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Autoprotease N^{pro}: Analysis of self-cleaving fusion protein

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ABSTRACT

A reversed phase high pressure liquid chromatography method was developed for determination of *in vitro* refolding and cleavage kinetics for the N^{pro} autoprotease fusion peptide EDDIE-pep6His using a TSK Super-Octyl column with a segmented acetonitrile gradient. Self-cleaving fusion proteins such as N^{pro} autoprotease fusion proteins consist of the single autoprotease N^{pro} and a target peptide or a target protein as fusion partner. Hence, three protein species are present after self-cleavage: the target peptide or protein, the single N^{pro} autoprotease and, in case of incomplete cleavage, residual N^{pro} fusion protein. Thus, for an accurate analysis the method must be standardized for three components in the presence of host cell impurities. For method validation, protein standards of EDDIE-pep6His and the single N^{pro} autoprotease EDDIE were prepared from inclusion bodies (IBs) by ion exchange, immobilized metal ion affinity, size exclusion, and reversed phase chromatography. A linear correlation was obtained for EDDIE-pep6His and EDDIE in the range from 95 to 730 μg/ml with a lower limit of quantification (LLOQ) and a lower limit of detection (LLOD) of 34.5 and 11.4 μg/ml, respectively, for EDDIE-pep6His and 39.6 and 13.1 μg/ml, respectively, for EDDIE. Finally, a fully automated batch refolding of EDDIE-pep6His from IBs was performed to demonstrate the applicability of this method. It was shown that the initial EDDIE-pep6His concentration in the refolding solution decreased from 194.3 to 83.8 μg/ml over a refolding time of 385 min resulting in a final refolding and cleavage yield of 50%.

1. Introduction

Self-cleaving fusion proteins such as N^{pro} autoprotease fusion proteins and related mutations are used for the production of recombinant peptides and proteins [1–4]. These fusion proteins are expressed in large quantities in inclusion bodies. Upon refolding, the autoprotease N^{pro} cleaves off its target protein or peptide [5,6]. Detection of the autoprotease, the cleaved target protein, and the residual fusion protein is required to monitor and quantify the expression rate, refolding, and protein purification. Ideally, all three protein species should be quantified in a single analytical method such as electrophoresis or chromatography.

Electrophoresis is frequently used for monitoring the progress of refolding. The sizes of unfolded and refolded self-cleaving fusion proteins are significantly different because the target protein is cleaved off upon refolding and the different species can be separated by SDS-electrophoresis. Additionally, self-cleaving fusion proteins generate two protein species with different molecular masses, as compared to the parent uncleaved fusion protein. Fusion proteins may aggregate or precipitate during the refolding process. Therefore, to identify optimal refolding conditions, the determination of the mass balance of all involved protein species is essential [8]. We have used electrophoresis to study N^{pro} fusion proteins, but have failed to obtain adequately quantitative data [1,7], especially for calculating the recovery of processed fusion protein with respect to the starting protein concentration. Quantitative data is difficult to obtain from gel electrophoresis studies because electrophoretic methods are difficult to standardize and their windows of linearity of molecular size are narrow.

Separation of different conformational states of proteins is possible with reversed phase HPLC [9,10]. In certain cases even intermediate forms can be separated. As a rule of thumb, the size of the molecule and thus the retention time increases in the following order: native protein $\ll$ apoform < acidic form $\ll$ thermally denaturated form $\ll$ GuHCl unfolded form [11]. Separation of a fusion protein from its cleaved fusion partner is even easier. The size and thus the retention time in reversed phase chromatography is significantly different, assuming that the target protein is large compared to the whole fusion protein. For small peptides this may not be the case, resulting in a difficult separation. Our N^{pro} autoprotease fusion peptide used in this work has a molecular

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mass of approximately 21 kDa, the target peptide has a molecular mass of approximately 2 kDa, and the single N^{pro} autoprotease therefore has a mass of 19 kDa. Identification of correct refolding conditions or the monitoring of refolding kinetics requires a relatively fast method. With conventional reversed phase HPLC the separation efficiency with a reasonable time/length of gradient is not achieved. For real time tracking of a refolding process, the reaction must be sampled every 15–30 min, requiring a method that is complete in less than 15 min. Samples cannot be frozen because they might change and refolding will still continue during freezing and thawing. Stopping the refolding in the drawn sample by, for example, acidification also might drastically alter the sample composition. Application of UHPLC which uses submicron particles, is an alternative methodology. With particles lower than 3 μm, sufficient resolution might be obtained in a reasonable time even under non-UPLC conditions [12]. TSKgel Super-Octyl is a material which has been used for the separation of proteins and thus is a logical choice for this separation problem. Such a method can also be used for high throughput screening to determine optimal conditions for refolding and for the subsequent purification procedures [13]. Moreover, high throughput methods are important for rapid process development [14–18] to relate the refolding process to the overall process scheme.

Proteins expressed as inclusion bodies are relatively pure, with up to 90% purity often being achieved [4]. This protein purity is the advantage which compensates for the time consuming refolding procedure. The protein in inclusion bodies is present in extremely high concentration, a kind of semi-solid state. For refolding, proteins must be diluted into refolding buffer to avoid protein aggregation. Aggregation follows usually a high order of reaction, at least an order of two and higher [4,19,20]. So protein refolding is performed at a concentration between 20 and 400 μg/ml. For the initial stages of the refolding procedure, the formed species are present in very low concentrations. When the refolding process reaches equilibrium, the residual fusion protein which has not been processed, is present in low concentration. Thus, the method must detect low levels of protein with great sensitivity.

We present here a RP-HPLC method based on the 2 μm material TSKgel Super-Octyl which enables the determination of *in vitro* refolding kinetics as well as the quantification of the N^{pro} autoprotease fusion protein EDDIE-pep6His. After method development, method calibration and validation was performed. For this purpose, protein standards were required which were not commercially available. Thus, they were prepared from IBs at laboratory scale. The applicability of the developed method was demonstrated by determining the *in vitro* refolding and cleavage kinetics of a batch refolding of EDDIE-pep6His.

2. Materials and methods

2.1. Equipment and chemicals

Chromatographic experiments were carried out on an ÄKTA Avant 25 (GE Healthcare, Uppsala, Sweden) controlled by UNICORN software version 6.1. The chromatographic media used, Source 30S, IMAC Sepharose HP, Superdex 75 pg, and Source 30RPC, were purchased from GE Healthcare (Uppsala, Sweden). All chemicals were purchased from Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany), if not otherwise stated.

2.2. N^{pro} fusion technology

In this work, a genetically optimized variant of the N^{pro} wild type autoprotease designated as EDDIE was used [1]. The N^{pro} fusion peptide used here was termed EDDIE-pep6His and consisted of EDDIE and a small model peptide called pep6His as fusion partner. EDDIE has 168 amino acids, a molecular weight of approximately 19 kDa and a pI of 6.59. EDDIE-pep6His has 184 amino acids, approximately 21 kDa and a pI of 6.63. pep6His is an artificial construct with no biological function consisting of 10 randomly selected amino acids and a 6-His-tag. Its molecular weight is approximately 2 kDa and its pI 6.55. Furthermore, it has no UV absorbance at 280 nm due to the lack of tryptophan, tyrosine and phenylalanine.

2.3. Recombinant protein expression and IB isolation

Both EDDIE-pep6His and EDDIE were overexpressed in *Escherichia coli* BL 21 with a pET30a plasmid (Novagen, Madison, WI, USA) containing the corresponding coding gene [1]. High density fed-batch cultivations of *E. coli* were performed on a 20-L scale with a semi-synthetic batch medium and subsequent C-limited fed-batch medium at 37 °C, pH 7.0.

Cells were harvested using a disk centrifuge (Pathfinder PSC 1-06-177; GEA GEA Westfalia Separator Group, Oelde, Germany) and resuspended in 50 mM Tris, 50 mM NaCl, 0.02% Tween at pH 8.0 with an Ultra Turrax (IKA, Staufen, Germany) to reach a dry matter of 30 g/L. The cell suspension was passed two times through a Panda 2 K homogenizer (GEA Niro Soavi S.p.A., Italy) at a pressure of 1000 bar. IBs were subsequently separated with a Pathfinder PSC 1-06-177 disk centrifuge. The pellet was washed twice with 20 mM Tris, 0.5 M NaCl, 0.02% Tween at pH 8.0 and then with 0.5 M NaCl. After each washing step, IBs were centrifuged with a Pathfinder PSC 1-06-177 disk centrifuge. After the final washing step the pellet was resuspended in water with an Ultra Turrax to reach a 25% IB-suspension and was stored at −20 °C.

2.4. IB dissolution

For the purification of the protein standards, the IB suspension was diluted 1:5 in dissolution buffer. In the case of EDDIE, the dissolution buffer contained 8 M urea, 50 mM sodium acetate (NaAc), 2 mM ethylenediaminetetraacetic acid (EDTA), 20 mM α-monothioglycerol (MTG) at pH 5.0 after dissolution of IBs. In the case of EDDIE-pep6His, the dissolution buffer contained 8 M urea, 50 mM glycine, 5 mM MTG at pH 9.0 after dissolution with IBs. IBs were allowed to dissolve for 30 min. The solution was then centrifuged for 10 min at 13.200 rpm, 4 °C using a Centrifuge 5415R (Eppendorf AG, Hamburg, Germany). The supernatant was removed after centrifugation with a 10 ml syringe (Omnifix®, B. Braun Melsungen AG, Germany) and filtered through a 0.22 μm filter unit (Millipore, Billerica, MA, USA). The protein concentration of the filtered protein solution was determined by UV measurement. The absorbance at 260 and 280 nm was measured with a Cary 50 UV-VIS Spectrophotometer (Varian, Palo Alto, CA, USA) calibrated with the respective solutions used for protein dissolution. The protein concentration was calculated according to Lambert–Beer's Law using the theoretical extinction coefficient of EDDIE-pep6His ($\varepsilon = 1.098$ at $1.0\,\mathrm{mg\,ml^{-1}}$ and 280 nm) and EDDIE ($\varepsilon = 1.199$ at $1.0\,\mathrm{mg\,ml^{-1}}$ and 280 nm), respectively.

For refolding experiments, EDDIE-pep6His IBs were dissolved according to the protocol above with the exception that a different dissolution buffer was used containing 8 M urea, 50 mM Tris, 15 mM MTG at pH 7.3 after dissolution with IBs.

2.5. Cation exchange chromatography (CIEC)

A HiScale 26/40 column (GE Healthcare) packed with Source 30S with an inner diameter of 2.6 cm and a bed height of 20 cm was used. The column was equilibrated for 2 CV at 450 cm/h with equilibration buffer containing 3 M urea, 20 mM NaAc, 20 mM MTG at pH