furthermore, the strategy works for intracellular as well as secreted production of the target protein. All enzymes showed enzymatic activity suggesting that the charged purification handle does not greatly affect the target protein. However, for certain appli-

cations a native target protein, devoid of additional amino acids, can be required. The system could then be designed with an enzymatic recognition sequence between the target protein and the Z_{basic}-purification handle. This would enable site specific proteolysis of the fusion protein and isolation of the native target protein. Several proteases has been described for site-specific proteolysis including Factor X_a and H64A subtilisin BPN′ [29].

Taken together these results show upon the power of using a purification handle genetically fused to the target protein for facilitated purification. Here we have shown that it is possible to improve the selectivity of IEC by the use of a highly charged fusion partner.

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