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A method for the prevention of thrombin-induced degradation of recombinant proteins

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Abstract

A new strategy to prevent degradation of recombinant proteins caused by non-specific cleavage by thrombin is described. We demonstrate that degradation due to non-specific cleavage of recombinant protein mediated by thrombin can be completely prevented by separation of thrombin from the recombinant protein on spin columns packed with heparin-sepharose. This method is generally applicable to all recombinant proteins that require the thrombin for the cleavage of affinity tags for purification. To our knowledge, this is the first report of an efficient and reliable method for the separation of residual thrombin from purified recombinant proteins.

Keywords

recombinant protein; thrombin; heparin; degradation; structure; non-specific

Large scale production and purification of pure recombinant proteins is the first important step towards understanding their structure-function relationship [1,2]. In general, recombinant proteins are bioengineered with affinity tag either at their N- or C-terminus [2]. These affinity tags not only facilitate easy purification of recombinant proteins, but are also known to aid in overexpression of proteins in soluble forms [3]. To eliminate downstream interference, many fusion vectors are designed such that the fusion partner (affinity tag) can be easily eliminated from the protein of interest after cell lysis. This is most often accomplished by the insertion of a protease-specific cleavage site between the affinity tag and the recombinant protein of interest [3]. Thrombin is one of the common and popular restriction proteases used for removal of affinity tags from recombinant fusion proteins [4,5]. It is preferred to other restriction proteases due to its relatively low cost and optimum cleavage activity in the pH range of 6–8. Thrombin, an endoprotease, is known to preferentially recognize the -Leu-Val-Pro-Arg-Gly-Ser-sequence and cleave at the Arg-Gly bond [6]. Despite its extensive use, thrombin has been reported to non-specifically cleave at secondary sites located in some overexpressed recombinant proteins [6]. Non-specific cleavage mediated by thrombin is quite common. However, to date there is no efficient experimental procedure to prevent the undesired non-specific cleavage of recombinant proteins caused by thrombin. Heterogeneous protein samples generated by non-specific thrombin catalyzed cleavage would pose a major hurdle for structural studies such as X-ray crystallography and NMR spectroscopy. In this study, we report a novel

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and efficient method to prevent thrombin mediated non-specific cleavage of recombinant proteins using heparin-sepharose.

Heparin is known to bind to thrombin and completely inhibit its activity on fibrinogen [6]. We measured the binding affinity (at 25 °C) of thrombin (Sigma, molecular mass ~ 30 kDa) for heparin (Sigma) using isothermal titration calorimetry. The binding isotherm representing the titration of 50 μ M thrombin [dissolved in 10 mM tris (pH 7.2) containing 20 mM NaCl] with heparin (1 mM) is sigmoidal, and saturates at a protein to heparin ratio of 1:1 (Fig. 1, panel A). The apparent binding constant characterizing the affinity between thrombin and heparin is estimated to be about 6.5 μ M. The K_d value is in good agreement with that reported earlier by Oslon et al. [7]. ITC experiments carried out at different concentrations of NaCl (in the range of 50 mM – 500 mM) showed complete loss of binding between thrombin and heparin at 350 mM NaCl (Fig. 1, panel B). The binding affinity of thrombin for heparin was further verified by loading 0.5 mL of thrombin (25 μ M to 100 μ M) solution [prepared in 10 mM tris (pH 7.2) containing 20 mM NaCl] on to a spin column packed with heparin-sepharose (bed volume of 1.5 mL). The heparin-sepharose spin column loaded with thrombin was incubated at 4 °C for 30 minutes. The spin column was subsequently centrifuged (at 4 °C) at 3000 rpm for 5 minutes and the eluate was collected in 1.5 mL eppendorf tubes. SDS-PAGE of 1.5 mL aliquots of the eluate obtained after centrifugation showed no detectable thrombin, suggesting that all of the thrombin used was bound to the heparin-sepharose resin (Fig. 1, panel C). The spin column with bound thrombin was eluted (at 4 °C) by centrifugation (at 3000 rpm) with a stepwise gradient of NaCl (50 mM to 1.5 M NaCl). SDS-PAGE of the eluate collected at different concentrations of NaCl showed the thrombin band (~ 30 kDa) in NaCl concentrations between 150 mM to 300 mM (Fig. 1, panel C). Densitometric scan of the SDS-PAGE gel showed that thrombin was maximally eluted in 250 mM NaCl (Fig. 1, panel D). These results provide clear evidence that the affinity of thrombin to heparin-sepharose can be exploited to achieve its separation from recombinant proteins following cleavage of affinity tag(s) used for purification.

We show an example of the purification of apoS100A13, a ~ 12 kDa calcium binding protein [8] to demonstrate the successful use of heparin-sepharose to remove thrombin present in recombinant protein preparations after affinity tag cleavage. A sample of apoS100A13 was overexpressed in *Escherichia coli* (BL21 pLysS) as a fusion protein with GST affinity tag (Fig. 2, panel A). After cell lysis, GST-apoS100A13 fusion protein was purified on glutathione-sepharose (GE Health Care), and subsequently buffer exchanged with 10 mM tris (pH 7.2) containing 20 mM NaCl using an Amicon concentrator (3 kDa cut-off membrane). Fifty milligrams of the GST-apoS100A13 was later incubated for 8 hours at 25 °C with 50 NIH units of thrombin. SDS-PAGE of the reaction product(s) revealed only three prominent bands corresponding to apoS100A13 (~ 12 kDa), GST (~ 25 kDa), and thrombin (~ 30 kDa) (Fig. 2, panel A). The band corresponding to the GST-apoS100A13 (~ 37 kDa) was observed to be completely missing on the SDS gel, suggesting that the cleavage of GST was complete (Fig. 2, panel A). The apoS100A13:thrombin:GST mixture was incubated again with glutathione sepharose to eliminate GST from the mixture. This was confirmed by SDS-PAGE analysis, which showed that apoS100A13 and thrombin were eluted in the wash buffer consisting of 10 mM tris (pH 7.2) containing 20 mM NaCl (data not shown). The mixture of apoS100A13 and thrombin was concentrated (at 4 °C) by ultrafiltration using a Centricon concentrator (3 kDa cut-off). SDS-PAGE of the S100A13:thrombin mixture, acquired after various time periods of storage at 25 °C, showed a major (M_r ~ 12 kDa) band corresponding to apoS100A13 and a minor band representing a product of thrombin degradation (Fig. 2, panel B). The gradual decrease in the intensity of the major band suggests that prolonged incubation of thrombin further degrades the protein and generates cleavage products of smaller length(s) (Fig. 2, panel B). In addition, the decrease in intensity of the minor band (below the apoS100A13 band) suggests further degradation, producing smaller peptides which were undetectable using SDS-

PAGE. Electrospray ionization (ESI) mass spectrometric data revealed that the protein was cleaved by thrombin at two sites at the C-terminal end (at Gly 94 and at Arg 88 (data not shown). Interestingly, the non-specific cleavage sites resemble the primary thrombin cleavage site, and therefore possibly represent non-specific secondary sites of cleavage for the enzyme. Non-specific thrombin cleavage was further assessed by 2D ^1H - ^{15}N HSQC spectral analysis of a purified ^{15}N labeled apoS100A13 sample acquired after various time periods of storage at 25 °C. Each crosspeak in the 2D ^1H - ^{15}N HSQC spectrum represents an amide resonance (except for proline) in a given conformation of the protein. Therefore, time-dependent protein degradation by thrombin can be easily monitored by the appearance of new crosspeaks in the ^1H - ^{15}N HSQC spectra of ^{15}N labeled recombinant protein. ^1H - ^{15}N HSQC spectrum of the apoS100A13 acquired immediately after purification showed the expected number of ^1H - ^{15}N crosspeaks and there were no crosspeaks corresponding to the degraded protein. However, several new peaks begin to appear in the ^1H - ^{15}N HSQC spectra of apoS100A13 collected after 24 hours of incubation of the protein with thrombin (Fig. 2, panel C). These peaks correspond to degradation products of apoS100A13 formed over time because it is only this protein in the mixture that is isotope enriched (Fig. 2, panel C). These results also clearly highlight the ability of thrombin to promote non-specific cleavage of the purified recombinant apoS100A13 sample.

We examined the possibility of using heparin-sepharose to eliminate thrombin from purified preparations of recombinant apoS100A13. Purified GST-cleaved apoS100A13 (50 mg), in 3 mL of 10 mM tris (pH 7.2) containing 20 mM NaCl, was incubated with 50 NIH units of thrombin for 8 hours. The products of the cleavage reaction were loaded into 1.5 mL heparin-sepharose spin columns pre-equilibrated at 4 °C with 10 mM tris (pH 7.2) containing 20 mM NaCl. The heparin-sepharose spin columns were subsequently centrifuged (at 4 °C) at 3000 rpm for 5 minutes and the eluate was collected in eppendorf tubes. SDS-PAGE of the eluate showed a single band corresponding to the molecular weight (~ 12 kDa) of apoS100A13, with no evidence of the thrombin band at ~30 kDa (Fig. 2, panel D). SDS PAGE gels, stained individually with silver and with Coomassie blue, revealed no low molecular weight (< 12 kDa) degradation bands even after the purified recombinant apoS100A13 protein sample had been stored for more than two weeks at 25 °C (Fig. 2, panel D). Similarly, ^1H - ^{15}N HSQC spectrum of the ^{15}N labeled apoS100A13 acquired after 24 hours of storage (at 25 °C) showed no crosspeaks representing the degradation products of the protein (Fig. 2, panel E). These results unambiguously show that degradation of recombinant proteins caused by non-specific thrombin mediated cleavage can be successfully prevented by separating thrombin from the protein of interest using heparin-sepharose.

In this study, we have clearly demonstrated that the heparin binding affinity of thrombin can be successfully exploited to prevent its non-specific cleavage activity on recombinant proteins. We find this method to be very reliable and generally applicable to all recombinant proteins that require the use of thrombin to cleave the affinity tag. Protein purification using heparin-sepharose has proved to be immensely useful in the preparation of expensive stable isotope enriched (^{15}N and $^{15}\text{N}/^{13}\text{C}$) samples for the determination of the three-dimensional solution structures of several other proteins [Cell division cycle 42 (Cdc42), Chromo domains (CD1, CD2 and CD3) from chloroplast signal recognition particle, C2A and C2B domains of rat synaptotagmin and basic fibroblast growth factor], as this method greatly reduces the risk of thrombin-induced non-specific cleavage of the proteins of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ITC, isothermal titration calorimetry; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ESI, Electrospray ionization; HSQC, heteronuclear single quantum coherence.

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Fig. 1.

Isothermal titration calorimetry representing the interaction of heparin with thrombin in panel **A**- 20 mM NaCl and panel **B**- 350 mM NaCl. The upper panel represents the raw data and the bottom panel is best-fit of the raw data, after subtracting the heat of dilution. The isothermogram fits best with an apparent binding constant value of $\sim 6.5 \mu\text{M}$. The concentration of the thrombin used was $50 \mu\text{M}$. Appropriate background corrections were made to account for the heats of dilution. The experiment was performed at 25°C in 10 mM tris (pH 7.2). Panel **C**- SDS-PAGE of the supernatant fractions collected from heparin-sepharose column using various concentrations of sodium chloride. Lane- **M** shows the molecular weight marker. Lanes- **1** to **18** depict protein bands contained in fractions collected in 0 mM, 20 mM, 50 mM, 100 mM, and 150 mM NaCl.

150 mM, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1000 mM, 1250 mM and 1500 mM NaCl. Panel **D** – Densitometric scan of the thrombin band shown in the SDS gel in panel – **C**. Thrombin elutes maximally in 250 mM NaCl.

Fig. 2.

Panel **A**- SDS-PAGE analysis of thrombin cleavage of GST-apoS100A13 fusion protein before and after thrombin cleavage. Lane- **M** represents the molecular weight marker. Lane-**1**, represents the GST-apoS100A13 fusion protein; Lane-**2**, represents the thrombin cleavage products of GST-apoS100A13 fusion protein. Panel **B**- SDS-PAGE analysis of apoS100A13 stored at 25 °C for – 0 hours (Lane-**1**), 6 hours (Lane-**2**), 12 hours (Lane-**3**), and 24 hours (Lane-**4**). Lane-**M** represents the molecular weight marker. Panel **C**- ^1H - ^{15}N HSQC spectrum of apoS100A13 containing residual thrombin. ^1H - ^{15}N HSQC spectra were acquired after 24 hours of incubation at 25 °C in 10 mM tris- d_6 (pH 7.2) containing 20 mM NaCl. Panel **D** – Coomassie-blue stained SDS-PAGE of apoS100A13 re-purified on heparin-sepharose

immediately after purification (0 hours, Lane-1); after storage at 25 °C for 14 days (Lane-2); Silver-stained SDS-PAGE of apoS100A13 re-purified on heparin-sepharose, and stored at 25 °C for 14 days is shown in Lane-3. Panel E - ^1H - ^{15}N HSQC spectra of apoS100A13 after removal of thrombin using heparin-sepharose. ^1H - ^{15}N HSQC spectra were acquired after 24 hours of incubation at 25 °C in 10 mM tris-d6 (pH 7.2) containing 20 mM NaCl. ^1H - ^{15}N HSQC spectra were acquired with 8 scans.