Synthetic Peptide Mimics of the Bowman-Birk Inhibitor Protein

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Abstract: Proteins of the Bowman-Birk inhibitor family of serine proteinase inhibitors interact with the enzymes they inhibit via an exposed surface loop that adopts the ‘canonical’ proteinase inhibitory conformation. The resulting non-covalent complex renders the proteinase inactive. This inhibition mechanism is common for the majority of serine proteinase inhibitor proteins and many analogous examples are known. A particular feature of the Bowman-Birk inhibitor protein, however, is that the interacting loop is a particularly well-defined disulfide-linked short β-sheet region. Moreover, synthetic peptides based on this region keep the same structure as the corresponding part of the full sized protein and also retain inhibitory activity. This review describes the background to inhibition by Bowman-Birk inhibitor proteins (and derived peptides) and shows how peptides based on the reactive site can be manipulated in order to generate potent proteinase inhibitors with redirected specificities.

BACKGROUND—SERINE PROTEASE INHIBITORS

Proteinases are ubiquitously distributed, having key roles in a diverse range of biological processes including the blood coagulation system, the complement cascade and hormone processing pathways [1]. Naturally occurring proteinase inhibitors act to regulate proteinase activity within these pathways; synthetic inhibitors are widely used to study proteinase action or for reason of therapeutic intervention [2]. Natural proteinase inhibitors are generally proteins [3,4] and are frequently larger than the proteinase they inhibit. Many serine proteinase inhibitor proteins have evolved an inhibitory region comprising an exposed peptide loop that adopts a common ‘canonical’ conformation [4,5]. This loop is frequently constrained by the presence of disulfide bridges and/or extensive hydrogen bonding networks that act to lock the structure into the correct canonical structure. It is thought that this region has the same conformation as that of a productively bound proteinase substrate [3,4], although this assumption has recently been questioned [6]. The primary contact region contains the scissile peptide bond P1-P1′ and a few flanking residues either side, typically P4-P2′ (nomenclature of Schechter & Berger [7]). The sequence of the reactive site determines the specificity of the inhibition and the identity of the P1 residue is considered to be the main determinant of specificity and frequently reflects the substrate specificity of the target proteinase [8,9].

One goal of inhibitor research has been the reduction in size and simplification of these inhibitor proteins to their minimal structural elements [10,11]. Such proteinomimetics should be amenable for targeting and optimizing towards a given proteinase, and also allow a closer dissemination of their specific sequence and/or structural requirements. More generally, there is a growing interest in both protein mimicry and the use of natural scaffolds as templates for altered activity [12,13]. Proteinomimetics offer the potential to combine the functional benefits both of macromolecular and of low-molecular weight systems: high potency coupled with the capacity for enormous synthetic diversity. One such template that has presented itself is that of the inhibitory loop region of the Bowman-Birk inhibitor.

THE BOWMAN-BIRK INHIBITOR FAMILY

Name and History

The Bowman-Birk inhibitor (BBI) family is a typical canonical serine proteinase inhibitors. They are found in the seeds of leguminous (dicots) and gramineous (monocot) plants [14,15]. The family is named after the workers who first isolated (Bowman, 1946 [16]) and characterised (Birk, 1963 [17]) a member of this family from soybean. Soybean BBI is perhaps the most studied member of this family and often referred to as ‘classical BBI’.

Biological Aspects

The precise physiological/functional role of plant proteinase inhibitors is not entirely clear, but it would appear that they represent a form of storage protein and/or are involved in protection of the seed or whole plant from pathogens [18,19].

Of considerable potential significance is the implicated anti-carcinogenic effect of BBI (reviewed recently by Kennedy...
Populations which consume relatively high concentrations of legumes (which are also rich in members of the Kunitz inhibitor family) in their diets have relatively low incidences of prostate, colon, breast and skin cancers [21]. BBI proteins are considered important factors in this since they are highly stable to both cooking temperature and digestion [22]. It is believed that the anti-carcinogenic effect is the result of inhibition of proteolytic activity involved in the induction and/or expression of transformed cells [23]. A novel serine proteinase activity in MCF7 human breast cancer cells, susceptible to inhibition by BBI has been identified [24].

**Structural Chemistry and Features**

The covalent structure of soybean BBI was first elucidated by Odani & Ikenaka [25] and is shown in Fig. 1. More recently, several BBI structures, both alone and complexed to trypsin, have been solved by X-ray crystallography or by NMR methods (Table 1). The three-dimensional structure of BBI is shown in Fig. 2.

The primary structure of soybean Bowman-Birk inhibitor protein showing the organisation of the seven disulphide bonds. The two 9-residue reactive site loops are shaded and the positions of the P_1 residues are indicated.

The BBI proteins are small cysteine rich (typically 60–90 amino acids) proteins with a high degree of sequence homology. A network of highly conserved disulphide bridges help to maintain a rigid structure. About 40 different members of this family have been isolated [14,15,26] (SWISSPROT databank release date 16 June 2000). Phylogenetic trees for BBI-type inhibitors have been presented by Ikenaka and Norioka [27] and more recently by Prakash et al. [15]. The majority of these proteins have a symmetrical ‘double headed’ structure consisting of two

<table>
<thead>
<tr>
<th>Source</th>
<th>Complexed?</th>
<th>Resolution (Å)</th>
<th>PDB code</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adzuki bean (<em>Paseolus angularis</em>)</td>
<td>Trypsin</td>
<td>2.3</td>
<td>1tab</td>
<td>[30]</td>
</tr>
<tr>
<td>Barley (<em>Hordeum vulgare</em>)</td>
<td></td>
<td>1.9</td>
<td>1c2a</td>
<td>[28]</td>
</tr>
<tr>
<td>Mung bean (<em>Vigna radiata</em>) (fragment)</td>
<td>Trypsin</td>
<td>2.1</td>
<td>1smf</td>
<td>[50]</td>
</tr>
<tr>
<td>Pea (<em>Pisum sativum</em>)</td>
<td></td>
<td>2.7</td>
<td>1pbi</td>
<td>[57]</td>
</tr>
<tr>
<td>Soybean PI-II (<em>Glycine max</em>)</td>
<td></td>
<td>2.5</td>
<td>1p12</td>
<td>[58]</td>
</tr>
<tr>
<td>Soybean (<em>Glycine max</em>)</td>
<td></td>
<td>2.8</td>
<td></td>
<td>[59]</td>
</tr>
<tr>
<td>Soybean (<em>Glycine max</em>)</td>
<td>Trypsin</td>
<td>NMR structure</td>
<td>1bbi and 2bbi</td>
<td>[40]</td>
</tr>
<tr>
<td>Soybean</td>
<td></td>
<td>2.3</td>
<td>1df6</td>
<td>[60]</td>
</tr>
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</table>
tricyclic domains. Each domain contains an independent ‘canonical’ proteinase binding site and one inhibitor molecule can form a 1:1:1 stoichiometric complex with two different proteinases [28].

**Fi. (2).** Structure of BBI.
(A) The structure of Soybean PI-II Bowman-Birk inhibitor [58] is displayed with the backbone represented as a tube. The two reactive site loops are coloured red and the disulphides are shown in yellow.
(B) Detailed structure of one reactive site loop from this inhibitor. This is oriented as (a) and indicates the location of the P2, P1, and P1' residues (on the ‘front side’ of the inhibitory loop [31]), the cisPro and the disulphide that bridges the loop.
### Table 2  Comparison of Inhibition Constants

This table shows the inhibitory activity of Bowman Birk inhibitors with various proteinase enzymes.

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>BBI source and isform (if known)</th>
<th>$K_i$ (nM)</th>
<th>Reactive site/s</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine trypsin</td>
<td>Soybean (2-IV)</td>
<td>0.14</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.61</td>
<td></td>
<td>[59]</td>
</tr>
<tr>
<td>Torresea caerensis (TcTi)</td>
<td>1</td>
<td></td>
<td>Lys-Ser, His-Ser</td>
<td>[62]</td>
</tr>
<tr>
<td>Dioclea glabra DgTi</td>
<td>0.5</td>
<td></td>
<td>Lys-Ser, His-Ser (Lys-ser reactive against Trypsin)</td>
<td>[63]</td>
</tr>
<tr>
<td>Soybean C-II</td>
<td>37</td>
<td></td>
<td>Ala-Ser, Arg-Ser</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td>[33]</td>
</tr>
<tr>
<td>Peanut B-III</td>
<td>2</td>
<td></td>
<td>Arg-Arg, Arg-Ser</td>
<td>[33]</td>
</tr>
<tr>
<td>Faba bean (FBI)</td>
<td>6.1</td>
<td></td>
<td>Lys-Ser, Tyr-Ser</td>
<td>[65,66]</td>
</tr>
<tr>
<td>Bovine chymotrypsin</td>
<td>Soybean (2-IV)</td>
<td>6.4</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td>[59]</td>
</tr>
<tr>
<td>TcTi</td>
<td>50</td>
<td></td>
<td>Lys-Ser, His-Ser</td>
<td>[62]</td>
</tr>
<tr>
<td>Peanut-B-III</td>
<td>12</td>
<td></td>
<td>Arg-Arg, Arg-Ser</td>
<td>[68]</td>
</tr>
<tr>
<td>Faba bean</td>
<td>44</td>
<td></td>
<td>Lys-Ser, Tyr-Ser</td>
<td>[65,66]</td>
</tr>
<tr>
<td>Soybean C-II</td>
<td>86</td>
<td></td>
<td>Ala-Ser, Arg-Ser</td>
<td>[34]</td>
</tr>
<tr>
<td>Rat mast cell chymase</td>
<td>Soybean</td>
<td>13.2</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[69]</td>
</tr>
<tr>
<td>Human mast cell chymase</td>
<td>Soybean</td>
<td>0.05</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[70]</td>
</tr>
<tr>
<td>Human mast cell tryptase</td>
<td>Soybean</td>
<td>no inhibition</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[70]</td>
</tr>
<tr>
<td>Human cathepsin G</td>
<td>Soybean (2-IV)</td>
<td>1.2</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[37]</td>
</tr>
<tr>
<td>Human duodenase</td>
<td>Soybean (2-IV)</td>
<td>4a</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[37]</td>
</tr>
<tr>
<td>Human leukocyte elastase</td>
<td>Soybean (2-IV)</td>
<td>28a</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[71]</td>
</tr>
<tr>
<td>Porcine pancreatic elastase</td>
<td>Soybean</td>
<td>11,000</td>
<td>Lys-Ser, Leu-Ser</td>
<td>[72]</td>
</tr>
<tr>
<td>Soybean ClII</td>
<td>110</td>
<td></td>
<td>Ala-Ser, Arg-Ser</td>
<td>[34]</td>
</tr>
<tr>
<td>Plasmin</td>
<td>TcTi</td>
<td>36</td>
<td>Lys-Ser, His-Ser</td>
<td>[62]</td>
</tr>
<tr>
<td>Factor XIIa</td>
<td>TcTi</td>
<td>1450</td>
<td>Lys-Ser, His-Ser</td>
<td>[62]</td>
</tr>
<tr>
<td>Thrombin, Factor Xa, human plasma kallikrein, porcine pancreatic and human urinary kallikreins</td>
<td>TcTi</td>
<td>no inhibition</td>
<td>Lys-Ser, His-Ser</td>
<td>[62]</td>
</tr>
</tbody>
</table>

- The $K_i$ value is 400 nM if the inhibitor is pre-complexed with trypsin.
- The $K_i$ value is 28 nM for the initial EI complex, but subsequently forms a 2.3 nM tighter complex.

In dicots, the molecular weight of the BBI is ~8000, whilst monocots have BBI inhibitors that may be divided into two further classes, one of size ~8000 with one reactive site (which thus far, have anti-tryptic activity) and the other of ~16000 with two reactive sites [15,29]. A repetitive sequence, of 4000 and 8000 respectively, suggests gene duplication [15,29].

In addition to the conserved disulphide bridge network, the majority of BBI inhibitors have a core reactive site loop of 9 residues (an 11 residue loop is found at one reactive site of the peanut inhibitors A-II, B-II and B-III [27]). The structure of this loop is shown in detail in Fig. 2b. These loops share proline at $P_3'$ and serine at $P_1'$ [27]. The 9-residue loop comprises, within the full protein, a short type VIb β-turn. Tsunogae et al. [30] described this inhibitory loop as a two-stranded antiparallel β-sheet comprising two regions separated by the cis-Pro at $P_3'$. The ‘back side’ ($P_3'$-$P_9$) acts principally to restrain the reactive-site region in an inhibitory conformation whilst not directly interacting with the enzyme. All contacts with the proteinase are via the ‘front side’ of the loop ($P_3$-$P_2'$). Of these interacting
residues, the P1' serine residue is well conserved, possibly due to its involvement in intramolecular hydrogen bonding within the inhibitor loop [31], and the P3 cysteine residue is essential for disulfide formation.

Activity and Specificity

Dual headed inhibitors typically display either anti-chymotrypsin/trypsin or trypsin/trypsin activity. In addition to these well-studied serine proteinases, activity against an increasing number of trypsin-like and chymotrypsin-like proteinases has been described (Table 2). This includes activity against 'janus' faced proteinases such as cathepsin G and duodenase.

In common with other canonical inhibitors, the identity of the P1 residue is considered to be the main determinant of the proteinase inhibited [3,8,9]. For trypsin binding domains, the P1 residue is lysine or arginine, for pancreatic elastase, alanine, and for chymotrypsin, the P1 residue is leucine or tyrosine. Semi-synthetic variants of BBI proteins have been generated, which suggest that Phe is the optimal P1 residue for chymotrypsin, and that inhibition follows the series P>W>L>M>V>A>desL [32]. Deimination of Arg to citrulline at both reactive sites destroyed trypsin activity in peanut B-III, but altered chymotrypsin activity only marginally [33]. At P1' inhibition of chymotrypsin by soybean BBI follows the series S>A>T>V>G >desL [34]. More recently, mutational variation has been made possible by cloning and expression in E. coli [35,36].

 Whilst dual headed BBI proteins are able to inhibit two proteinases at the separate reactive sites, there are exceptions and measured alteration in the relative affinity when one site is already occupied. Soybean BBI inhibits duodenase with $K_i$ 4 nM, but 400 nM when pre-complexed with trypsin [37]. Peanut inhibitor was found to be devoid of anti-chymotryptic activity when pre-complexed with trypsin, and vice-versa [38]. Though the two reactive sites are approximately 40 Å apart [28] it is possible that in some instances there is steric hindrance if two independent proteinases attempt to bind.

Minimised Proteinase Inhibitors Based on BBI

The realisation that classical BBI could form a 1:1:1 complex with two separate proteinases led early workers to determine if they could dissect these two independent activities. Hogle and Liener [39] first demonstrated that activity was dependent upon the disulphide content. Controlled reduction resulted in complete loss of activity when an average of four bonds was broken. The positions of disulphide bridges in the soybean inhibitor were determined by a combination of cyanogen bromide and pepsin treatment [25]. Whilst the anti-tryptic activity remained the same, this method resulted in a loss of about 80% of the anti-chymotrypsin activity. The anti-tryptic region was constrained by two disulphides (Cys8-Cys24 and Cys58-Cys62) and the anti-chymotryptic region by one (Cys32-Cys51). Two polypeptide bridges (residues 25-31 and 52-57) connect these regions. The later 2D-NMR structure of BBI [40] supports this observation since it shows a larger variation in the structure of the anti-chymotrypsin domain. This implies that there are fewer stabilising tertiary interactions in that domain. Similar cleavage of other BBI proteins has similarly resulted in fragments with varying chemical and proteolytic stability [27].

INHIBITION BY BBI BASED PEPTIDES

The retention of activity following fragmentation stimulated several workers to investigate the minimal requirements necessary to reproduce this activity synthetically. Nishino et al. [41] first synthesised a cyclic peptide with activity based on the core nine residues of the anti-tryptic loop (residues 14-22). Since then several publications have described mono, bi and tri-cyclic peptides designed to mimic the activity of the BBI. In general, the peptides analysed have the following nine amino acid core:

```
---C----T/A---X---S/A---X---P---P/A---Q---C---
```

Nine-mer loops with P1 Lys were found to inhibit trypsin with a $K_i$ value of around µM [42]. Similar results were also reported by Terada et al. [43]; in addition they reported that changing the P1 residue allowed alteration of specificity with Lys or Arg inhibiting trypsin whilst Leu or Tyr allowed inhibition of chymotrypsin and subtilisin. More extended sequences that include a second disulfide show some improvements in terms of lowered $K_i$ and/or improved stability towards hydrolysis [44-46]. The activity is found to be dependent on the ends of the loops being disulphide linked as in the parent protein. Non-cyclic peptides have little or no activity [47]. Maeder et al. [48] have described an 11-mer peptide that displayed a lower $K_i$ value against trypsin than was found in the studies using 9-mer sequences. It appears that having an additional residue each side of the 9-mer core improves the inhibition properties and the stability of the peptide [46]. The disulfide itself appears only necessary to organise the structure; in the whole protein it is possible to remove this disulfide without impairing activity [36], although here the remaining structure is presumably sufficient to organise the reactive site loop.

Structural Analysis

The NMR structure of BBI peptides has been reported [49,50]. This study, which is at relatively low resolution, indicates that the peptide is organised in solution. An X-ray structure of the complex between a peptide fragment and trypsin reveals that BBI peptides adopt the same overall
conformation when bound to the active site as does the corresponding region of the parent protein [50]. We have recently solved the structure of a monocyclic BBI peptide in solution at high resolution, and find that the structure is remarkably close to that found in the BBI protein. It appears that the disulphide-linked 9-residue motif, having a cisPro and internal hydrogen bonding, represents a stable folding unit. The retention of structure in the synthetic peptides accounts for their continued activity as inhibitors.

**Hydrolytic Stability**

BBI peptides act as competitive inhibitors of their target proteinases. Poor hydrolysis rates have been reported for some BBI based peptides. Hydrolysed peptides are unable to inhibit trypsin or regenerate intact inhibitor [42]. The susceptibility of peptides to hydrolysis is sequence-dependent and does not necessarily correlate with inhibitory potency [26,46,47,51,52].

**Systematic Variation at a Single Position**

A number of studies have analysed the contribution of a single residue position within BBI peptides by systematic comparison of multiple peptides differing only at that locus.

**The P₁ Position**

Many workers have constructed BBI-based peptides that have incorporated P₁ variation [42-45,47]. The results show that changes to the P₁ residue can result in large changes in specificity, which are broadly in line with the substrate.

![Fig. (3). Bead library schematic.](image)

An 11-residue BBI-derived sequence was attached to synthesis resin via a pentaglycine spacer. Randomisation was incorporated at the positions marked Xaa and consisted of all DNA-encoded amino acids except Cys, but including Nle, to generate an 8,000 component library [53].
specificity of the proteinase in question. For example, trypsin inhibitors tend to have at P1 Arg or Lys, as would be found for trypsin substrates. This correlation of the P1 identity for inhibitors and substrates is commonly found in proteinase inhibitors in general [3,8,9].

**The P2 Position**

The role of the P2 residue within the BBI loop has been investigated by McBride et al. [26]. In this study, inhibition of chymotrypsin was monitored within the sequence SCXFSIPPPQCY (cyclised via the cysteines) where X was replaced by 26 different amino acids. It was found that Thr was the optimal residue at this position, with the side chain of this residue having a dual role in intraloop hydrogen bonding (via the –OH group) and in making hydrophobic interactions with the enzyme (via the –CH3 group) [28]. This result is supported by the library studies described below, where in a randomised library only sequences having a P2 Thr residue were selected as chymotrypsin inhibitors [53].

**The P2’ Position**

The role of this residue in trypsin binding has been analysed within the sequence SCTKSXPPQCY [51]. A total of 21 peptides were compared; the optimal residue was found to be Ile, which resulted in a Ki value of 9 nM.

**Analysis of Activity Using Peptide Libraries**

Advances in combinatorial and multiple peptide synthesis have allowed the creation and analysis of synthetic libraries of BBI-based peptides. Using a BBI-derived template sequence tethered to synthesis resin, strategic positions likely to be important for interaction with proteinases could be varied. In the first study of this kind, McBride et al. [53] constructed a library having 8,000 variations by targeting P2, P1 and P2’ for randomisation with all DNA-encoded amino acids except cysteine but including norleucine. Screening was performed using biotinylated chymotrypsin and beads that showed chymotrypsin binding were visualised by an enzymatic colour reaction utilising alkaline phosphatase-conjugated Streptavidin. Approximately 1 in 1,000 beads showed binding and the sequence of 13 beads was determined by direct Edman degradation of the active bead. Ten out of these sequences showed activity following resynthesis; the remainder had Ki values of ~20 nM for chymotrypsin and revealed a strong consensus. At P2 all sequences had Thr; P1 was Tyr or Phe; P2’ was Ile, Leu or Nle. The preference for aromatic residues at P1 is consistent with the substrate preference of chymotrypsin [54]. These results are also in accord with previous work where the P1 residue alone was varied [47].

In an extension of this work, a similar 8,000 component library was constructed having randomisation at P4, P1 and P1’ [55]. This second-generation library, which included the optimal Thr residue at P2, was screened for chymotrypsin binding to identify twelve active sequences. The consensus inhibitor sequence had at P4 Nle, P1 Phe and P2’ Ile, which showed a Ki value of 3.5 nM. The success of this approach can be appreciated by the fact that this is a better inhibition constant than is found for any full length BBI protein against this proteinase (Table 2). Screening of this P4, P1 and P1’ bead library against human leukocyte elastase (HLE) has also been described [52]. A total of 23 active binding beads were analysed in this study, with 21 of these being found to have Ala at P1 and the remaining two Thr. The consensus sequence had at P4 Nle, P1 Ala and P2’ Ile; the Ki for this sequence against HLE was 65 nM.

All of these studies with bead-immobilised peptides require pre-incubation of the bead with the proteinase. This means that sequences liable to rapid hydrolysis are not revealed, even if they are reasonable inhibitors. Specifically, sequences with P1 Trp were not selected as chymotrypsin inhibitors [55] and P1 Val was not identified amongst selected HLE binders [52] even though such sequences demonstrated low Ki values: in each case, however, these sequences were far more rapidly turned over than those selected.

**CONCLUSIONS**

Small cyclic peptides based on the reactive site of BBI protein retain the structure and activity of the canonical loop region from the parent protein. This almost unique circumstance allows the construction of highly potent proteinase inhibitors that build on the design principles found in natural proteinase inhibitors but which are easily synthesized. The potency of these short peptide loops makes it surprising, perhaps, that nature bothers to produce full-length proteins to act as inhibitors. In this regard, a recent publication by Luckett et al. [56], is extremely interesting. They describe a new inhibitor isolated from sunflower, SFTI-1, which is found to be a 14-residue fully cyclic peptide, which also incorporates a disulphide. The sequence of this peptide is shown below:

```
P1
Gly Arg Cys Thr Lys Ser Ile
Asp
Pro Phe Cys Ile Pro Phe
```

The structure incorporates exactly the same 9-residue disulphide-linked sequence found in the BBI-based peptides. However, it additionally ties together the ends of the peptide to produce a second 7-residue sequence. It seems likely that this would produce a more rigid structure and might account for the reported 0.1 nM Ki value, which is about two orders of magnitude lower than that found for analogous peptides that only contain the 9-residue loop [47].
ACKNOWLEDGEMENT

This work was supported by the BBSRC.

ABBREVIATIONS

BBI = Bowman-Birk inhibitor
Nle = Norleucine
$P_n$ = The $n$th residue of a substrate or inhibitor on the carbonyl side of the bond hydrolysed by a proteinase.
$P_n'$ = The $n$th residue of a substrate or inhibitor on the amino side of the bond hydrolysed by a proteinase.

REFERENCES