Factor Xa cleavage of fusion proteins

Elimination of non-specific cleavage by reversible acylation

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A method is described for the elimination of non-specific cleavage of fusion proteins by factor Xa. Putative non-specific cleavage sites C-terminal to lysyl residues are blocked by reversible acylation by 3,4,5,6-tetrahydrophthalic anhydride prior to cleavage. After cleavage, the acyl groups are removed quantitatively by exposure to slightly acidic conditions. This method employs no harsh reagents or conditions, and may be generally applicable to factor Xa cleavage of fusion proteins.

Fusion protein cleavage; Factor Xa; Protein modification

1. INTRODUCTION

Efficient production of specific proteins by recombinant DNA techniques often requires expression of a fusion protein. To release the desired protein, the fusion protein must be cleaved at a specific site, which cannot also occur within any of the proteins to be made. Specific proteases can be used for this purpose.

The expression vector pLcIIFX created by Nagai and Thøgersen [1,2] contains, at the junction between the partial cII sequence and the site of the sequence of the protein to be expressed, a sequence encoding the tetrapeptide recognition sequence of blood coagulation factor Xa for prothrombin (Ile-Glu-Gly-Arg) [3]. Factor Xa cleaves the fusion protein (CIIFX-protein) at the C-terminal side of the tetrapeptide sequence, liberating authentic recombinant protein [1]. This expression system has been used for the production of several eukaryotic proteins in *E. coli* [2,4], including globins [5–7].

Factor Xa has been observed to cleave at additional sites other than the tetrapeptide recognition sequence in some fusion proteins [2,5], including wild type and mutant CIIFX-porcine myoglobin (pMb) used in this study. Non-specific cleavage of the pMb fusion protein is shown here to be due to factor Xa, to be likely to occur at lysyl residues, and to be inhibited by the reversible acylation of the fusion protein amino groups by THPA. Both acylation by THPA and deacylation may be achieved under gentle conditions [8,9]; this is therefore a method of potentially general use for the protection of fusion proteins from non-specific cleavage after lysyl residues by factor Xa.

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Abbreviations: THPA, 3,4,5,6-tetrahydrophthalic anhydride; pMb, porcine myoglobin; P37Y, 37Pro⟶Tyr

2. EXPERIMENTAL

2.1. *Construction of pLcIIFX-porcine myoglobin mutants*

Porcine myoglobin was expressed from the M13mp18FXpmyo template [7]. Most of the studies were carried out on a mutant form (P37Y), in which the template was altered by site-directed mutagenesis [10,11]. Mutant clones were identified by sequencing along their entire length [12]. The 600-bp *Bam*HI/*Hin*dIII digest fragment was ligated into pLcII [2]. The ligation mixture was transfected into *E. coli* QY13; clones were selected by small-scale protein induction assay [2].

2.2. *Induction, expression and purification of mutant fusion protein*

Protein synthesis and inclusion body preparation were according to Nagai and Thøgersen [2]. The fusion protein was solubilized in urea-PED (8 M urea, 50 mM sodium phosphate (pH 8.0), 1 mM EDTA, 1 mM DTT), clarified by centrifugation and purified by gel filtration on a Sephacryl S-200 column pre-equilibrated with urea-PED. The concentrations of fusion protein samples were determined from their absorbance at 280 nm in 6 M guanidinium chloride, 20 mM sodium phosphate (pH 6.5), using extinction coefficients derived from the tryptophan and tyrosine content [13] (ϵ_{280} = 13.9 $mM^{-1} \cdot cm^{-1}$).

2.3. *THPA acylation of mutant fusion protein*

THPA acylation was achieved according to Gibbons and Schachman [8,9]. THPA (0.5 M in acetone) was added, with stirring, to the fusion protein sample at 4°C. If necessary, 0.5 M NaOH was added dropwise to maintain a pH of 8–8.5. After 30 min, the sample was dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0), 0.5 M urea, 1 mM $CaCl_2$ at 4°C.

2.4. *Preparation of protease inhibitor-treated factor Xa*

Separate 15 μl fractions, each containing 9 μg factor Xa, were incubated with one of the following protease inhibitors prior to inclusion in a fusion protein cleavage reaction:

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(i) 5 U hirudin (type H7380, 500 U/mg, Sigma Chemical), at final reaction conditions of 50 mM Tris-HCl (pH 7.4), for 15 min at 37°C [14,15]. The degree of contaminant bdellin (trypsin-plasmin inhibitor) activity [16] in the hirudin preparation was determined by assaying the activity of trypsin solutions, according to Kassell [17], after preincubation with the hirudin preparation for 15 min at 37°C in 0.2 M triethanolamine (pH 7.8), 10 mM $CaCl_2$. Comparison with titration curves of trypsin inhibitor activity for pure bdellins [18] permitted estimation of the bdellin content of the hirudin preparation.

(ii) 12.5 nmol E-64 (Sigma Chemical), prepared in 10% DMSO, 0.1% Triton X-100, for 15 min at 25°C [19].

(iii) 5 U bovine anti-thrombin III (Sigma Chemical), at final reaction conditions of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.3), for 15 min at 37°C [20].

(iv) 30 μg BPTI (Bayer AG), at final reaction conditions of 67 mM sodium phosphate (pH 8.0), for 15 min at 25°C [21,22].

2.5. *Cleavage of fusion protein with factor Xa*

Non-THPA acylated CIIFX-pMb fusion proteins are only partially soluble at 0.45 mg/ml after dialysis against 50 mM Tris-HCl (pH 8.0), 0.5 M urea, 1 mM $CaCl_2$, whereas THPA acylated fusion proteins are fully soluble after dialysis. Cleavage with factor Xa was performed at 28°C at an enzyme to substrate ratio of 1:80 (w/w).

2.6. *Removal of THP groups*

Dialysis for 24–40 h at 4°C and pH 6 is a general method for the quantitative removal of THP groups from modified proteins [8,9]. pMb precipitates under these conditions, however, as this pH is close to the p*I* of the protein, and quantitative deacylation was not observed. Quantitative removal of THP groups from pMb can be produced using conditions where the protein remains soluble, such as dialysis against 0.1 M acetic acid for 20 h, or addition of guanidinium chloride to 5 M and incubation at 4°C for 20 h.

2.7. *Purification of porcine myoglobin*

If the removal of acylating groups by dialysis was not quantitative, the deacylated species was purified by anion exchange chromatography on a DEAE-Sepharose CL6B column (40 cm × 1 cm diameter), equilibrated with 4.5 M urea, 25 mM Tris-HCl (pH 7.6). The sample was loaded in the same buffer, and eluted with a 200 ml linear gradient of 0–0.1 M NaCl.

2.8. *Electrophoretic analysis*

Samples from cleavage reactions were analyzed by SDS-polyacrylamide gel electrophoresis, according to Schaegger and Von Jagow [23]. A 16.5% T, 3% C separating gel was used in all instances, with a 10% T, 3% C spacer gel.

The degree of THPA acylation of samples was determined by polyacrylamide gel electrophoresis [24], with 8 M urea included in gel mixtures and samples [8].

3. RESULTS AND DISCUSSION

Cleavage of unmodified CIIFX-pMb (P37Y) with factor Xa generates several protein species by non-specific cleavage, in addition to authentic myoglobin (Fig. 1a). The rate of non-specific cleavage is not affected significantly by E-64 (Fig. 1b), a specific inhibitor of cysteine proteases, or by hirudin (Fig. 1c), a specific inhibitor of thrombin. The hirudin preparation was found to contain bdellins at 25–30% by weight (a level comparable to those reported for other crude preparations [16]), therefore non-specific cleavage is not due to plasmin activity. However, both anti-thrombin III (a specific inhibitor of thrombin and factor Xa), and BPTI (a general inhibitor of serine proteases) inhibit all cleavage (Fig. 1d, e). It is likely, therefore, that non-specific cleavage is due to factor Xa, not to proteases present as contaminants.

The degree of non-specific cleavage varies between wild type and mutant myoglobin species, with mutant CIIFX-pMb (P37Y) the most susceptible. This implies a dependence of cleavage not only on primary sequence, but also on conformation and accessibility. The molecular weights of the non-specific cleavage products were determined by SDS-polyacrylamide gel elec-

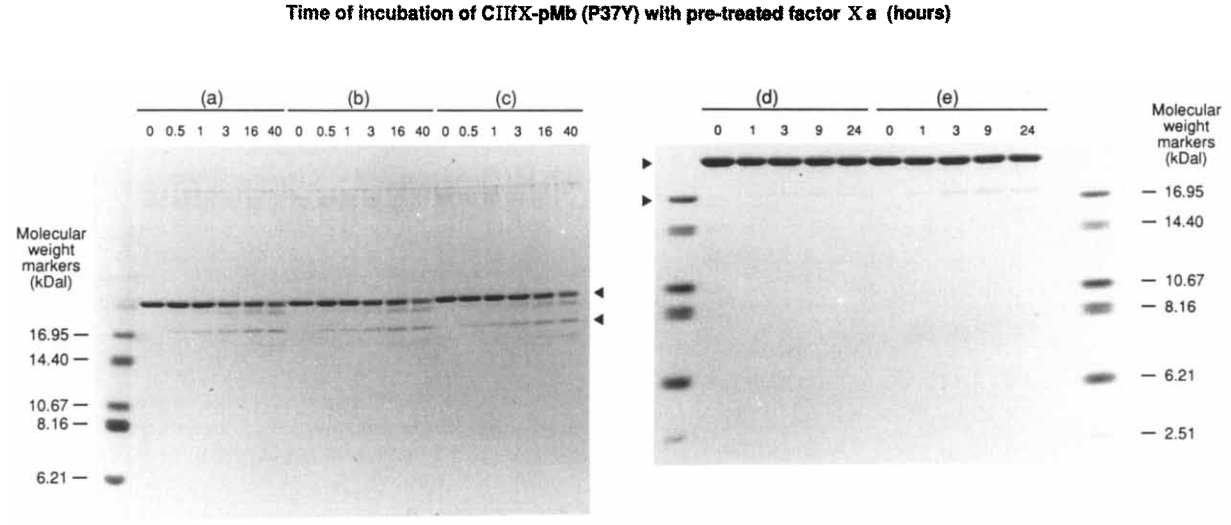

Fig. 1. SDS-PAGE of species generated by cleavage of unmodified CIIFX-pMb (P37Y) by protease inhibitor-treated factor Xa. The inhibitors used, and their concentration per 9 μg aliquot of factor Xa were: (a) no treatment; (b) 12.5 nmol E-64; (c) 0.5 U hirudin; (d) 0.5 U anti-thrombin III; (e) 30 μg BPTI. The molecular weights of cleavage products are given in Table I. Treatments a, b, c and treatments d, e were run on different gels; in each case, the upper arrowhead identifies the fusion protein (approx. mol. wt. 21200 kDa) and the lower arrow identifies pMb (P37Y) (approx. mol. wt. 17400 kDa).

Table I

Major cleavage products generated on digestion of unmodified CIIFX-pMb (P37Y) by factor Xa

Polypeptide molecular weight[a]	Putative cleavage site at the peptide bond C-terminal to:
21380 ± 200[b]	None
20230 ± 320	Lys145 (or Arg139)
18790 ± 310	Lys133
17700 ± 290[c]	FXa cleavage site
15740 ± 290	FXa cleavage site and Lys145 (or Arg139)
15170 ± 70	Lys96 or Lys98

[a] Error limits are the standard deviation in molecular weight ($n = 5$)
[b] CIIFX-pMb (P37Y) fusion protein
[c] Authentic pMb (P37Y)

trophoresis. Putative cleavage sites were identified from the calculated molecular weights (Table I), which suggested that cleavage was occurring at lysyl residues.

The primary sequences of pMb [25,26] and β-globin [27,28] were compared to identify a possible basis for the non-specific cleavage that occurs with the former, but not the latter. There is a significant difference between β-globin and pMb in the number of lysyl residues they contain (11 and 19, respectively), and in their distribution throughout the protein. In pMb, 4 regions may be identified in which clustering of lysyl residues occurs, giving sequences with multiple positive charge. Only one such region occurs in β-globin, suggesting that factor Xa was cleaving at sites of high positive charge.

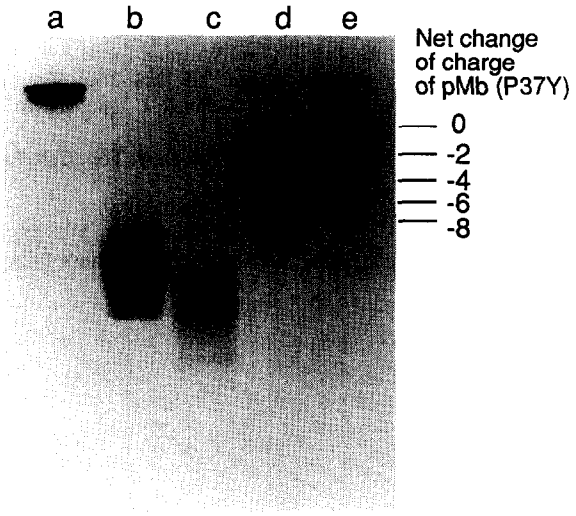

Fig. 2. Comparison of the degree of deacylation achieved by dialysis against 0.1 M acetic acid and dialysis against 50 mM citrate buffer (pH 5.8), by urea-PAGE of dialysates: (a) unmodified fusion protein CIIFX-pMb (P37Y); (b) THP acylated fusion protein; (c) acylated pMb (P37Y) generated by factor Xa cleavage; (d) acylated pMb (P37Y) after dialysis against 50 mM citrate buffer (pH 5.8) for 20 h at 4°C; (e) acylated pMb (P37Y) after dialysis against 0.1 M acetic acid for 20 h at 4°C.

The possibility that non-specific cleavage was occurring at lysyl residues led to attempts to minimize it by reversible acylation of the fusion protein. The most readily reversible acylation reagent is THPA [8]. Acylation of the CIIFX-pMb species with THPA leads to a change of net charge of −2 for each amino group acylated [8]. Electrophoretic analysis of acylated samples demonstrated primarily the expected species, but also some with changes of net charge of −3, −5, etc. The presence of fusion protein with a change of net charge of −1, prior to blocking (Fig. 2a), indicates that another covalent modification, such as spontaneous deamidation [29] or carbamylation by cyanate on the degradation of urea [30], occurs in addition to any possible acylation by contaminants of the commercial THPA preparation and is at least partly responsible for the generation of these species. The minor species are separated from unmodified pMb by anion exchange chromatography.

A stoichiometry of 0.8 THPA molecules per free amino group was empirically determined to be sufficient to eliminate non-specific cleavage (Fig. 3). The observation that THP acylation with a stoichiometry of less than 1 is sufficient to suppress cleavage is unlikely to be applicable to all proteins. Native pMb contains 4 clusters of lysyl residues; it is possible that either THP acylation of all lysyl α-amines in such clusters is sterically unfavored, or that partial acylation of each cluster is sufficient to eliminate potential cleavage within that region.

Generally, THP groups can be removed very easily, without the need for harsh reagents or conditions [8,9], which makes this method preferable to trifluoroacetylation [31] or acylation by citraconic anhydride [32] for masking amino groups. However, dialysis at pH 5.8–6.0 leads to precipitation of pMb and pMb (P37Y), and non-denaturing polyacrylamide gel electrophoresis of the dialysate in the presence of

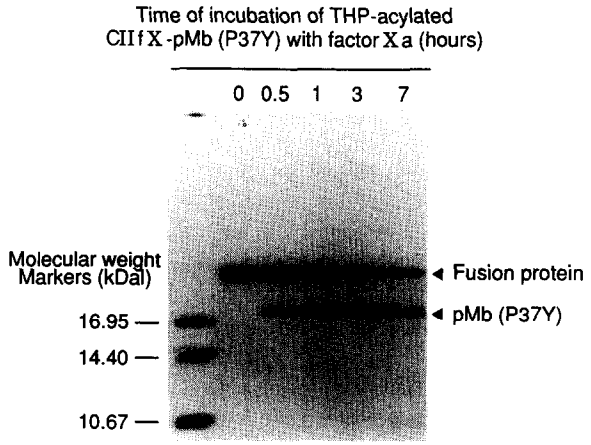

Fig. 3. SDS-PAGE of the products of cleavage of THP acylated CIIFX-pMb (P37Y) (0.8 THPA molecules per free amino group) by factor Xa.

urea shows that deacylation is not quantitative (Fig. 2d). Quantitative deacylation of the pMb species therefore requires different conditions from those generally applicable to other proteins, and was achieved by either dialyzing against 0.1 M acetic acid, or by incubating in 5 M guanidinium chloride at 4°C and pH 5.8–6.0 (Fig. 2e). After removal of minor charged species by anion exchange chromatography, the yield of pMb (P37Y) from deblocking by either acetic acid or guanidinium chloride incubation is typically 0.70 mg/g packed cells (wet weight). This compares favorably with 0.18 mg/g yielded by partial cleavage of non-acylated CIIFX-pMb (P37Y) (Fig. 1a), if fully quantitative separation of cleavage products is achieved.

Acylation by THPA may be suitable as a general method for eliminating non-specific cleavage of CIIFX-fusion proteins by factor Xa. Where the p*I* of the fusion protein is less than 8 (the pH at which factor Xa cleavage is routinely executed), the change in net charge on acylation by THPA may also increase the solubility of the fusion protein, accelerating the rate of cleavage.

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