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Structure, Biochemistry and Comparative Aspects of Mammalian Seminal Plasma Acrosin Inhibitors

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SEMINAL acrosin inhibitors are natural antagonists of the acrosomal proteinase "acrosin" important for the spermatozoon to penetrate the zona pellucida, the innermost layer surrounding the vitellin membrane of the egg. They have first been discovered by Haendle *et al.*[1] by their ability to inhibit pancreatic trypsins. Their presence has been demonstrated for many mammalian species like man,[2] pig,[3–6] guinea pig,[3] bull,[7] hamster,[8] rat,[9] rabbit,[10] mouse[1] and others. During the last decade attempts have been made to isolate and purify these acid- and heat-stable, low molecular-weight proteinase inhibitors. Classical methods for protein separation have been used as well as the modern technique of affinity chromatography on insoluble trypsin resin.[11]

The preparation of homogenous inhibitors is complicated by the occurrence of numerous isoinhibitors in one species *and* by the occurrence of multiple chromatographic forms. The isoinhibitors are natural mutants and gene products of different genes in a heterogeneous population. They differ by single amino acid substitutions in their amino acid sequence (Table 1). The multiple chromatographic forms are generated during processing and handling of the seminal plasma, e.g. by proteolytic attack of the polypeptide chain by aminopeptidases,[3] by acid hydrolysis of Asp–Pro peptide bonds[12] or amide groups,[13] and by a natural and introduced microheterogeneity in the carbohydrate portion of inhibitors with glycoprotein nature.[14]

The amino acid compositions of several acrosin inhibitors from seminal plasma (and from spermatozoa) of man, boar and guinea pig are presented in Table 1. All natural protein-constituent amino acids have been found. The total number of amino acids per unit of inhibitor molecule corresponds to about sixty residues. Thus, the molecular weight of these seminal inhibitors is about 6000–7500 with respect to their protein part.

In contrast to the human seminal inhibitor[2] the boar inhibitor is a glycoprotein.[3,6] Several multiple chromatographic forms of this glycoprotein have been separated by ion equilibrium chromatography on SE-Sephadex C-25 and by electrophoresis on cellulose acetate folio.[6] Their amount and elution pattern varies with pretreatment of the seminal plasma. Each of the active boar inhibitors recovered after electrophoretic separation gave an amino acid composition identical with one of the isoinhibitors A, A_1 or B.[6] However, the total

TABLE 1. AMINO ACID COMPOSITIONS OF SEMINAL ACROSIN INHIBITORS

Amino Acid	Boar isoinhibitors from plasma[a]			Boar spermatozoa[a]	Guinea pig vesicles[b]					Human plasma HUSI-II	
	A	A_1	B		d	e	f	b	β	G-1	G-2
Aspartic acid	7	7	7	7	6	6	6	6	5	6	6
Threonine	4	4	4	4	4	4	4	1	1	3	3
Serine	5	5	5	5	5	6	5	2	3	4	4
Glutamic acid	5	5	5	5	4	4	4	10	9	4	3
Proline	3	3	3	3	2	3	2	5	4	5	5
Glycine	5	5	5	5	5	5	5	6	5	6	5
Alanine	2	2	2	2	0	1	0	1	1	1	1
Valine	1	1	1	1	3	3	3	3	3	1	1
Half-cystine	6	6	6	6	6	6	6	6	6	6	6
Methionine	1	1	1	1	1	1	1	0	0	2	2
Isoleucine	2	2	2	2	1	1	1	4	3	4	4
Leucine	2	2	2	2	3	3	3	5	4	2	2
Tyrosine	3	3	3	3	4	4	4	2	1	2	3
Phenylalanine	5	5	5	5	3	4	3	0	0	1	1
Lysine	5	4	6	4	4	5	5	1	2	3	3
Histidine	3	3	3	3	3	3	3	2	2-3	2	2
Arginine	5	6	6	5	4	4	4	6	6	4	5
Tryptophan	1	1	1	(1)	0	0	0	0	0	0	0
Total	65	65	67	65	58	63	59	60	55-56	56	56
Carbohydrates	+	+	+	+	–	–	–			–	–
Molecular weight	~11,500	~11,500	~12,000	~11,500	6687	7217	6815	6772	6275-6412	6186	6319

[a]Tschesche *et al.*, 1974.[6]

[b]Fink *et al.*, 1971. Shown are the compositions of the acrosin-trypsin-plasmin inhibitors, d, e and f and of the acrosin-trypsin inhibitors b and β.[3]

[c]Schiessler *et al.*, 1974, this volume.

TABLE 2. CARBOHYDRATE COMPOSITION OF BOAR SEMINAL PLASMA ISOINHIBITORS[a]

Monosaccharides[b]	Isoinhibitors				
	A	A[c]	A_1	B	B[c]
Fucose	0.05	0.91	0.74	0.05	0.86[e]
Mannose	1.84	1.67	2.92	1.83	1.29
Galactose	1.98	2.96	5.92	1.55	1.38
Glucose	4.14[d]	0.66	0.96	1.28	1.34
Galactosamine	2.27	2.38	5.16	2.49	2.44
Glucosamine	3.40	2.80	7.96	1.35	2.87
Sialic acid	0.05	0.09[f]	0.41	0.05	0.05
Total	13–15	13	25	9–10	12–13

[a]Calculated for total m.w. 11,000.
[b]Determined after HCl–methanolysis, acetylation, and silylation by gas chromatography.
[c]Other peak from SE-Sephadex separation of isoinhibitors.
[d]Sample dialyzed for 60 hr against 25 l of dest. water prior to carbohydrate determination.
[e]Determined by cystein–H_2SO_4 method according to Dische and Shettles.
[f]Determined by resorcinol method according to Svennerholm.

carbohydrate content of the different chromatographic forms varied considerably (Table 2). The differences in the total number of monosaccharide units ranges from about ten to almost twenty-five residues per inhibitor molecule. This explains the discrepancies and multiple molecular weight forms of 7000 up to 13,000 m.w. of the boar seminal inhibitor separated by gel filtration in several laboratories.[5,6] In addition the monosaccharide composition of individual forms presented in Table 2 is subject to change and indicates a microheterogeneity of the carbohydrate moieties.

STRUCTURE OF SEMINAL ACROSIN INHIBITORS

A. *The Boar Seminal Plasma Inhibitor*

The amino acid sequence of the sixty-five residues of boar seminal plasma inhibitor A_1 was determined.[12] The inhibitor was subjected to performic acid oxidation and arginine-directed tryptic cleavage after citraconylation of the ϵ-amino groups of the lysine residues. Five arginine peptides and the C-terminal peptide were separated and purified by gel filtration and ion equilibrium chromatography. The twenty-six-residue peptide was further degraded by two lysine-directed tryptic cleavages, as indicated in Fig. 1. All tryptic peptides were sequenced in an automatic Beckman sequencer by a modified dimethyl-allylamine/trifluoroacetic acid buffer program.[12] All phenylthiohydantoins were identified by chemical ionization mass spectrometry using isobutane as reactant gas. This procedure allowed unequivocal assignment of all amides.

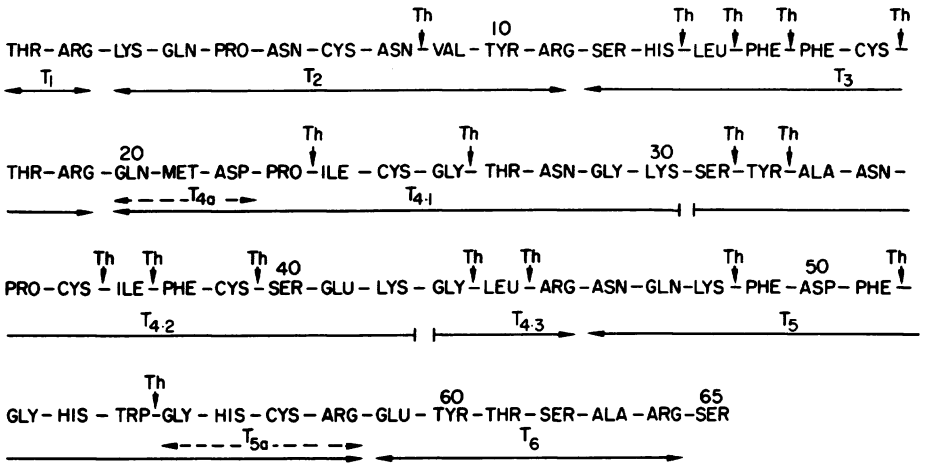

FIG. 1. The linear amino acid sequence of the acrosin inhibitor A_1 from boar seminal plasma. The arrows indicate the peptides after arginine-directed tryptic cleavage. The break points mark additional cleavages after lysine-directed cleavages of peptide T-4. The broken lines indicate peptides from limited acid hydrolysis of the Asp–Pro bond and from unspecific tryptic cleavage at Trp 54. The peptides were ordered on the basis of overlap peptides from a thermolytic digest.

Particularly noteworthy is the sensitivity of the Asp 22–Pro 23 peptide bond to acid hydrolysis. Cleavage of this bond due to acidic pretreatment of the inhibitor was found to occur in an extent of 5–10%, giving rise to two additional peptides as indicated in Fig. 1. An unspecific tryptic hydrolysis at tryptophan 54 was observed in a peptide not previously subjected to performic acid oxidation.

The tryptic peptides were ordered in the linear sequence given in Fig. 1 on the basis of overlap peptides obtained from a thermolytic digest of the native seminal inhibitor. A detailed description of the structure determination will be published separately.[12]

Three disulfide containing peptides were isolated and purified to homogeneity from the thermolytic digest of the native seminal inhibitor. The combination of the half-cystines is based on the amino acid composition of the isolated peptides each containing a single disulfide bridge, on three cycles of Edman degradation, and on the amino acid composition of the peptide halves after performic acid oxidation. The disulfide bridges are formed by the half-cystine residues I and V (Residues 7 and 39), II and IV (Residues 17 and 36), and III and VI (Residues 25 and 57). Thus, the polypeptide chain folds into a globular and compact covalent structure pictured in Fig. 2. Three disulfide bridged loops are formed which have been designated A, B and C. The chain folding is equal to that of the pancreatic secretory trypsin inhibitors.[15,16] A detailed discussion of the homologies between these structures is subject of the last subjection.

Two carbohydrate moieties have been detected in the boar seminal inhibitor A_1. Their probable attachment sites are indicated in Fig. 2. Their attachment through O-glycosidic linkages to Ser 12 and Ser 62 is based on the amino acid composition of the protein part after basic sodium borohydride induced β-elimination, on the isolation of the carbohydrate containing tryptic arginine peptides T-3 and T-6, on the results of Edman degradation of peptide T-3, and on the degradation of peptide T-6 by aminopeptidase K and carboxypeptidase B.[12,14]

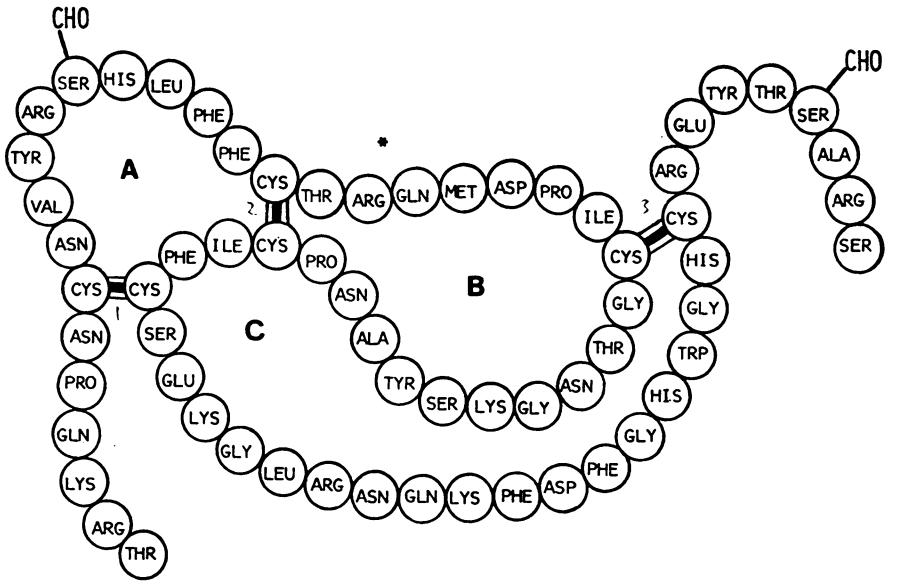

FIG. 2. Schematic representation of the covalent structure of the boar seminal plasma acrosin inhibitor demonstrating the pairing of the disulfide bridges. The probable attachment sites of the carbohydrate chains are indicated by –CHO. The reactive site peptide bond is formed by the amino acids Arg 19–Gln 20 in the B-loop as indicated by *.

B. *The Guinea Pig Seminal Vesicles Inhibitor*

The *N*-terminal amino acid sequence of the acrosin inhibitor e_2 from guinea pig seminal vesicles and of a tryptic peptide from the C-loop of the inhibitor were sequenced by conventional methods using the "dansyl-technique".[17] The amides have not been assigned. The amino acid sequence was elucidated for the first twenty-four residues including the reactive site peptide bond Lys 19–Glx 20 and for a tryptic octapeptide aligned in the positions 49 through 56 on the basis of its homology to the boar seminal inhibitor. The alignment is presented in Fig. 5. The homology between the inhibitors from boar seminal plasma and from guinea pig seminal vesicles is obvious from the alignment of the sequenced fragments and from the composition of the not yet sequenced C-terminal part of the tryptic peptide (Residues 25 through 32).

From the amino acid sequence data of these two seminal acrosin inhibitors we are tempted to assume that all low molecular-weight, acid- and heat-stable acrosin inhibitors secreted into the mammalian seminal plasma are members of the same family of inhibitor proteins.

INHIBITORY PROPERTIES

Association Constants

The association constants of some of the seminal inhibitors with acrosin of the same species as well as with trypsin have been determined using the titration method[18] and are given in

TABLE 3. ASSOCIATION CONSTANTS $(M/L)^{-1}$

Inhibitor Source	Enzymes			Reference
	Boar acrosin	Human acrosin	Bovine trypsin	
Boar seminal plasma				
Isoinhibitor A	1.4×10^9		3.2×10^9	Tschesche *et al.*, 1974[6]
Guinea pig seminal vesc.				
Acrosin-trypsin-plasmin inhibitor			3.5×10^9	Fink, 1970; Fritz *et al.*, 1970[11,17]
Acrosin-trypsin inhibitor			5×10^8	Fink, 1970; Fritz *et al.*, 1970[11,17]
Human seminal plasma				
HUSI–II		9×10^{10}		Schiessler *et al.*, 1974[2]

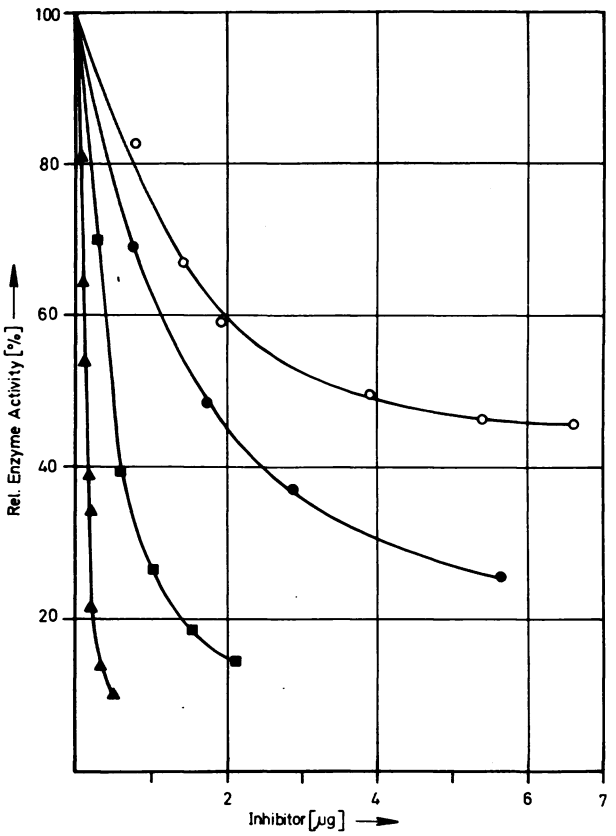

FIG. 3. Titration curves of boar acrosin with the boar seminal acrosin inhibitor (▲) and the pancreatic secretory trypsin inhibitors from sheep (■), pig (●), and dog (○). Preincubation time of enzyme and inhibitor: 30 min in 0.2 M triethanolamine/HCl-buffer, pH 7.8 at 25°C.

Table 3. The values are in the 10^9–10^{10} liters/moles range. The homology between the seminal acrosin inhibitors and the pancreatic trypsin inhibitors from dog, pig and sheep explains their cross inhibitory activity against boar acrosin[19] (Fig. 3). The most effective inhibitor, however, is still the boar seminal inhibitor indicating a species specificity and close mutual relationships in the evolution of enzyme–inhibitor systems.

Reactive Sites

The reactive site of the boar inhibitor was identified as the peptide bond Arg 19–Gln 20 by partial proteolytic cleavage of this particular bond at acid pH using boar acrosin and subsequent isolation of the two polypeptide chains.[6] On the basis of its homology to the boar inhibitor the reactive site of the guinea pig inhibitor is the bond Lys 19–Glx 20 (Fig. 5). Thus, the boar inhibitor is an Arg-type inhibitor[3,6] while the guinea pig inhibitor is of the Lys-type and susceptible to inactivation by acylation.[3]

Partial Proteolysis

The partial proteolytic cleavage of the reactive site peptide bond Arg 19–Gln 20 of the boar inhibitor by boar acrosin at pH 3.75 established a thermodynamic equilibrium with 76 ∓ 2% virgin inhibitor (peptide bond intact) and 24 ∓ 2% modified inhibitor (this bond hydrolyzed).[6]

A constant of hydrolysis of K_{hyd} = 0.33 was calculated. The same value for K_{hyd} was determined by several laboratories for the partial proteolysis of the pancreatic secretory trypsin inhibitors by trypsin.[20,21]

Temporary Inhibition

The reliberation of proteolytic activity from an enzyme inhibitor complex was designated temporary inhibition[22] and was first investigated on the pancreatic secretory trypsin inhibitors and trypsin.[13,23–25] We have observed the same phenomenon for the system boar seminal inhibitor and pancreatic trypsin.[6] However, the system boar seminal inhibitor and boar acrosin was found to be more stable[6] (Fig. 4). Isolation of more highly purified boar acrosin with higher specific activity[26] enabled us to study the phenomenon more carefully.

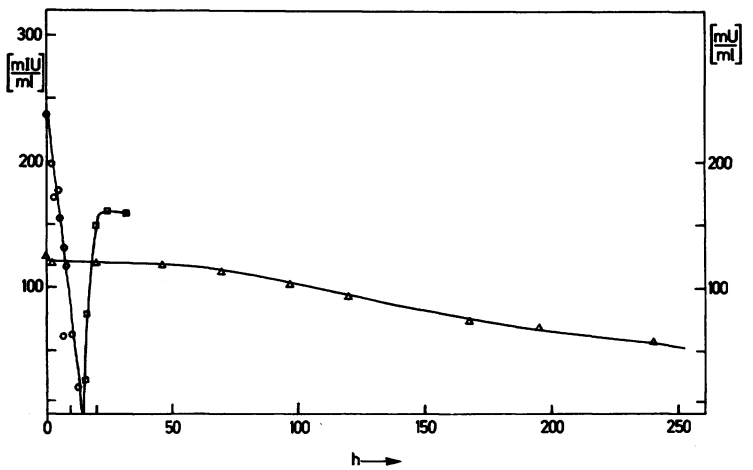

FIG. 4. Temporary inhibitory effect of the acrosin inhibitor from boar seminal plasma against boar acrosin and bovine pancreatic trypsin. Molar ratio at zero time: inhibitor trypsin = 1.5:1 and inhibitor boar acrosin = 1.7:1; in 0.2 M triethanolamin/HCl-buffer, pH 7.8 (0.02 M $CaCl_2$) at 25°C (trypsin) and 22°C (acrosin), respectively. Residual inhibitory activity against trypsin (–○–); tryptic activity reappeared (–□–); inhibitory activity against boar acrosin (–△–).

The inhibition is not permanent but can be described by the term "pseudopermanent", since the inhibition remains unchanged either at a pH of 7.8 or 3.75 of incubation for at least 45 hr at room temperature but then drops depending on pH. This is in contrast to the 4–6 times higher specific activity of boar acrosin compared to porcine (or bovine) trypsin that leads to expect a more rapid inactivation. The much slower inactivation probably reflects the more stringent steric requirements of the larger enzyme acrosin (m.w. 38,000) to attack a rigid substrate like the inhibitor. Whether the phenomenon of inactivation of the seminal inhibitor by acrosin is of any physiological significance, e.g. during the process known as capacitation, at present remains unknown.

HOMOLOGIES AND EVOLUTION

A homology between the inhibitors from boar seminal plasma and from boar pancreatic juice is obvious from the alignment of both amino acid sequences (Fig. 5). Maximal homology is obtained only if three deletions are placed in the region of the A-loop of the pancreatic inhibitor I,[27–29] e.g. in the positions 15, 17 and 18. The homology then is based on seventeen identical amino acid residues in corresponding positions and on a great number of conservative substitutions. Especially all half-cystine residues are in homologous positions. Thus, the B- and C-loop contain identical numbers of twenty and thirty-three amino acid residues, respectively, while the A-loop is extended by three residues. Since one of the carbohydrate moieties is attached to the A-loop, this loop obviously is oriented towards the surface of the molecule. Both carbohydrate chains are fairly apart from the reactive site obviously not interfering with the enzyme complex formation. From the absence of carbohydrates in the pancreatic inhibitor it is obvious that the sugar moieties are without significance for the inhibitory function. The same argument holds true for the eight C-terminal and the three N-terminal amino acid residues in the seminal inhibitor which by comparison to the pancreatic inhibitor I and the des–Thr–Ser–Glu–Pro–Arg-inhibitor I[30,31] are unimportant for the inhibitory activity.

The boar seminal and the boar pancreatic inhibitor are products of the same ancestral gene that doubled during evolution and evolved independently. Both homologous inhibitors are secretory proteins, however, one is a glycoprotein, the other a plain protein. On this basis the argument that the carbohydrates are important structural elements to allow active transport through all membranes becomes somewhat arbitrary. This situation resembles the one of the cow colostrum inhibitor, which is a glycoprotein,[32,33] and the bovine trypsin-kallikrein inhibitor (Kunitz), which is a plain protein. However, the latter inhibitor is an intracellular component and not secreted from the tissue and thus the situation is not comparable.

The comparison of the structure of the seminal inhibitors can be extended to non-mammalian inhibitors as presented in Fig. 5. These proteinase inhibitors are altogether members of a single family of genetically related proteins of which the bovine pancreatic secretory trypsin inhibitor (Kazal's inhibitor)[34] was the first representative to be discovered. The structure of the boar and guinea pig seminal inhibitors is homologous to the human,[35] bovine,[36] porcine,[27–29] and ovine pancreatic secretory inhibitors,[16,37] and to the two domains of the twice as long (doubled gene) dog submandibularis inhibitor[38] or the three domains of the three times larger (tripled gene) Japanese quail inhibitor,[39] or the already elaborated partial sequences of Bdellin B-3 from leeches.[40] All amino acid sequences have been aligned to give maximal homology. This implies that all half-cystines occupy correspond-

Boar seminal							Thr	Arg	Lys	Gln	Pro	Asn	Cys	–	–	–	–	–	Asn	Val	Tyr	Arg	Ser	His	Leu
Guinea pig seminal					Phe	Ala	Pro	Ser	Lys	Val	Asx	Ser	Cys	–	–	–	–	–	Arg	Pro	Asx	–	Ser	Asx	–
Porcine pancreatic I					Thr	Ser	Pro	Gln	Arg	Glu	Ala	Thr	Cys	–	–	–	–	–	Thr	Ser	Glu	Val	Ser	–	Gly
Bovine pancreatic					Asn	Ile	Leu	Gly	Arg	Glu	Ala	Lys	Cys	–	–	–	–	–	Thr	Asn	Glu	Val	Asn	–	Gly
Ovine pancreatic					Asn	Ile	Leu	Gly	Arg	Glu	Ala	Lys	Cys	–	–	–	–	–	Thr	Asn	Glu	Val	Asn	–	Gly
Human (Kazal)					Asp	Ser	Leu	Gly	Arg	Glu	Ala	Lys	Cys	–	–	–	–	–	Tyr	Asn	Glu	Leu	Asn	–	Gly
Dog submandibularis (I)	Gly	Pro	Pro	Pro	Ala	Ile	Arg	Gly	Arg	Glx	Val	Asx	Cys	–	–	–	–	–	Ser	Asx	Tyr	Lys	Gly	Lys	Gly
Dog submandibularis (II)													Cys	–	–	–	–	–	Thr	Glu	Tyr	–	Asx	Asx	–
Japanese quail (I)									Val	Glu	Val	Asp	Cys	Ser	Arg	Phe	Pro	Asn	Thr	Thr	Asn	Glu	Glu	–	Gly
Japanese quail (II)									, Val,	Thr)	Met	Asp	Cys	Ser	Arg	Tyr	Pro	Asn	Thr	Thr	Ser	Glu	Asp	–	Gly
Japanese quail (III)									Val	Ser	Val	Asp	Cys	Ser	Glx	Tyr	Pro	Lys	Pro	Ala	–	–	–	–	–
Bdellin B-3										Asp	Thr	Glu	Cys	–	–	–	–	–	–	–	–	–	–	–	–

Boar seminal	–	–	–	Phe	Phe	Cys	Thr	Arg	Gln	Met	Asp	–	Pro	Ile	–	Cys	Gly	Thr	Asn	Gly	Lys	Ser	Tyr	Ala	Asn	Pro	Cys
Guinea pig seminal	–	–	–	Arg)	(Met	Cys	Thr	Lys)	(Glx	Leu	Asx	–	Pro	Val,	–	Cys,	Gly,	Thr,	Asx,	Gly,	His,	Thr,	Tyr)				
Porcine pancreatic I	–	–	–	–	–	Cys	Pro	Lys	Ile	Tyr	Asn	–	Pro	Val	–	Cys	Gly	Thr	Asp	Gly	Ile	Thr	Tyr	Ser	Asn	Glu	Cys
Bovine pancreatic	–	–	–	–	–	Cys	Pro	Arg	Ile	Tyr	Asn	–	Pro	Val	–	Cys	Gly	Thr	Asp	Gly	Val	Thr	Tyr	Ser	Asn	Glu	Cys
Ovine pancreatic	–	–	–	–	–	Cys	Pro	Arg	Ile	Tyr	Asn	–	Pro	Val	–	Cys	Gly	Thr	Asp	Gly	Val	Thr	Tyr	Ala	Asn	Glu	Cys
Human (Kazal)	–	–	–	–	–	Cys	Thr	Lys	Ile	Tyr	Asn	–	Pro	Val	–	Cys	Gly	Thr	Asp	Gly	Asp	Thr	Tyr	Pro	Asn	Glu	Cys
Dog submandibularis (I)	–	Ser	Glu	Ile	Ala	Cys	Pro	Arg	Leu	His	Glx	–	Pro	Ile	–	Cys	Gly	Thr	Asp	His	Lys	Thr	Tyr	Ser	Asn	Glx	Cys
Dog submandibularis (II)	–	Thr	–	–	–	Cys	Thr	Met	Asp	Tyr	Asx	Arg	Pro	Leu	Tyr	Cys	Gly	Ser	Asp	Gly	Lys	Asn	Tyr	Ser	Asn	Lys	Cys
Japanese quail (I)	Lys	Asp	Glu	Val	Val	Cys	Pro	Asp	Glu	Leu	Arg	–	Leu	Ile	–	Cys	Gly	Thr	Asp	Gly	Val	Thr	Tyr	Asn	His	Glu	Cys
Japanese quail (II)	Lys	Val	Thr	Ile	Leu	Cys	Thr	Lys	Asp	Phe	Ser	–	Phe	Val	–	Cys	Gly	Thr	Asp	Gly	Val	Thr	Tyr	Asp	Asn	Glu	Cys
Japanese quail (III)	–	–	–	–	–	Cys	Pro	Lys	Asp	Tyr	Arg	–	Pro	Val	–	Cys	Gly	Ser	Asp	Asp	Lys	Thr	Tyr	Ser	Asn	Lys	Cys
Bdellin B-3	–	–	–	–	Val	Cys	Thr	Lys	Glu	Leu	His	–	Arg	Val	–	Cys	Gly	Ser	Asp	Gly	Val	Thr	Tyr	Asp	Asn	Glu	Cys

Boar seminal	Ile	Phe	Cys	Ser	Glu	Lys	Gly	Leu	Arg	Asn	Gln	Lys	Phe	Asp	Phe	Gly	His	Trp	Gly	His	Cys	Arg	Glu	Tyr	Thr	Ser	Ala	Arg	Ser
Guinea pig seminal													Phe	Thr	Phe	Ser	His	Tyr	Gly	Arg									
Porcine pancreatic I	Val	Leu	Cys	Ser	Glu	Asn	Lys	Lys	Arg	Gln	Thr	Pro	Val	Leu	Ile	Gln	Lys	Ser	Gly	Pro	Cys								
Bovine pancreatic	Leu	Leu	Cys	Met	Glu	Asn	Lys	Glu	Arg	Gln	Thr	Pro	Val	Leu	Ile	Gln	Lys	Ser	Gly	Pro	Cys								
Ovine pancreatic	Leu	Leu	Cys	Met	Glu	Asn	Lys	Glu	Arg	Gln	Thr	Pro	Val	Leu	Ile	Gln	Lys	Ser	Gly	Pro	Cys								
Human (Kazal)	Val	Leu	Cys	Phe	Glu	Asn	Arg	Lys	Arg	Gln	Thr	Ser	Ile	Leu	Ile	Gln	Lys	Ser	Gly	Pro	Cys								
Dog submandibularis (I)	Met	Leu	Cys	Ala	Phe	Thr	Leu	Asp	Lys	Lys	Phe	Glx	Val	Arg	Lys	Leu	Gln	Asp	Thr	Ala	Cys	Asp	Ile	Glu					
Dog submandibularis (II)	Ser	Phe	Cys	Asn	Ala	Val	Lys	Ser	Arg	Gly	Thr	Ile	Phe	?	Leu	Ala	Lys	His	Gly	Glu	Cys								
Japanese quail (I)	Met	Leu	Cys	Phe	Tyr	Asn	Lys	Glu	Tyr	Gly	Thr	Asn	Ile	Ser	Lys	Glu	Gln	Asp	Gly	Glu	Cys	Gly	(Glu,Pro, . . .						
Japanese quail (II)	Met	Leu	Cys	Ala	His	Asn	Val	Val	Gln	Gly	Thr	Ser	Val	Gly	Lys	Lys	His	Asp	Gly	Glu	Cys	Arg	Lys	Glu	Leu	Ala	Ala		
Japanese quail (III)	Asn	Phe	Cys	Asn	Ala	Val	Val	Glu	Ser	Asn	Gly	Thr	Leu	Thr	Leu	Asn	His	Phe	Gly	Lys	Cys								
Bdellin B-3																													

FIG. 5. Alignment of the homologous amino acid sequences of the boar seminal plasma acrosin inhibitor (top line)(12) and the partial sequence of the guinea pig seminal vesicles inhibitor(17) with the sequences of the porcine,(27–29) bovine,(36) ovine(16,37) and human(35) pancreatic secretory trypsin inhibitor, and with the two domains of the dog submandibularis inhibitor,(38) the three domains of the Japanese quail inhibitor,(39) and the partial sequence of Bdellin B-3 from leeches.(40)

TABLE 4. RING SIZE VARIATION OF THE A-LOOPS IN THE FAMILY OF KAZAL-TYPE INHIBITORS

Inhibitor	Number of A-loop residues
Japanese Quail I	18
Japanese Quail II	18
Japanese Quail III	9
Boar Seminal	11
Guinea pig Seminal	
Pancreatic	8
Dog Submandibularis I	13
Dog Submandibularis II	8
Bdellin B-3	1

ing positions and that a varying number of deletions had to be placed between the first and the second disulfide bridge forming the A-loop. The A-loop presents a surprisingly variable region in all inhibitors. This explains why the homology between the boar seminal and the boar pancreatic inhibitor was not obvious from the amino acid sequence of the first twenty N-terminal residues.[6,41] The number of amino acid residues forming the A-loop varies from eighteen in the two domains of the Japanese quail inhibitor over eleven in the boar and nine in the guinea pig seminal inhibitor down to just one residue in the Bdellin B-3 sequence. The variations in the ring size of the A-loops have been documented in Table 4. The data favor the hypothesis that except for the hydrophobic residue P_4 preceding the second half-cystine bridge this A-loop is unimportant for maintaining the proper confirmation and the inhibitory activity. In contrast to the residue and ring size variation in the A-loop a strict preservation of the sizes of the B- and C-loops is striking. Thus, the B- and C-loops are the key structural elements of this family of proteinase inhibitors.

The homology between the seminal and the pancreatic inhibitors documents an ancestral genetic relationship. This conclusion and the observation that biological systems like the acrosin–acrosin inhibitor or the trypsin–pancreatic trypsin inhibitor system evolve in mutual relationship allows us to anticipate that acrosin is an enzyme structurally closely related to trypsin.

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