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Enzymatic cleavage of fusion proteins using immobilised protease 3C

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Abstract

A strategy for efficient cleavage of fusion proteins using an immobilised protease has been developed. Protease 3C from coxsackie virus was recombinantly produced in *Escherichia coli* and covalently immobilised onto a solid support. Thereafter, Z_{basic} tagged fusion proteins, with a specific cleavage sequence between the domains, were flown through the proteolytic column and circulated until complete cleavage. Subsequently, the processed protein solution was applied on a cation exchanger. Thereby, removal of the released, positively charged fusion tag, Z_{basic}, was done by adsorption to the matrix while the target proteins were recovered in the flow through. Interestingly, the columns were shown to be reusable without any measurable decrease in activity. Moreover, after storage in 4 °C for two months the activity was almost unaffected.

Keywords: Immobilised protease; Z_{basic}; Protease 3C; Cleavage

The use of purification tags has become a major breakthrough for the purification of recombinant proteins. Many diverse proteins can now be purified in a high throughput fashion using general schemes [1]. However, the intended use of the protein may sometimes require the removal of the purification tag as it can influence the expected properties of the target protein, interfere with the three-dimensional structure and mediate unwanted immunogenic responses.

Tag removal can either be performed by chemical or enzymatic methods [2]. Chemical hydrolysis requires rather harsh conditions that often denatures the protein and can cause chemical modifications on the side chains [3]. Additionally, chemical cleavage sites are only specified by one or two residues and are therefore often found within the target protein, which limits chemical methods to the release of peptides or smaller proteins.

Even though considerably more expensive, enzymatic proteolysis is usually preferred since it can be performed under physiological conditions and generally gives a more selective cleavage. A large repertoire of proteases with different specificity and varying length of the recognition sequence is now available, e.g., Tobacco Etch Virus (TEV)[1] protease [4], Thrombin [5], Factor X_a [6], Urokinase [7], and Protease 3C [8].

Even though recombinant production in bacteria and selective recovery through the use of purification tags has improved the availability, the costs of the proteases are still considerable. The subsequent removal of the protease and the released tag is another important issue. However, this can also be facilitated by having a purification tag fused to the protease, preferable the same as the one that is to be released from the target protein [9]. Another elegant strategy is to immobilise the protease on a solid support [10–12] and thereby also be able to reuse the same protease several times to reduce the production cost.

The coxsackie virus protease 3C is a site specific protease that recognises the octapeptide LEALFQ/GP and cleaves between the Gln and Gly residues. Previously, protease 3C has been produced as a fusion to the positively charged purification tag, Z_{basic} [13] and thereafter been successfully used for cleavage of a fusion protein [9].

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[1] *Abbreviations used:* TEV, tobacco etch virus; TSB, tryptic soy broth.

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In this paper, the Z_{basic} tagged protease 3C is covalently immobilised onto an NHS activated matrix to accomplish reuse of the enzyme. The hereby obtained proteolytic column is then repeatedly used for cleavage of Z_{basic} tagged fusion proteins. After completed cleavage, the released Z_{basic} tag is separated from the target protein by capture on a cation exchanger. Thereby a highly pure target protein is obtained in a suitable buffer in the flow through.

Materials and methods

All recombinant work was performed using *Escherichia coli* strain RRIΔM15 [14]. Recombinant DNA techniques were performed essentially as described by Sambrook et al. [15]. Synthetic oligonucleotides were purchased from MWG-Biotech AG (Edersberg, Germany). DNA and restriction enzymes were purchased from New England Biolabs (Hertz, UK) and used according to the manufacturer's recommendations. Base composition of the constructed vectors was verified by cycle sequencing using MegaBACE 1000 DNA sequencing system (Molecular Dynamics, Sunnyvale, CA). *E. coli* strain BL21DE3 (Novagen Inc., Madison, WI) was used for protein expression. All ion-exchange experiments were performed on an Akta Explorer 100 system (GE Healthcare, Uppsala, Sweden).

Expression vectors

The fusion protease, Z_{basic}3C, was expressed using the vector pT7Zb3C [9]. The first substrate protein was expressed using the vector pT7ZbQGKlenow [9] encoding Z_{basic} and Klenow linked by the heptapeptide EALFQ/GP (denoted QG) which is site-specifically cleaved by protease 3C. The second substrate protein was expressed using the vector pT7ZbZtaqZwt encoding Z_{basic}-QG-$Z_{taq}Z_{wt}$ with the 3C cleavage site in between the first two moieties. This vector was constructed by PCR-amplification of the gene for Z_{wt} using the primers: FARO18: CCCAAGCTTGTCGACGATGACAACAAATTCAACAA (*Sal*I) and RIJE9: CCCCTCGAGTTATTTCGGCGCCTGAGCATC (*Xho*I) on the template pET28-Z. Amplification of the gene encoding Z_{taq} was performed using the primers: ZSUB1: CCCCGAATTCCGTAGACAACAAATTCAACAA (*Eco*RI) and ZSUB2: TTTTAAGCTTTTAGTCGACTTTCGGCGCCTGAGCATC (*Sal*I) on the template pKN1Ztaq(4:5). The both amplified genes were then restricted using *Sal*I/*Xho*I and *Eco*RI/*Sal*I, respectively, before they were ligated into the plasmid pT7ZbQG [9] that was cut with *Eco*RI and *Xho*I. Base composition of the resulting construct was verified by cycle sequencing.

Production and purification of Z_{basic} fusion proteins

BL21(DE3) cells harbouring the expression vectors were grown overnight at 37 °C in 2 ml inoculation plates containing 1 ml (30 g L^{-1}) Tryptic soy broth (TSB) (Difco, Detroit, MI) supplemented with 5 g L^{-1} yeast extract (Difco) and 50 μg ml^{-1} kanamycin. On the following morning, the cultures were inoculated to 100 ml fresh medium and growth continued with shaking until an $OD_{600\,nm}$ of 1 was reached. Protein production was induced by addition of isopropyl-β-D-thiogalactoside (Apollo Scientific Ltd, Bredbury, Stockport, UK) to a final concentration of 1 mM and production continued for 20 h at 37 °C. After expression, the cell suspension was gently harvested (4000*g*, 8 min, 4 °C), re-suspended in 30 ml of 50 mM sodium phosphate, pH 7.5, and disrupted in an ice-bath using sonicator (Vibra cell, Sonics and Materials, Danbury, CT) at 60% duty cycle for 3 min with 1.0 s pulses. Insoluble material was removed by centrifugation at 10,000*g* for 10 min at 4 °C, followed by filtration through a 0.45 μm filter. A volume of 10 ml of the lysate was loaded on to a 5 ml HiTrap S HP (GE Healthcare) previously equilibrated with 10 CV of running buffer (50 mM sodium phosphate, 200 mM NaCl, pH 7.5). After the loading step, unbound material was washed out with 10 CV of running buffer and weakly bound material was eluted with 500 mM NaCl. Z_{basic} tagged proteins were eluted using a linear gradient from 500 mM to 1 M NaCl. Substrate fusion proteins were then dialysed into 50 mM sodium phosphate, pH 7.5.

Amine-coupling conjugation of protease 3C

The fusion protease, Z_{basic}3C, was dialysed into coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and concentrated in an Ultrasart Cell to 2.5 mg/ml using ultrafiltration membranes with nominal cut off of 10 kDa (Sartorius GmbH, Gottingen, Germany). A volume of 8 ml (20 mg) was circulated for 2 h (0.25 ml/min) through a 1 ml NHS activated HiTrap HP column (GE Healthcare) using a P1 pump (GE Healthcare). Coupling, washing, and deactivation were performed according to the manufacturer's instructions. Coupling efficiency was calculated after measuring the absorbance of the desalted flow through.

Cleavage reactions with immobilised protease 3C

The cleavage reaction was carried out at 4 °C in continuous mode with the substrate solution fed continuously through the column with immobilised protease (0.25 ml/min). A volume of 3 ml of the substrate fusion protein (approximately 30 nmol) was circulated through the column. A reducing agent (5 mM β-mercaptoethanol) was added as required for 3C protease activity [16]. The circulated sample was analysed on a 10–20% Criterion Precast Gel (BioRad, Hercules, CA) after every 30 min and the cleavage yield was calculated by densitometer scanning of the gels. Each column was reused five times in a time span of 7 days and then reused again after storage at 4 °C during two months.

Cation-exchange removal of released Z_{basic}

After completed cleavage, the protein sample was loaded onto a 1-ml HiTrap S HP cation-exchange column (GE