Phage Display Selection of P1 Mutants of BPTI Directed against Five Different Serine Proteinases

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The P1 position of protein inhibitors and oligopeptide substrates determines, to a large extent, association energy with many serine proteinases. To test the agreement of phage display selection with the existing thermodynamic data, a small library of all 20 P1 mutants of basic pancreatic trypsin inhibitor (BPTI) was created, fused to protein III, and displayed on the surface of M13 phage. The wild type of displayed inhibitor monovalently and strongly inhibited trypsin with an association constant of $K_a = 3 \times 10^{11} \text{M}^{-1}$. The library was applied to select BPTI variants active against five serine proteinases of different specificity (bovine trypsin and chymotrypsin, human leukocyte and porcine pancreatic elastases, human azurocidin). The results of enrichment with four proteinases agreed well with the available thermodynamic data. In the case of azurocidin, the phage display selection allowed determination of the P1 specificity of this protein with the following frequencies for selected P1 variants: 43% Lys, 36% Leu, 7% Met, 7% Thr, 7% Gln.

Key words: BPTI / Phage display / Protein inhibitors / Protein library / Serine proteinases.

The phage display strategy enables the presentation of large peptide and protein libraries on the surface of phage particles, from which molecules with desired functional property(ies) can be rapidly selected (Barbas and Burton, 1993). The great advantage of this method is a direct linkage between an observed phenotype and an encapsulated genotype, which allows for a rapid determination of selected sequences. The phage display approach has become a powerful tool in generating highly potent biomolecules (Nedwidek and Hecht, 1997), including searching for specific antibodies (Nygren and Uhlen, 1997), and exploring protein–protein interactions (Allen et al., 1995).

Protein inhibitors of serine proteinases that form canonical complexes with their target enzymes (Bode and Huber, 1992) are an excellent system to apply combinatorial procedures offered by phage display. In particular, the family of Kunitz inhibitors homologous to BPTI has been already extensively studied using this approach. As a result, many amino acid sequences that ensure strong and/or specific binding to a number of serine proteinases have been found (Roberts et al., 1992; Dennis and Lazarus, 1994a and b). Interestingly, unexpected results of selection have also been observed for kallikrein (Markland et al., 1996), thrombin (Markland et al., 1996) and chymotrypsin (Kossiakoff et al., 1993; Scheidig et al., 1997). In particular, high frequencies of the variants bearing Asn, His or Arg at P1 have been found for chymotrypsin (Kossiakoff et al., 1993; Scheidig et al., 1997). These side chains are known to bind about $10^3$ to $10^4$-fold weaker than optimal Tyr and Trp (Lu et al., 1997). Surprisingly, neither Tyr nor Trp have been observed at all after the selection.

We are currently involved in applying site-directed mutagenesis and phage display methods to select BPTI variants against several serine proteinases and to determine respective association energies. As the P1 side chain provides up to 40 – 70% of the total association energy to the complex formation (Lu et al., 1997; Qasim et al., 1997), we decided first to analyze a small library of P1 mutants of BPTI to determine whether thermodynamically optimal side chains can be selected. The enrichments were performed on four serine proteinases (bovine trypsin and chymotrypsin, porcine pancreatic and human leukocyte elastases) for which high quality thermodynamic data on binding to inhibitors with 20 different amino acids at P1 are already available (Lu et al., 1997). We also performed the screening on human azurocidin, a serine proteinase-like protein of unexplored specificity, which during evolution lost the catalytic triad (Ser195Gly, His57Ser).
BPTI gene (pAED4+BPTI plasmid) was a kind gift from Dr. P.S. Kim). Next, the fragment coding for the ompA signal sequence and antibody light chain was removed from the phagemid by restriction digestion. The resulting phagemid, pComb3H+BPTI, was confirmed by DNA sequencing to contain an in-frame fusion of pelB leader, BPTI and phage pll280-406 genes, and tested for the ability to produce infective phages that present BPTI.

In order to verify whether BPTI presented on the phage surface (BPTI<sup>D</sup>) retains its strong inhibitory activity toward trypsin, we measured the association constant for BPTI<sup>D</sup> – bovine β-trypsin interaction using standard procedures (Lu et al., 1997; Lazdunski et al., 1974) with Tos-Gly-L-Pro-L-Arg-pNA as a substrate. The determined constant, <i>K<sub>a</sub> = 3 · 10<sup>11</sup>M<sup>-1</sup></i>, is approximately 60-fold lower than the value for the interaction between BPTI and trypsin (Lazdunski et al., 1974). The decreased <i>K<sub>a</sub></i> value could be caused by a slower complex formation due to immobilization of inhibitor on the phage surface. A similar decrease in the association constant has been reported for peptide hormone-receptor interaction (Li et al., 1995). Nevertheless, BPTI<sup>D</sup> is still a very strong inhibitor of trypsin. We also verified an expected monovalent display of BPTI with 1.25 inhibitor molecules per phage particle by comparing the inhibitor concentration with the infective phage titer. pComb3H+BPTI served as a template for constructing a library of P1 BPTI variants.

BPTI and many other canonical protein inhibitors are known to change their specificity against serine proteinases due to point mutations in the primary binding loop. The 10<sup>5</sup>-fold dynamic range of association constants for coded amino acids introduced at the P1 position was observed for different proteinase-inhibitor complexes (Lu et al., 1997; Qasim et al., 1997).

The library was designed to contain all 20 P1 mutants of BPTI. The NNS scheme (N = G, A, T, C; S = G, C; all equimolar) was chosen for mutagenesis. Library construction was performed by double PCR (Bank, 1993). Diversity of the pool was verified by DNA sequencing of randomized pComb3H+BPTI, which showed equimolar distribution of introduced nucleotides. Sequencing of individual clones from the population of the initial library and pools after target proteinase selection (together about 250 clones) proved that all amino acid residues, except for proline, were represented. The absence of proline at the P1 position of sequenced clones may be caused by a very low folding yield of this mutant. The P1 Pro BPTI was the only one of 17 different P1 mutants expressed in Escherichia coli that did not exhibit correct pairings of disulfides after oxidative refolding from inclusion bodies (unpublished results from our laboratory).

We tested the efficiency of the P1 library selection using five different proteinases. The enzymes used for enrichments were chosen based on genuine differences in their P1 specificity spectra. Trypsin shows narrow specificity for the basic side chains of Lys and Arg, while the large and hydrophobic pocket of chymotrypsin is much less restrictive and is able to bind many different apolar side chains. Porcine pancreatic (PPE) and human leukocyte (HLE) elastases were chosen to test whether subtle differences in their specificities (at the level of several-fold) against small and branched hydrophobic side chains could be observed. Finally, azurocidin, which is the catalytically inactive form of a serine proteinase with a well-defined S1 pocket (Petersen et al., 1993), was chosen to explore its specificity, which cannot be determined using the classical peptide substrate assay approach.

We aimed to select the library under equilibrium conditions. The library enrichments were performed in microcentrifuge tubes on the slurry of a target protein coupled to Sepharose 4B (50 mg slurry containing 10–100 pmol of active enzyme) (Pepper, 1992), which was best suited for our approach. The reliability of the chosen method was verified by comparing the elution profile of wild-type BPTI phages to the background of helper phage elution (Figure 1). The screening involved up to 3 rounds of enrichment on an immobilized target protein. After each cycle of enrichment about 15 individual clones were sequenced to...
monitor the changes in amino acid occurrence at the P1 position.

The amino acids found at the randomized P1 position of the BPTI inhibitor after each round of selection on different target enzymes are summarized in Table 1. In general, the results show good agreement with known binding affinities. In the case of trypsin, the first round resulted in the selection of both positively-charged amino acids at P1 position. The P1 Lys and Arg BPTI variants are known to interact with trypsin at least 10^3-fold stronger, compared to other P1 mutants (unpublished results from our laboratory). The second round of enrichment led to the consensus residue Lys. Here, lack of Arg is unexpected, although 3-fold stronger binding of Lys to bovine trypsin, compared to the Arg side chain, has been reported (Zbyryt and Otlewski, 1991). Chymotrypsin does not exhibit as strong a preference for a particular type of amino acid side chain as trypsin. After two rounds of selection, 5 different amino acids (Trp, Leu, Met, Lys, and Gln) were observed. All of them bind relatively strongly to chymotrypsin when analyzed in turkey ovomucoid third domain (OMTKY3) scaffold (Lu et al., 1997). However, the presence of Gln, which binds about 10^3-fold weaker than (absent) Phe and Tyr, is surprising. Both Phe and Tyr were present after the first round of selection. The selection constants were determined in 50 mM Tris-HCl, 20 mM CaCl2, 0.01% Triton X-100, pH 8.0 at 22 °C (HNE buffer additionally contained 0.5 mM NaCl) using a developed procedure (Otlewski and Zbyryt, 1994; Lu et al., 1997). The K_s values for azurocidin-Lys15 BPTI was determined through measurement of association and dissociation rate constants by surface plasmon resonance on a Pharmacia BIACore Lötös and Johnsson, 1990). All measurements were performed on free inhibitor mutants expressed in E. coli, as described (Krokoszynska et al., 1998).

Table 1. The P1 Residues of BPTI Variants Obtained from Sequencing of Individual Clones after Selection on Target Proteinases.

<table>
<thead>
<tr>
<th>Target proteinase</th>
<th>Round</th>
<th>Observed amino acids at P1 residue</th>
<th>K_s [M^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None^a</td>
<td>2Ser, 2Lys, 1Val, 1Ile, 1Leu, 1Tyr, 1Gly, 1His</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>1</td>
<td>10Lys, 3Arg, 1Cys, 1Gln, 1Leu</td>
<td>K^{Lys} = 1.7 \times 10^{3d}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10Lys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>11Lys, 5Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2*</td>
<td>16Lys</td>
<td></td>
</tr>
<tr>
<td>PPE</td>
<td>1</td>
<td>2Phe, 2Arg, 1Trp, 1Thr, 1Tyr, 1Ser, 1Leu, 1Met, 1Lys</td>
<td>K^{Phe} = 8.0 \times 10^{2}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8Lys, 2Thr, 1Val, 1Ile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4Lys, 3Thr, 1Ala, 1Ile, 1Gln</td>
<td></td>
</tr>
<tr>
<td>HLE</td>
<td>1</td>
<td>5Lys, 4Ile, 3Val, 2Arg, 1Met, 1Thr, 1Tyr</td>
<td>K^{Val} = 6.1 \times 10^{5}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7Val, 7Ile, 3Lys</td>
<td>K^{Ile} = 1.5 \times 10^{5}</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>1</td>
<td>11Lys, 2Leu, 2Gln, 2Tyr, 1His, 1Phe, 1Met, 1Arg</td>
<td>K^{Leu} = 1.2 \times 10^{9}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14Lys, 3Trp, 2Gln, 1Met, 1Lys</td>
<td>K^{Met} = 8.7 \times 10^{7}</td>
</tr>
<tr>
<td></td>
<td>2*</td>
<td>19Lys, 6Lys, 3Trp, 2Gln, 2Met</td>
<td></td>
</tr>
<tr>
<td>Azurocidin</td>
<td>1</td>
<td>9Lys, 4Ile, 2Thr, 1Gln</td>
<td>K^{Lys} = 1.5 \times 10^{9}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10Lys, 1Met, 1Leu, 1Gln</td>
<td>K^{Met} = 2.9 \times 10^{7}</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>6Lys, 5Lys, 1Met, 1Thr, 1Gln</td>
<td>K^{Ile} = 2.1 \times 10^{7}</td>
</tr>
</tbody>
</table>

^a The pool of the initial (unselected) library.

^b The values of association constants for proteinase-inhibitor interactions are given for the most strongly selected P1 variants. Association constants were determined in 50 mM Tris-HCl, 20 mM CaCl_2, 0.01% Triton X-100, pH 8.0 at 22 °C (HNE buffer additionally contained 0.5 mM NaCl) using a described procedure (Otlewski and Zbyryt, 1994; Lu et al., 1997). The K_s value for azurocidin-Lys15 BPTI was determined through measurement of association and dissociation rate constants by surface plasmon resonance on a Pharmacia BIAcore Lötös and Johnsson, 1990). All measurements were performed on free inhibitor mutants expressed in E. coli, as described (Krokoszynska et al., 1998).

^c Rounds denoted as 1*, 2* are the screening performed on a given target with the phage pool obtained after one round of enrichment on PPE.


The library selections were performed on the target proteins covalently immobilized to Sepharose 4B by the method of Pepper (1992). Each round of selection was composed of binding of the phage library (10^11 cfu) to the target protein performed overnight in a microcentrifuge tube in 10 mM phosphate buffer, pH 7.4, containing 300 mM NaCl and 0.05% Triton X-100, washing of unbound phages with incubation buffer and then 100 mM NaCl, 0.05% Triton X-100, pH 7.0. The selected variants were eluted with 50 mM glycine-HCl, 100 mM NaCl, pH 2.5 and the fractions were neutralized with 1 mM Tris-HCl, pH 8.3. The small portion of the eluted library was used to infect E. coli XL1-Blue for isolation of individual clones for DNA sequencing. The rest of the enriched pool was propagated in cells of the E. coli SURE strain for subsequent rounds of selection.
no acids exhibiting the strongest binding to this enzyme. Though thermodynamically not large, the differences between the specificities of HLE and PPE were correctly reflected in the results of the library selection.

The positive results obtained with four proteinases encouraged us to probe the specificity of azurocidin, which, although catalytically inactive, is known to bind wild type (P1 Lys) BPTI (Petersen et al., 1993). Two rounds of selection of the P1 BPTI library showed strong affinity of azurocidin for P1 Lys but also for moderately large, uncharged P1 amino acids (Leu, Thr, Met, Gln). In this respect enrichment on azurocidin resembles that on chymotrypsin.

Of five enzymes tested, final selection on both elastases provided a rather coherent picture, agreeing well with known data on their P1 side chain preferences. However, the absence of Arg after the final selection on trypsin, and, in the case of chymotrypsin, lack of Tyr and Phe, were unexpected. Therefore, to further check the selection efficiency, we performed additional round(s) of enrichment starting from the phage pool after one cycle of enrichment on PPE. This pool of phages seemed to represent all amino acids quite well, including Arg, Lys, and Tyr, Phe, Trp, Leu, which are optimal for trypsin and chymotrypsin, respectively. We felt, however, that such preselection should have slightly changed the frequencies of individual amino acids, and, perhaps, would lead to a different selection pattern. Additionally, we performed this test for azurocidin in order to confirm its specificity spectrum.

Results from a direct selection could be confirmed after one (azurocidin) or two (trypsin, chymotrypsin) additional rounds of enrichment beginning with the PPE-preselected pool. In the case of azurocidin and trypsin, exactly the same amino acids occurred with rather similar frequencies (Table 1). The results on chymotrypsin are also in this respect coherent, although relative frequencies of Lys and Leu were reversed.

In this study we tested the efficiency of phage display selection using a set of well-defined proteinase-protein inhibitor interactions known to exhibit a very broad range of association energy. In general, for each of the proteinases studied, the selection of P1 amino acids from the small library of P1 BPTI variants was consistent with the available thermodynamic data for OMTKY3 mutants (Lu et al., 1997). Lack of full agreement may result from many factors, which were also suggested by others, including different amino acid representation due to a different number of codons, nonspecific binding, steric hindrances, nonideal selection conditions. The last point may be important for this study. Very high association constants, up to $10^{13}$ M$^{-1}$, are featured by most of the serine proteinase-protein inhibitor complexes. They are associated with extremely low dissociation rate constants ($k_{on}$) and, therefore, very long half-lives of individual complexes (up to weeks). This may lead to kinetic selection: all variants with dissociation half-lives longer than a few hours ($k_{off}$ below $10^{-4}$ s$^{-1}$) can be selected if the incubation time is not extraordinarily long. Since all inhibitors bind with rather similar association rate constants, ($k_{on}$) about $10^6$ M$^{-1}$ s$^{-1}$, kinetic selection can occur for inhibitors binding with association constants above about $10^{10}$ M$^{-1}$. Finally, it is currently not sure whether results of BPTI selection presented in this report can be directly compared with $K_i$ values determined for OMTKY3 P1 mutants (Lu et al., 1997). Structures of chymotrypsin complexes of P1 Lys variants of OMTKY3 (PDB code 1HJA) and BPTI (Scheidig et al., 1997) show completely different conformations of Lys side chain in the S1 pocket. The strong selection preference observed for P1 Lys BPTI agrees with two additional hydrogen bonds formed in the S1 pocket by the side chain of this mutant. We can expect that P1 side chains of other variants can also adopt different conformations and, hence, different binding affinities and selection patterns.

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References


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