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A new and general procedure for refolding mutant Bowman-Birk-type proteinase inhibitors on trypsin-Sepharose as a matrix with complementary structure

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The trypsin-reactive subdomain of the Bowman-Birk-inhibitor of serine proteinases is shown to be highly vulnerable to perturbations induced by single amino acid replacements. Dramatic deviations from a stoichiometric 1:1 ratio to the chymotrypsin-reactive subdomain used as an internal standard occur with all variants after renaturation in solution. A stoichiometric 1:1 ratio of subdomains is achieved via a new refolding procedure on trypsin-Sepharose as a matrix with complementary structure.

Bowman-Birk inhibitor; Proteinase; Enzyme inhibitor; Trypsin-Sepharose; Protein engineering; Protein folding

1. INTRODUCTION

The kinetic analysis of protein variants modified via genetic techniques has become a widely accepted tool for analyzing relationships between structure and activity in proteins [1]. However, even very slight changes in the amino acid sequence of a protein might affect other structural elements important for its functional properties. Deleterious effects on the stability [2] and refolding properties [3] of a protein should always be considered. Unambiguous distinction of structural from functional mutants is therefore required for a clear-cut interpretation of results.

The Bowman-Birk inhibitor (fig.1) of serine proteinases [4,5] was considered to be a very suitable protein for this purpose because it contains two closely aligned subdomains with distinct trypsin- and chymotrypsin-inhibitory activities. Both subdomains are not completely independent of each other as regards their activities [6] and spectroscopic properties [7]; however, the chymotrypsin-reactive part is more sensitive in this respect.

We have recently described an approach through the use of recombinant DNA techniques to a Bowman-Birk-type proteinase inhibitor via chemically synthesizing the gene [8] and decided to start with site-directed mutagenesis in the trypsin-reactive head while using the chymotrypsin-reactive subdomain as an internal standard for the detection of structural perturbations. The serious problems due to refolding that were encountered during the work on the variants, however, prompted us to devise a modified protocol to deal with such difficulties.

2. MATERIALS AND METHODS

All materials and general methods, details of expression, CNBr cleavage, refolding procedure for parent Bowman-Birk-type proteinase inhibitor and technique for assaying residual trypsin activities have been described [8]. The final concentration of trypsin in assays was 5.83×10^{-9} M. Chymotrypsin-inhibitor complexes were formed in 100 μl buffer [50 mM Tris-Cl (pH 7.5), 20 mM $CaCl_2$] for 15 min and residual enzyme activities were assayed by the method of Erlanger et al. [9] using *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) as synthetic substrate. Bovine chymotrypsin, containing a minimum of 95% of active material, and the synthetic substrate were obtained

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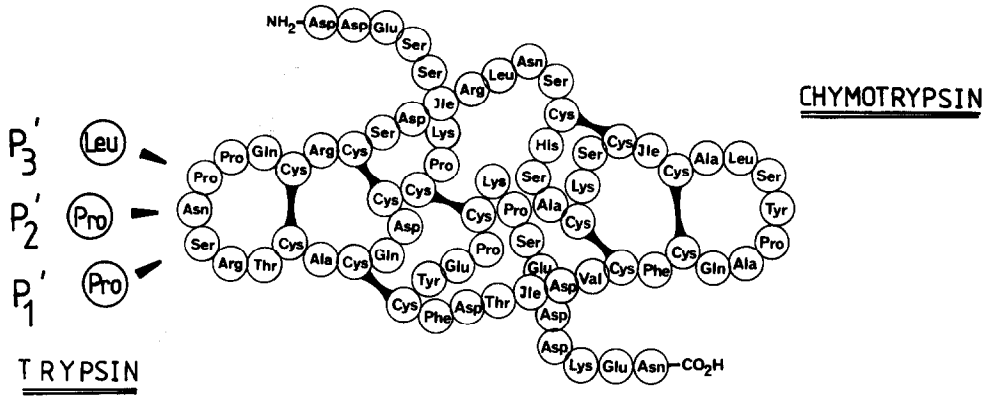


Fig.1. Covalent structure of the parent Bowman-Birk-type proteinase inhibitor. Single amino acid replacements in the trypsin-reactive subdomain are shown.

from Sigma. The final chymotrypsin concentration in the assays was 4.16×10^{-8} M. Stock solutions of inhibitor were appropriately diluted for assays of residual proteinase activities.

The amounts of inhibitor (in μg) were calculated from the expressions

$$I = [E^o]VM_r/R$$

and

$$R = V^{eq}/V^{tot}$$

where $[E^o]$ represents the final concentration of proteinase (in mol/l), V the final volume of the enzyme assays (6×10^{-4} l), M_r denoting the value for the inhibitor M_r (8×10^9 μg/mol), V^{eq} the volume of the aliquot of diluted stock solution at the equivalence point and V^{tot} the volume of diluted stock solution corresponding to the total amount of inhibitor. The equivalence points for titrations with chymotrypsin were evaluated by using the graphic method of Dixon [10] as shown in fig.2b,d, since the ratio $[E^o]/K_i$ [11] under the present assay conditions is within the range for flat inhibition curves [12]. The parent protein [8] was verified as giving a stoichiometric 1:1 ratio of both proteinase-reactive subdomains.

The variants were expressed in *E. coli* W 3110 as fusion proteins to a β-galactosidase fragment and cleaved with CNBr as described in [8]. An IMA disintegrator (IMA, Zeppelinheim, FRG) operating at 5000 rpm was used for disintegration of cells instead of a French press.

Refolding and affinity chromatography for mutant proteinase inhibitors were performed as follows: 32 mg crude CNBr cleavage mixture were incubated in 240 μl reduction/denaturation buffer, containing 2 M Tris-Cl (pH 8), 0.2% EDTA, 6 M guanidinium chloride and 2 M 2-mercaptoethanol at 37°C for 18 h, and then added dropwise to 240 ml renaturation buffer, comprising 80 mM Tris-Cl (pH 8), 0.1 mM EDTA and 0.2 mM oxidized glutathione at 37°C with vigorous stirring. The refolding mixture was shaken for 60 min at 37°C, trypsin-Sepharose [8] added and shaking continued at 32°C for 90 min. The matrix was transferred to a column and washed with 50 ml of 0.1 M sodium borate (pH 7.6) containing 10 mM $CaCl_2$, 50 ml of 10 mM $CaCl_2$ and 10 mM HCl in the case of P_1'-Pro and 5 mM HCl for P_2'-Pro and P_3'-Leu variants, respectively, at 4°C. The P_1'-Pro variant was eluted with 100 mM HCl, the others being eluted with 50 mM HCl at a flow rate of approx. 0.2 ml/min. 5-ml fractions were collected, 80-μl aliquots being assayed for residual trypsin activities (cf. fig.3).

The active fractions were pooled, diluted with water and lyophilized. The samples gave single bands at 8 kDa on SDS gels and single active peaks on reversed-phase HPLC with retention times which are in accordance with that of the parent protein [8].

3. RESULTS

Gene sequences coding for the following three variants: P_1'-Ser ⟶ Pro, P_2'-Asn ⟶ Pro and P_3'-Pro ⟶ Leu in the trypsin-reactive subdomain of the Bowman-Birk-type proteinase inhibitor (fig.1) were constructed by site-directed modification of the published [8] synthetic gene in order to address several questions on the mechanism [13] of proteinase inhibitors. These single amino acid replacements do not occur in any of the natural inhibitors of the Bowman-Birk family [5]. Details of their construction, biochemical characterization and kinetic properties will be published somewhere else.

The gene sequences encoding these variants were cloned into the *Eco*RI and *Sal*I restriction sites of plasmid pLZP WB1 and expressed as fusion proteins with a β-galactosidase fragment in *E. coli* W 3110 as described for the parent inhibitor [8]. Up to 400 mg of crude fusion-proteins were obtained from 1 l of bacterial culture. The resulting fusion proteins were treated with cyanogen bromide in 70% formic acid and used for refolding experiments without further purification [8].

Experiments on renaturation in solution under the conditions described for the parent protein [8] were unsuccessful with the variants, however: the relative amount of trypsin-reactive material was reduced dramatically reaching zero in the case of the P_1'-Pro-variant, or a small fraction of the chymotrypsin-reactive subdomain in the other two variants (cf. a,c in figs 4–6).

The majority of the material comprising the trypsin-reactive subdomain is apparently distributed in the form of non-active conformers. This indicates the occurrence of local structural perturbations in the trypsin-reactive region of these variants.

All attempts at improving this unfavourable ratio through systematic variation of the refolding conditions were without success. Experiments on refolding in the presence of thioredoxin from *Corynebacterium nephridii* according to Pigiet and Schuster [14] also failed to yield the correct levels for the trypsin-reactive subdomain. Nevertheless, refolding in the presence of trypsin-Sepharose combined with subsequent affinity chromatography, as described in section 2 and fig.3, was found to improve this disappointing situation dramatically. This is demonstrated on comparison of the titration curves of the P_1'-Pro variant refolded in the presence (fig.2a) and absence (fig.2c) of trypsin-Sepharose. In contrast, the chymotrypsin-titration curves in fig.2b,d are virtually superimposable. This shows that the amount of the internal standard is not affected by the presence of trypsin-Sepharose within experimental error.

The results of all titration experiments on both proteinase-reactive subdomains obtained via refolding in the respective absence and presence of trypsin-Sepharose are summarized in figs 4–6. The amounts of trypsin- and chymotrypsin-reactive material correspond to the heights of filled and hatched bars, respectively. The relative amounts of

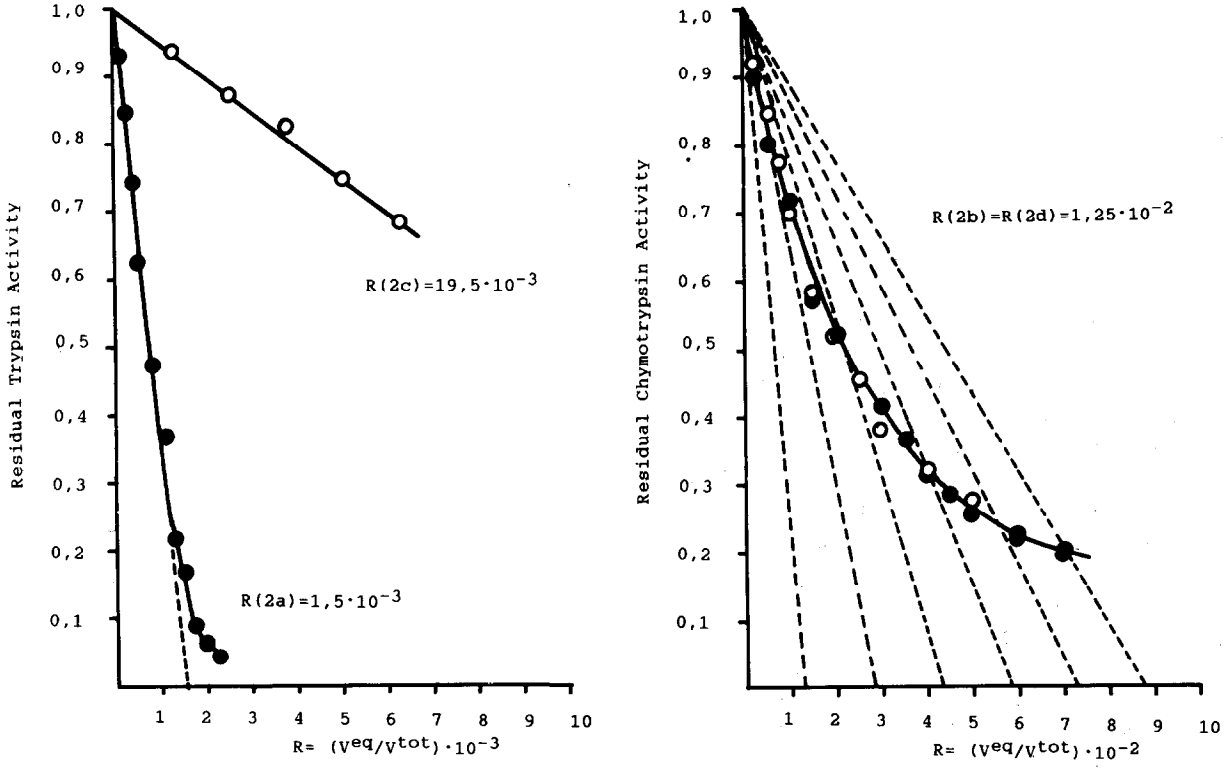


Fig.2. Titration of both subdomains of the P_1'-Pro variant of the Bowman-Birk-type inhibitor. Refolding in the presence of trypsin-Sepharose (●): titration with trypsin (curve 2a) and chymotrypsin (curve 2b). Refolding in the absence of trypsin-Sepharose (○): titration with trypsin (curve 2c) and chymotrypsin (curve 2d).

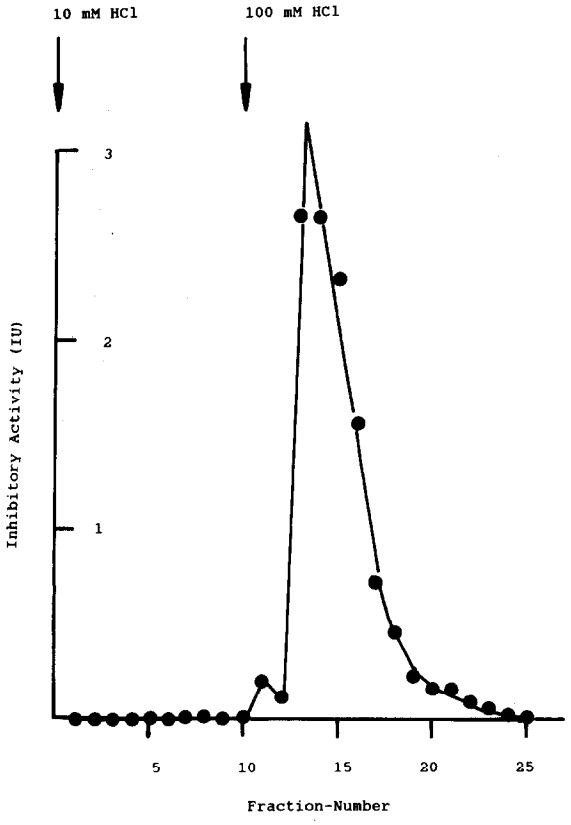
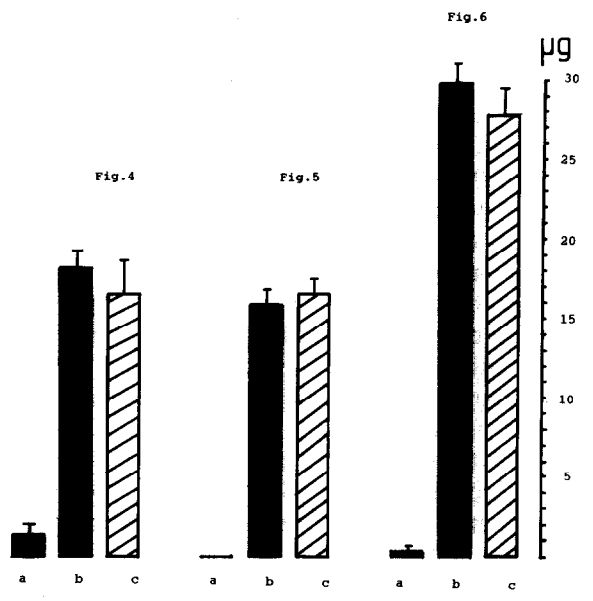


Fig.3. Affinity chromatography of the P$_1'$-Pro variant after refolding in the presence of trypsin-Sepharose. One inhibitory unit (IU) is defined as the amount of inhibitor (in μg) required to inhibit trypsin at a concentration of 1 μg/ml.



Figs 4–6. (From left to right) Summary of titration experiments in both proteinase-reactive subdomains of the P$_1'$-Pro (fig.4), the P$_2'$-Pro (fig.5) and P$_3'$-Leu (fig.6) variants. Amount of: (a) trypsin-reactive material after refolding in solution without trypsin-Sepharose as described [8]; (b) trypsin-reactive material after refolding in the presence of trypsin-Sepharose; (c) chymotrypsin-reactive material (the corresponding titration curves do not depend on the refolding method as shown in fig.2b,d).

trypsin-reactive and chymotrypsin-reactive subdomains are shifted toward the correct values, within experimental error, for the stoichiometric ratio (1 : 1) on performing the refolding procedure in the presence of trypsin-Sepharose.

Neither of the two proteinase-reactive activities was detectable in the ultrafiltered flow-through fractions from affinity columns combined with all buffers used to remove nonspecifically bound proteins.

This clearly demonstrates that the stoichiometric ratio of 1 : 1 was not the result of a simple affinity chromatography step on trypsin-Sepharose. The relative increase in trypsin-inhibitory material after refolding and affinity chromatography on trypsin-Sepharose and the constant amounts of chymotrypsin-reactive material – which is detectable only in the specifically eluted protein – rule out this possibility.

4. DISCUSSION

One of the major problems faced during the course of protein engineering studies is to detect and to control refolding problems in a protein modified by site-directed mutagenesis. In this respect, the Bowman-Birk inhibitor of serine proteinases turned out to be an excellent model protein.

The present article on the refolding protocol using trypsin-Sepharose as a matrix with a complementary structure for the renaturation of mutant proteinase inhibitors, provides the first description of such a technique. This method should also be of interest for general use in protein engineering studies on other proteinase inhibitors from different families. The general approach, however, may be of even broader significance. The activation of inactive mutant enzymes by specific antibodies has been reported previously [15] and might be used similarly.

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