STRUCTURAL HOMOLOGY OF A TRYPSIN-PLASMIN INHIBITOR FROM LEECHES (BDELLIN B-3) WITH SECRETORY TRYPSIN INHIBITORS FROM MAMMALS

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1. Introduction

Besides the occurrence of the thrombin-specific inhibitor hirudin [1,2], considerable amounts of trypsin-plasmin inhibitors (named bdellin according to a suggestion of R. Marx, Munich) are present in the salivary glands as well as in other organs of the leech *Hirudo medicinalis* [3,4]. Especially high concentrations are found in the region of the outer sexual organs [5]. The bdellins exhibit also strong inhibitory activity towards the trypsinlike proteinase acrosin present in the acrosomes of spermatozoa [6].

Comparison of a partial sequence of bdelling B-3, one of the various multiple forms isolated in highly purified form, revealed a surprisingly strong homology to distinct portions of the sequences [13,19,20] of the pancreatic secretory trypsin inhibitors (PSTI). On the other hand, the PSTIs (or Kazal-type inhibitors) were shown recently to be structurally homologous to the seminal acrosin inhibitors [21], the double-headed elastase-trypsin inhibitor from dog submandibular glands [22], and the multiheaded proteinase inhibitors form eggs of the Japanese quail [23]. Hence, the structural principle of this sort of polypeptides is common to proteinase inhibitors from relatively simple as well as from highly specialized organisms. The Kazal-type inhibitors are especially suitable, therefore, for studies in respect to the evolution and the mode of adaptation of the inhibitors to the specificity requirements of proteinases. In the latter case, gene duplication and thus the formation of double- or multi-headed inhibitors seems to be a prerequisite for the extension of the inhibition specificity.

The wide distribution of another type of inhibitors of broad specificity was recently established. Inhibitors which are structurally homologous to the prototype BPTI [7–10] (basic pancreatic trypsin inhibitor or Kunitz inhibitor from bovine organs) are the bovine colostrum inhibitor [11], isoinhibitor K from the snail *Helix pomatia* [12], snake venom toxin I and K from *Dendroaspis polylepis* [14] and inhibitor II from *Vipera russeli* [15], the inhibitor from turtle egg white [16] and inhibitor II from sea anemones [24].

Remarkably, hirudin, the thrombin-specific inhibitor form the leech does not show much similarity to either of the two inhibitor groups [17]. The same holds true for a trypsin-chymotrypsin inhibitor from *Ascaris lumbricoides* [18], an intestinal parasite of mammals.

2. Experimental and results

Trypsin-plasmin inhibitors from the leech hirudo medicinalis were prepared as a sub-fraction from commercially available samples of 'hirudin' (Medimpex, Budapest). Using chromatographic methods [4], several related bdellins could be obtained. Bdellin B-3 exhibits the highest specific inhibitory activity.

Because from a total of 5.5 μmol of Bdellin B-3 all sequence studies had to be done, nanomole

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Table 1
Bdellin B-3 and bdellin B-3 peptides derived by tryptic and chymotryptic cleavages after performic acid oxidation

Position	1–46	1–8	9–21	13–46	13–21	22–46[a]
Cleavage[b]	–	try	try	try	try/chy	try/chy
n =	46	8	4	34	9	25
$CySO_3H$	5.60	2.09	–	4.01	1.12	2.83
Asx	5.13	1.01	–	4.00	0.93	3.07
Thr	3.84	1.68	–	1.97	0.86	1.11
Ser	1.97	–	–	1.93	0.96	–
Glx	5.93	1.14	1.01	4.03	–	4.03
Pro	–	–	–	–	–	–
Gly	4.10	–	–	3.75	2.14	1.61
Ala	3.92	–	–	3.73	–	3.73
Cys	–	–	–	–	–	–
Val	4.01	1.00	–	3.14	2.00	1.14
Met	–	–	–	–	–	–
Ile	–	–	–	–	–	–
Leu	1.98	–	0.79	1.12	–	1.12
Try	0.99	–	–	0.76	0.66	0.10
Lys	1.12	0.91	–	–	–	–
His	4.94[c]	–	1.00	5.55	–	5.55
Arg	1.11	–	1.00	–	–	–

Amino acid composition in moles per mole.
[a] Calculated by difference of peptides (13–46) minus (13–21)
[b] Cleavage by trypsin (try) or trypsin and chymotrypsin (try/chy)
[c] In stored fractions; in freshly prepared fractions 6 histidine residues were found

methods were applied for peptide chromatography [25], amino acid analysis [26] and sequence studies [27].

The amino acid composition of the inhibitor is shown in table 1, first column. By its mol. wt. of 4968, bdellin B-3 is the smallest endoproteinase inhibitor of protein nature found so far.

The polypeptide chain of bdellin B-3 presumably is crosslinked by three disulfide bridges. In contrast to other inhibitor molecules of comparable size, bdellin B-3 contains no proline residue. Only lysine, one arginine and one tyrosine residue are present, but no methionine residue.

Enzymatic cleavage with trypsin and/or chymotrypsin resulted in the liberation of peptides characterized by their amino acid compositions, see table 1, columns 2–5. Application of automatic [27] and manual degradation led to the partial sequence shown in fig.1, lower line.

When bdellin B-3 is compared to inhibitors of the Kazal type (fig.1, upper line), corresponding sequences can be clearly seen. The locations of the internal cysteine residues fit well into the structural scheme of the PSTIs. By analogy, Cys-4 should link to Cys-28, Cys-6 to Cys-25 and Cys-14 to the cysteine residue at or near the C-terminus of bdellin B-3, resulting in the formation of three loops (see below).

The reactive site residue of bdellin B-3 is lysine in position 8. When Lys-8 is altered by chemical modification (maleylation of the ϵ-amino group), the inhibitory activity is completely lost. Around this position, amino acid sequences correspond to sequences around position 18 of the PSTIs, known to be the reactive center in the other known inhibitors (e.g., see legend of fig.1).

Cleavage behind Lys-8 occurring to an extent of 15–20% in the course of the isolation by affinity chromatography does not diminish the inhibitory potency. The resulting two polypeptide fragments must be held together by the adjacent cystine bridges, as is known to be true for other inhibitors [16].

With respect to a further comparison of PSTI

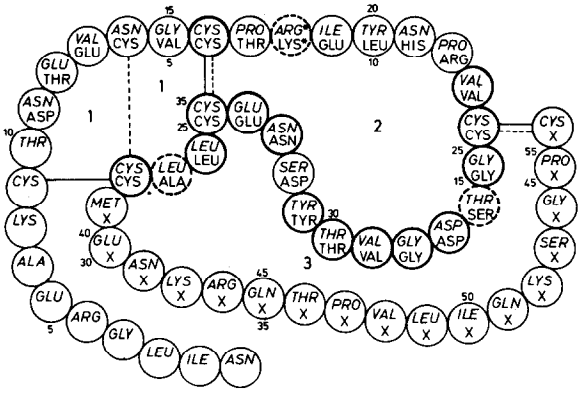

Fig.1. Structural homology between bdellin B-3 from leeches (lower line, roman type) and the bovine pancreatic secretory trypsin inhibitor [19] (upper line, italics). Thickened circles: homologous positions; broken circles: isopolar substitutions; thin circles: non-homologous positions or positions of unknown homology. Uninterrupted straight lines: S–S bridges in the bovine inhibitor. Broken straight lines: hypothetical cystine bridges in bdellin B-3. 1, 1, 2 and 3: loop 1 of the bovine inhibitor and bdellin B-3, respectively, loops 2 and loops 3. Positions 29–46 of bdellin B-3: (Asx, Thr, Ser, Glx_3, Gly_2, Ala_3, Val, His_5). OH. Comparison of bdellin B-3 reactive site (*) sequence with those of other inhibitors: bdellin B-3, positions 5–10: –Val–Cys–Thr–Lys*–Glu–Leu–. Human PSTI [17], pos. 15–20: –Gly–Cys–Thr–Lys*–Ile–Tyr–. Seminal acrosin inhibitor [22], pos. 16–21: –Phe–Cys–Thr–Arg*–Gln–Met–.

tertiary structures, the free N-terminal parts as well as the first loop of the various inhibitors show great differences both in the nature of amino acids and in the length of the polypeptide chains [13,19–21]. Of special interest is the shortening of loop 1 in the case of bdellin B-3 (fig.1). In contrast, the lengths of the other parts of the polypeptide chains apparently are identical with the only exception of the boar seminal plasma inhibitor [21], the C-terminus of which is elongated by eight amino acid residues over the last Cys compared to the other inhibitors.

The location of the last of the six cysteine residues is still unknown, as is the amino acid sequence at the C-terminus. Because of the differing character of the amino acids known to be present in this region in bdellin B-3 and in other inhibitors, only little additional homology may be expected. Besides the experimental confirmation of the presumed locations of the cystine bridges, however, the remaining sequence studies are of interest especially with respect to the positions of not less than five histidine residues in this part of the molecule of bdellin B-3.

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