A NEW KUNITZ-TYPE INHIBITOR FROM BOVINE SERUM AMINO ACID SEQUENCE DETERMINATION

Elmar WACHTER, Karin DEPPNER, Karl HOCHSTRASSER+, Katrin LEMPART+ and Reinhard GEIGER*

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität, Goethestr. 33 8000 München 2, +Biochemisches Labor der Klinik und Poliklinik für Hals-, Nasen- und Ohrenkranke der Universität, Marchioninistr. 15, 8000 München 70 and **Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität, Nußbaumstr. 20, 8000 München 2, FRG*

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1. Introduction

The acid-stable antitryptic activity of bovine serum is separated by gel chromatography into two fractions with apparent relative molecular mass (M_r) of 30 000 and M_r 8000. For the human system we have demonstrated that the low molecular weight inhibitor M_r 8000 (HI-8) stems from the physiological inhibitor M_r 30 000 (HI-30) present in human serum and urine (1–3). The inhibitor HI-8 represents the antitryptic active domain. In vitro, the active domain is released from the parent inhibitor HI-30 by limited tryptic proteolysis via an intermediate with M_r 14 000 (HI-14). Based on these results in the human system, we attributed the two fractions we obtained from bovine serum to an analogous parent inhibitor with M_r = 30 000 (BI-30) and an active domain with an M_r 8000 (BI-8). However, a N-terminal amino acid sequence determination proved that we had isolated a new Kunitz-type inhibitor from bovine serum. The inhibitor differs significantly in amino acid sequence from the active domains derived from the physiological inhibitors HI-30 and BI-30 present in human and bovine serum. The primary structure reveals pronounced homology to the basic trypsin–kallikrein inhibitor from bovine tissues [4]. The inhibitor shows no immunological cross-reactivity with the tissue inhibitor.

Here we present the primary structure of the new Kunitz-type inhibitor. The sequence allows a prediction of kallikrein inhibition.

2. Materials and methods

2.1. *Chemicals and reagents*

Trypsin treated with *N*-*p*-toluolsulfonyl-L-phenylalanine-chloromethyl ketone (TPCK), *N*-α-benzoyl-L-arginine-*p*-nitroanilide was obtained from Merck, Darmstadt. Porcine kallikrein and antibodies directed to the bovine basic pancreatic trypsin–kallikrein inhibitor (Trasylol®) were gifts from Bayer AG, Elberfeld. Human plasma kallikrein was isolated according to [5]. Human urinary kallikrein was isolated as in [6]. CNBr-activated Sepharose and Sephadex G-75 was from Pharmacia, Freiburg. Bio-Gel P-30 was from Bio-Rad, Gauting. Phenylene diisothiocyanate was from Eastman, Heidelberg. Phenylisothiocyanate and CNBr was from Fluka, Neu-Ulm. *N*-α-Benzoyl-arginine-ethyl ester and carbobenzoxy-L-Tyr-*p*-nitrophenyl ester were from Serva, Heidelberg. D-Val–L-Leu–L-Arg-*p*-nitroanilide was a gift from AB Kabi, Mölndal. Succinyl-L-Phe-*p*-nitroanilide was from Boehringer, Mannheim. All other reagents were of analytical grade and used as purchased.

2.2. *Isolation of the inhibitor from bovine serum*

Bovine serum (100 l) was treated with 70% $HClO_4$ to reach a final conc. 3% $HClO_4$. The supernatant was neutralized with 5 N KOH. $KClO_4$ was removed and the supernatant was concentrated to 5 l by rotary evaporation. The solution contained an antitryptic activity of 560 inhibitor units. The pH of the inhibitor solution was adjusted with 2 N NaOH at pH 7.8. Trypsin–Sepharose was added to the solution until the antitryptic activity was completely complexed.

The immobilized enzyme–inhibitor complex was washed with 5 l of 0.2 M triethanolamine/HCl buffer (pH 7.8) 0.2 M in NaCl. The inhibitory active material was released from the support by addition of 0.2 N KCl/HCl solution (pH 1.5). The recovery of antitryptic activity was 80%. The solution was concentrated by ultrafiltration on an Amincon UM-2 membrane and was subjected to gel filtration on Sephadex G-75. The antitryptic activity of the eluate was determined as in [7]. Three active fractions were identified. The fraction eluting with M_r 8000 was concentrated and desalted on Bio-Gel P-2 with 0.4 M ammonium acetate (pH 6.8) and was lyophilized repeatedly.

2.3. *Amino acid sequence determination*

2.3.1. CNBr-cleavage

Inhibitor (1 μmol) was dissolved in concentrated formic acid, 1 M in CNBr. The concentration was adjusted to 80% formic acid by addition of water to attain 1 ml final vol. The reaction was allowed to proceed for 24 h at 20°C in the dark. The fragment peptides were size-fractionated on a column of Bio-Gel P-30 minus 400 mesh developed with 80% formic acid. Amino acid analysis was performed after hydrolysis of peptides with 5.7 N HCl for 24 h at 105°C or 60 min at 160°C. Hydrolysates were analyzed on a Durrum D-500 (Palo, Alto). The *N*-terminal residues of peptides were determined by the dansylation method [8].

2.3.2. Immobilization of the inhibitor and the fragment peptides on activated porous glass supports

The inhibitor was attached via ε-amino groups to the *p*-phenylenediisothiocyanate derivative of 3-aminopropyl glass [9]. Reduction and aminoethylation of the cystine bridges was performed at the support-fixed inhibitor [10]. The CNBr-peptides with C-terminal homoserine were attached via the lactone derivative to 3-aminopropyl glass. The C-terminal peptide of the inhibitor, lacking homoserine was attached via the carboxylic group of the C-terminal Ala to 3-aminopropyl glass. The carboxylic function was activated by *N*-ethyl,*N'*-dimethylaminopropylcarbodiimide · HCl and *N*-hydroxybenzotriazol [11]. Edman degradation was performed by a home-built solid phase sequencer closely related to the one originally designed by Laursen [12,13]. The degradation program, the processing of the released anilinothiazolinones and the identification of the phenylthiohydantoins is detailed in [14].

2.4. *Assays of enzyme and inhibitor activity*

The inhibition of trypsin was tested with *N*-α-benzoyl-D,LArg-*p*-nitroanilide as substrate [7]. Inhibition of porcine pancreatic kallikrein was tested with *N*-α-benzoyl-L-Arg-ethyl ester. Inhibition of kallikrein from human plasma was tested with *N*-α-benzoyl-Pro–Phe–Arg-*p*-nitroanilide [15]. Kallikrein from human urine was assayed with *N*-carbobenzoxy-L-Tyr-*p*-nitrophenyl ester [16] and D-Val–L-Leu–L-Arg-*p*-nitroanilide. The values obtained were corrected for spontaneous hydrolysis. The inhibition constants were calculated according to [17].

3. Results and discussion

3.1. *Characterization of the acid stable proteinase inhibitors from bovine serum*

The supernatant of bovine as well as of human serum contains antitryptic activity after treatment with $HClO_4$. The antitryptic activity is separated by gel chromatography into two fractions for bovine serum with M_r 30 000 and M_r 8000. Only one fraction with M_r 30 000 is yielded in human serum (fig.1A,C). We have demonstrated that the human inhibitor is related to the inter-α-trypsin inhibitor [1–3]. Limited tryptic proteolysis of the human inhibitor leads via an intermediate with M_r 14 000 (HI-14) to an antitryptic active domain with M_r 8000 (HI-8). Limited proteolysis of the bovine antitryptic active fractions results in a similar pattern of active fractions. The distribution of the fractions, however, is strikingly different, despite constant experimental conditions. In the human system, only small amounts of the active domain with M_r 8000 are obtained, whereas in the bovine system this fraction is considerably more pronounced (fig.1B,D).

Until these experiments, the bovine active fraction with M_r 8000 had been considered to be a derivative released via intermediates from the bovine inter-α-trypsin inhibitor, although the amount of inhibitoric activity was puzzling when compared with the human system.

To complete the primary structure of the active domain from the bovine inter-α-trypsin inhibitor, the material corresponding to M_r 8000 was subjected to Edman degradation. The N-terminal sequence indi-

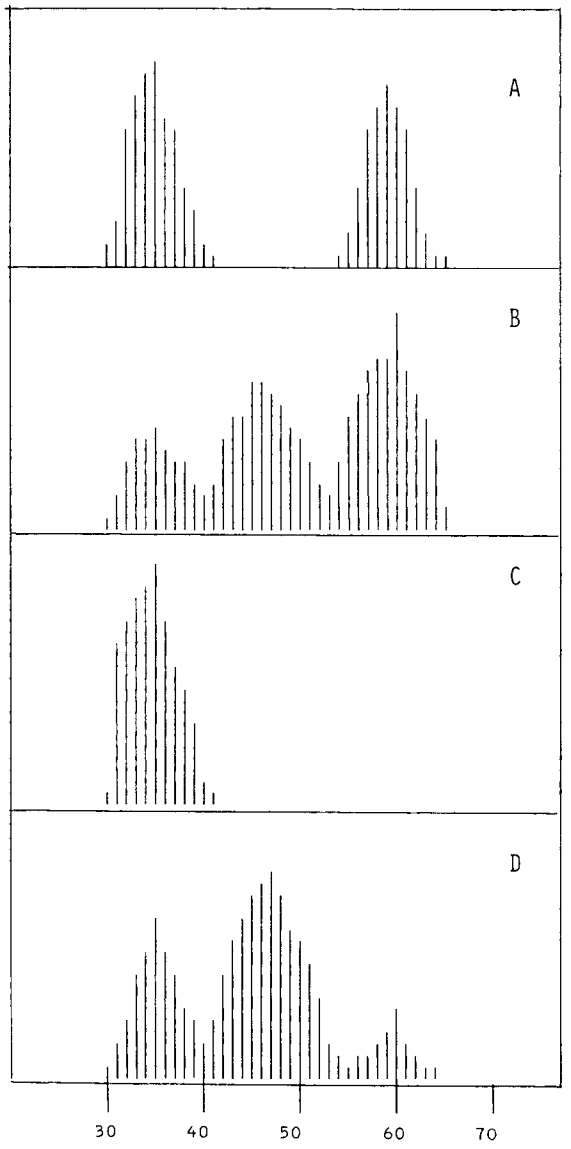

Fig.1. Gel filtration on columns of Sephadex G-75: dimension 200 × 3 cm; buffer 0.05 M borate (pH 8.0), 0.2 N NaCl; fraction vol. 15 ml; antitryptic activity (ordinate) was detected from aliquots as in [7]. (A) Supernatant of bovine serum after treatment with $HClO_4$; (B) bovine inhibitors isolated via trypsin–Sepharose; (C) supernatant of human serum after treatment with $HClO_4$; (D) human inhibitors isolated via trypsin–Sepharose.

cated that we had, in fact, isolated from bovine serum a new Kunitz-type inhibitor very closely related to the well known bovine pancreatic trypsin–kallikrein inhibitor [4], yet quite different to the antitryptic active domain derived from the bovine inter-α-trypsin inhibitor [18].

3.2. *Amino acid sequence determination*

The pattern for the sequence determination and the peptide alignment is presented in fig.2. The first step consisted in immobilizing the inhibitor at the activated support via the ε-amino groups of lysines within the polypeptide chain and the α-amino group of the N-terminus. The coupling yield was almost quantitative. Reduction and aminoethylation of the three disulfide bridges was performed using the support-fixed inhibitor. This procedure is time-saving and limits the possible gaps in the sequence. A modification of cysteines to aminoethylcysteine prior to the immobilization generates additional sidechain amino groups, i.e., additional anchor-points and possible gaps in the sequence. Furthermore, gaps must be interpreted as lysine or aminoethylcysteine, thus leading to ambiguities. In contrast, gaps obtained by the selected modification method may be attributed exclusively to lysine residues in the chain. The Edman degradation run up to aminoethylcysteine in pos. 40. The N-terminal Thr remained bound to the support.

To complete the sequence, the potential of the solid phase method of coupling selectively CNBr-peptides was utilized. From the degradation of the whole inhibitor one Met was settled at pos. 20 followed by Ile (fig.2). The bovine pancreatic trypsin inhibitor (BPTI) as well as the inhibitor from cow colostrum [4,19] contains a Met rather close to the C-terminus. Therefore it was tempting to expect a Met in the serum inhibitor too. CNBr-cleavage of the serum inhibitor resulted, in fact, in 3 fragment peptides after gel chromatography on Bio-Gel P-30. The largest fragment, B2, is characterized by a N-terminal Ile. The peptide B1 has a N-terminal Thr. The degradation of this peptide established the sequence already obtained from the previous degradation. Degradation of the peptide B2 established the second Met in pos. 54. The remaining hexapeptide B3 lacks homoserine and must be attributed, therefore, to the C-terminus of the inhibitor. Now the alignment of the fragment peptides was easy. Some ambiguite remained for res. 33 occupied by Glu in contrast to BPTI where this residue is Gln. The possibility that the $HClO_4$-treated serum inhibitor has been deamidated almost completely can not be excluded.

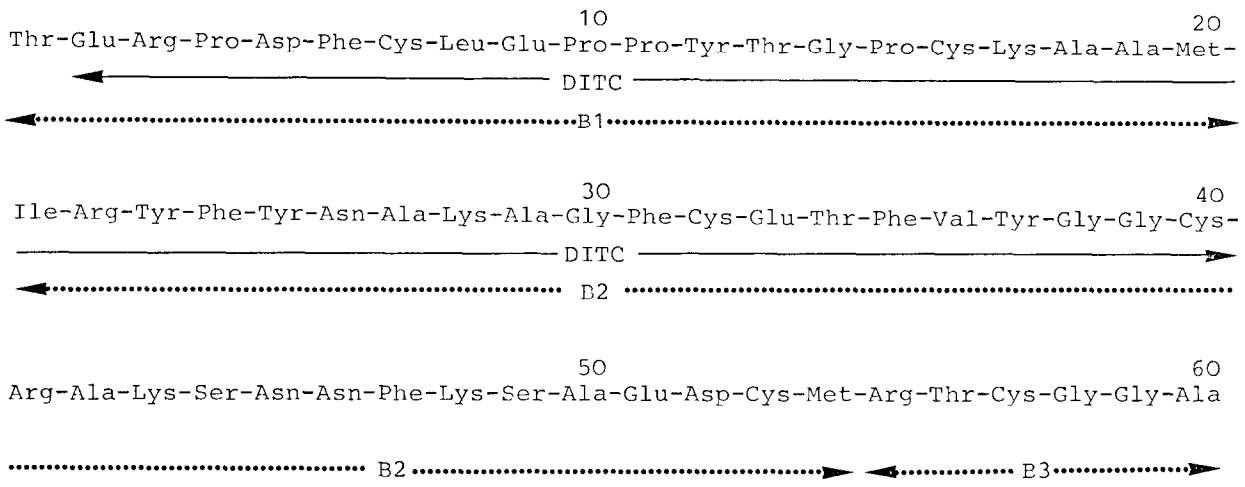

Fig.2. Pattern for amino acid sequence determination and alignment of peptides. DITC designates the degradation performed with the whole inhibitor immobilized to the *p*-phenylenediisothiocyanate derivative of 3-aminopropyl glass. B designates peptides generated by CNBr-cleavage.

3.3. *Inhibitory properties and immunological relationship*

The sequence of the bovine serum inhibitor indicates a strong structural homology to the known Kunitz-type inhibitor from bovine tissues [4]:

BI-8$^+$	T	E	R	P	D	F	C	L	E	P	P	Y	T	G	P	C	K	A	A	M	I	R	Y	F	Y	N
BPTI			R	P	D	F	C	L	E	P	P	Y	T	G	P	C	K	A	R	I	I	R	Y	F	Y	N
BI-8$^+$	A	K	A	G	F	C	E	T	F	V	Y	G	G	C	R	A	K	S	N	N	F	K	S	A	E	D
BPTI	A	K	A	G	L	C	Q	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D
BI-8$^+$	C	M	R	T	C	G	G	A																		
BPTI	C	M	R	T	C	G	G	A																		

Only 4 or 5 residues are changed. The number of replacements is dependent on the ambiguity associated with residue 33. The homologous Kunitz-type inhibitors released from the human and bovine inter-α-trypsin inhibitor are 'monovalent' inhibitors. Inhibition of chymotrypsin and plasmin is weak. BPTI is a broad specific inhibitor. A comparison of the bovine serum inhibitor with BPTI shows that inhibition of trypsin, chymotrypsin, plasmin, pancreatic and urinary kallikrein are almost identical. The K_i-values for porcine pancreatic kallikrein–inhibitor complexes are compared in fig.3. Plasma kallikrein inhibition is weak, similar to BPTI.

The structure–activity relationships of some Kunitz-type inhibitors and kallikreins from different sources was investigated in [20]. Inhibitors with basic residues in positions 17 and 39 (numbering according to BPTI) or 17 and 19 are strong inhibitors for glandular kallikreins. Inhibitors lacking the basic residues either in position 39 and/or 17 but possessing a basic residue in position 19 are still fairly strong kallikrein inhibitors. The Kunitz-type inhibitor from cow colostrum and the inhibitors derived from the human inter-α-trypsin inhibitor have no basic residue in any of these positions and do not form complexes with kallikreins. In the bovine serum inhibitor position 39 is occupied by Arg and is, in fact, a potent kallikrein inhibitor.

The bovine active domain released from the inter-α-trypsin inhibitor however, has a basic residue in pos. 39, Lys, but is no kallikrein inhibitor. One may conclude that in those cases when pos. 39 is responsible for kallikrein inhibition, this residue must not only be basic, but Arg.

The serum inhibitor shows no immunological cross-reactivity to antibodies directed to BPTI. Both inhibitors differ due to the altered sequence in the contact area to kallikrein [24]. The result affirms the assumption that the sequence, i.e., the contact area surrounding the active site in pos. 15, common to all inhibitors, is the main antigeneity-determining site.

The inhibitory properties of the bovine serum inhibitor were predicted from the elucidated sequence in connection with the pronounced homology to known and well-characterized Kunitz-type inhibitors.

Inhibitor	Source	12	13	14	15	16	17	18	19	20		34	35	36	37	38	39	40	$K_i \times 10^{-9}$ [M]
BPTI	bovine lung	-Gly-	Pro-	Cys-	Lys-	Ala-	ARG-	Ile-	ILE-	Arg-	----	-Val-	Tyr-	Gly-	Gly-	Cys-	ARG-	Ala-	0.8
SAI-5-II	sea anemone	-Gly-	Pro-	Cys-	Arg-	Ala-	ARG-	Phe-	PRO-	Arg-	----	-Ile-	Tyr-	Gly-	Gly-	Cys-	ARG-	Gly-	0.8
NNV-II	snake venom	-Gly-	Leu-	Cys-	Lys-	Ala-	ARG-	Ile-	ARG-	Ser-	----	-Ile-	Tyr-	Gly-	Gly-	Cys-	GLY-	Gly-	1.1
HHV-II	snake venom	-Gly-	Leu-	Cys-	Lys-	Ala-	TYR-	Ile-	ARG-	Ser-	----	-Ile-	Tyr-	Gly-	Gly-	Cys-	GLY-	Gly-	8.3
HPI_K	snail	-Gly-	Pro-	Cys-	Lys-	Ala-	SER-	Phe-	ARG-	Gln-	----	-Ile-	Tyr-	Gly-	Gly-	Cys-	ARG-	Gly-	27.7
CTI	colostrum	-Gly-	Pro-	Cys-	Lys-	Ala-	ALA-	Leu-	LEU-	Arg-	----	-Thr-	Tyr-	Gly-	Gly-	Cys-	GLN-	Gly-	----
BI-8^+	bovine serum	-Gly-	Pro-	Cys-	Lys-	Ala-	ALA-	Met-	ILE-	Arg-	----	-Val-	Tyr-	Gly-	Gly-	Cys-	ARG-	Ala-	12.0
HI-14-I	human ITI	-Gly-	Pro-	Cys-	Met-	Gly-	MET-	Thr-	SER-	Arg-	----	-Gln-	Tyr-	Gly-	Gly-	Cys-	MET-	Gly-	----
HI-14-II	human ITI	-Gly-	Pro-	Cys-	Arg-	Ala-	PHE-	Ile-	GLN-	Leu-	----	-Pro-	Tyr-	Gly-	Gly-	Cys-	GLN-	Gly-	----
BI-14-I	bovine ITI	-Gly-	Pro-	Cys-	Leu-	Gly-	LEU-	Phe-	LYS-	Arg-	----	-Leu-	Tyr-	Gly-	Gly-	Cys-	MET-	Gly-	----
BI-14-II	bovine ITI	-Gly-	Pro-	Cys-	Arg-	Ala-	PHE-	Ile-	?	- ? -	----	-Ser-	Tyr-	Gly-	Gly-	Cys-	LYS-	Gly-	----

Fig.3. Amino acid sequences of the proposed contact areas of structurally homologous Kunitz-type proteinase inhibitors with porcine pancreatic kallikrein in the enzyme–inhibitor complex and K_i values of the complexes. Numbering of residues is according to BPTI.
Abbreviations: BPTI, trypsin–kallikrein inhibitor from bovine tissues [4,20]; SAI-5-II, trypsin–kallikrein inhibitor from sea anemone [21]; NNV-II and HHV-II, trypsin–kallikrein inhibitor from snake venom [22]; HPI_K, trypsin–kallikrein inhibitor from *Helix pomatia* [23]; CTI, trypsin inhibitor from cow colostrum [19]. BI-8^+, trypsin–kallikrein inhibitor from bovine serum. HI-14-I, N-terminal Kunitz-type domain of the trypsin inhibitor released from the inter-α-trypsin inhibitor, inactive [2]; HI-14-II, active domain of the inhibitor [1]; BI-14-I, N-terminal Kunitz-type domain released from the bovine inter-α-trypsin inhibitor, inactive [18]; BI-14-II, active domain of the inhibitor

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