ISOLATION OF A TRYPSIN INHIBITOR (KUNITZ INHIBITOR) FROM BOVINE OVARY BY AFFINITY CHROMATOGRAPHY THROUGH TRYPSIN–SEPHAROSE

Jacqueline CHAUVET and Roger ACHER
Laboratory of Biological Chemistry, University of Paris VI, 96, Bd. Raspail, 75-Paris 6e, France

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1. Introduction

A basic trypsin inhibitor (Kunitz inhibitor) has been isolated from bovine pancreas [1–4], lungs [5], parotid glands [6], liver [7], and this polypeptide has been thought to be present in several other tissues of the ox [8]. This paper describes the isolation of Kunitz inhibitor from ovary by affinity chromatography and its characterization by amino acid analysis and partial sequence determination.

2. Results

2.1. *Purification of ovary inhibitor*

Inhibitor has first been purified by Sach et al. [9] under conditions previously described for pancreatic trypsin inhibitor. However, the purified material (Batch 10.099 RB: Choay) showed several components by paper electrophoresis [10] so that further purification was necessary. Because affinity chromatography of trypsins on ovomucoid–Sepharose was successful [11], a chromatography of ovary inhibitor on trypsin–Sepharose was attempted. Such a procedure has previously been used by Kassel and Marciniszyn [12] for isolation of pancreatic inhibitor. Activation of Sepharose was performed according to Cuatrecasas et al. [13]: 15 ml of Sepharose 4 B (Pharmacia) are filtered on a sintered glass, washed with water and resuspended in 40 ml of water; cyanogen bromide (2 g dissolved in 3 ml of water) is added. The pH is immediately adjusted to 11 by titration with 5 N NaOH and maintained for 10 min. After filtration on a sintered glass funnel, the activated Sepharose is rapidly washed with about 1 l of cold water and then with the same volume of cold 0.05 M sodium borate buffer pH 9. The activated Sepharose is suspended in 20 ml of borate buffer and 300 mg of pig trypsin (Batch CB 526: Choay), dissolved in 5 ml of the same buffer containing 0.01 M $CaCl_2$, are quickly added; the mixture is gently stirred at 4° for 18 hr. Trypsin–Sepharose is then washed with 1 l of 0.05 M borate buffer pH 9 containing 0.3 M NaCl and 0.01 M $CaCl_2$. A column of 1.5 × 7.5 cm is set up and equilibrated with 0.1 M acetate buffer pH 4.0 containing 0.3 M NaCl and 0.01 M $CaCl_2$. After about 400 ml, the absorbance at 280 nm is negligible and trypsin–Sepharose is ready for use.

18 mg of partially purified ovary inhibitor dissolved in 2 ml of acetate buffer pH 4.0, are placed on the column; trypsin–Sepharose is washed with the same buffer, and 3 ml fractions are collected. After 75 ml, no inhibitory activity was detected but an inactive material is found in the effluent (fig. 1); this product is desalted by gel-filtration on Sephadex G-25 with 0.1 M acetic acid and freeze-dried (2.3 mg). Inhibitor is eluted 0.1 M HCl containing 0.5 M NaCl and 0.01 M $CaCl_2$ (pH 1.2). After about 30 ml, inhibitor appears in the effluent; active fractions are pooled (24 ml), dialysed against water (3 changes of 100 ml, 30 min each) and desalted by passage through a column (0.9 × 61 cm) of Sephadex G-10 using 0.1 M acetic acid. The lyophilized material weighs 8.8 mg.

2.2. *Characterization of ovary inhibitor*

The amino acid composition of ovary inhibitor has been determined according to Spackman et al. [14]. Samples of 50–60 nmoles of polypeptide were hydrolyzed in an evacuated, sealed tube with 6 N HCl for 48 hr at 110°. Amino acid analysis was performed

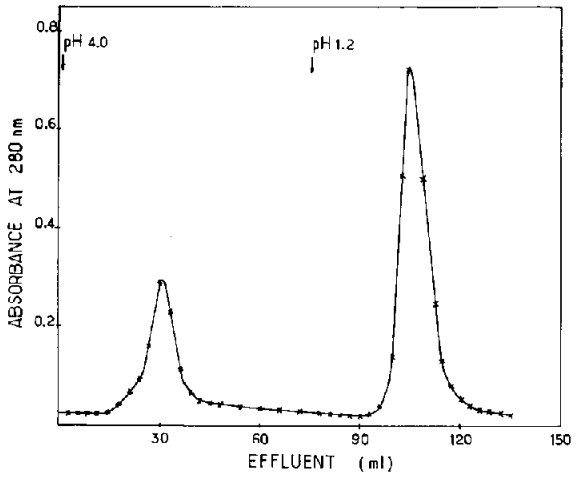

Fig. 1. Affinity chromatography of ovary trypsin inhibitor on trypsin–Sepharose (details in the text).

on an automatic analyzer Spinco 120 B fitted with a high-sensitivity cell. Cystine and methionine contents were determined as cysteic acid and methionine sulfone, respectively, on oxidized samples. Table 1 shows the experimental results for ovary inhibitor and the amino acid composition found for pancreatic trypsin inhibitor. It is clear that the two products have identical amino acid compositions.

Comparison between pancreatic and ovary inhibitors has been achieved by analysis of the peptides obtained by trypsin hydrolysis of performic acid oxidized products. Digestion by trypsin and isolation of tryptic fragments by peptide mapping were carried out as previously described [15]. The same 9 tryptic peptides found for pancreatic inhibitor were detected. Tryptic peptides were analyzed and results are given in table 2. Homologous peptides from both inhibitors have identical amino acid compositions. On the other hand peptides T_3, T_4 and T_5 were subjected to Edman degradation by using the paper strip variant [16, 17]. About 250 nmoles of each were employed. The sequences of the first residues (2, 5 and 3, respectively) were those expected.

Automatic Edman degradation [17] has been performed on inhibitor with a SOCOSI sequencer Model PS 100. Either native or carboxamidomethylated inhibitors were used. Carboxamidomethylated derivative (CAM-inhibitor) was prepared by reducing inhibitor (25 mg dissolved in 4 ml of 0.1 M borate buffer pH 8.1 containing 8 M urea) with dithiothreitol (10 mg in 0.1 ml of buffer). After 18 hr at room temp., iodoacetamide (20 mg) is added and the pH is maintained at 8.1 for 15 min with 0.1 M NaOH. The pH is lowered to 3.0 with 0.1 M acetic acid. Urea and salts are removed by passage through a column of Sephadex G-25 equilibrated with 0.1 M acetic acid and CAM-inhibitor is lyophilized (21 mg).

Table 1
Comparison of amino acid compositions of trypsin inhibitors from bovine pancreas and ovary (molar ratio, using leucine as reference).

Amino acid	Inhibitor from pancreas (number of residues per mole)	Inhibitor from ovary	
		Non-oxidized (65 nmoles)	Performic acid oxidized (48 nmoles)
Lys	4	3.72	3.60
Arg	6	6.10	6.05
Asp	5	5.15	5.09
Thr	3	2.42	2.61
Ser	1	1.03	0.96
Glu	3	3.03	3.17
Pro	4	3.52	4.00
Gly	6	6.12	6.17
Ala	6	6.24	6.22
Cys*	6	5.67	6.60
Val	1	0.97	1.13
Met*	1	0.76	1.05
Ile	2	1.45	1.61
Leu	2	2.00	2.00
Tyr	4	3.73	3.04
Phe	4	4.03	4.13

* Determined as cysteic acid and methionine sulfone, respectively, in the performic acid oxidized sample.

Merck reagents (Kit 8012 for sequential analysis) were used for Edman degradation. The program is virtually identical to that described by Edman and Begg [18]. About 600 nmoles were used either for native inhibitor or for CAM-inhibitor. Phenythiohydantoins of amino acids were identified by thin-layer chromatography [19]. Except for steps 1 and 17 where arginine derivative, non-extracted by chlorobutane, could not be identified, the amino acids expected for Kunitz inhibitor were found until isoleucine no. 18 after which no phenylthiodantoin was obtained. It is of interest to note that Edman degrada-

Table 2

Comparison of tryptic peptides of trypsin inhibitors isolated from bovine ovary and pancreas (number of residues per mole of peptide)*.

Amino Acid	T_1		T_2		T_3		T_4		T_5		T_6		T_7		T_8		T_9	
	Ovary 37 nm	Pancreas	Ovary 86 nm	Pancreas	Ovary 92 nm	Pancreas	Ovary 34 nm	Pancreas	Ovary 30 nm	Pancreas	Ovary 23 nm	Pancreas	Ovary 24 nm	Pancreas	Ovary 52 nm	Pancreas	Ovary 58 nm	Pancreas
Lys	0.67	1	–	–	–	–	0.94	1	–	–	1.00	1	0.93	1	–	–	–	–
Arg	0.73	1	1.56	1	1.53	1	–	–	1.16	1	–	–	–	–	0.97	1	–	–
$CySO_3H$	2.00	2	–	–	–	–	–	–	2.31	2	1.23	1	–	–	1.02	1	1.30	1
Asp	0.93	1	–	–	–	–	1.00	1	–	–	–	–	1.70	2	0.94	1	–	–
Thr	0.85	1	–	–	–	–	–	–	0.84	1	–	–	–	–	–	–	1.09	1
Ser	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.78	1	–	–
Glu	0.96	1	–	–	–	–	–	–	1.00	1	–	–	–	–	1.16	1	–	–
Pro	3.80	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gly	1.00	1	–	–	–	–	–	–	3.34	3	–	–	–	–	–	–	2.51	2
Ala	–	–	1.00	1	–	–	0.97	1	0.97	1	0.99	1	–	–	1.00	1	1.00	1
Val	–	–	–	–	–	–	–	–	0.89	1	–	–	–	–	–	–	–	–
$MetO_2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.91	1	–	–
Ile	–	–	–	–	2.00	2	–	–	–	–	–	–	–	–	–	–	–	–
Leu	1.00	1	–	–	–	–	–	–	0.67	1	–	–	–	–	–	–	–	–
Tyr**	0.77	1	–	–	–	–	1.73	2	0.66	1	–	–	–	–	–	–	–	–
Phe	0.95	1	–	–	–	–	1.00	1	1.12	1	–	–	1.00	1	–	–	–	–
Total number of residues	15		2		3		6		13		3		4		7		5	

* The amounts of peptides subjected to hydrolysis are indicated for ovary inhibitor (nm: nanomole).

** Tyrosine is partially destroyed when hydrolysis is performed on polypeptides eluted from paper.

tion is not stopped by the half cystine no. 5 in native inhibitor although this residue is bound in a 5–55 disulfide bridge. Similar observation was made with the half-cystine no. 1 of neurohypophysial hormones [20].

3. Discussion

Ovary inhibitor has the same amino acid composition as pancreatic inhibitor (Kunitz) gives the same tryptic peptides and has a similar N-terminal sequence. It can be concluded that both inhibitors are identical. Kunitz inhibitor has so far been chemically identified in five organs, namely pancreas, lungs, parotid glands, liver and ovary, and is likely present in most tissues. However its physiological function is not clear. It could play a general role in intracellular metabolism. If the function is really to inhibit a trypsin-like enzyme, its presence could indicate that of a trypsin-like protease. Up to now Kunitz inhibitor has only been found in ox and sheep [21] and its distribution in mammals or other vertebrates remains to be investigated.

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