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QUARTERLY

Antiproteolytic activity of goose pancreas: Purification, inhibitory properties and amino-acid sequence of a Kazal type trypsin inhibitor*

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A trypsin inhibitor of Kazal type has been isolated from goose pancreas by affinity chromatography on immobilized anhydrotrypsin, anion exchange and reverse phase HPLC. It inhibits bovine β-trypsin with the association constant (K_a) of $5.99 \times 10^8\ M^{-1}$. The complete amino-acid sequence was determined following CNBr treatment. The protein comprised a total of 69 amino-acid residues, corresponding to a molecular mass of 7.7 kDa. The P_1-P'_1 reactive site bond of the inhibitor was localized at position Lys25-Met26. The amino-acid sequence of GPTI shows extremely high homology to that of other inhibitors isolated from pancreas of birds.

The pancreatic secretory trypsin inhibitor (PSTI) was first described by Kazal [1] as a product of the acinar cells of the pancreas. Since then, PSTIs, also termed Kazal type trypsin inhibitors, have been purified from pancreas or pancreatic juice of various mammals, and their primary structures have been elucidated [2–6].

Pancreatic secretory trypsin inhibitors are known for their special character of inhibition: the enzymatic activity of trypsin reappears from the trypsin-PSTI complex as incubation proceeds [7]. This phenomenon has been called *temporary inhibition*.

The physiological role of PSTI was initially considered to be protection from premature activation of zymogen within the pancreas and the pancreatic juice. When trypsinogen is activated accidentally in the pancreas, PSTI inhibits trypsin activity to prevent activation of digestive enzymes in the pancreas. In the duodenum duct, the temporary inhibition property of PSTI allows recovery of the inhibited trypsin.

PSTI-like proteins have also been demonstrated in various tissues, including malignant tissues, therefore it has been proposed that they might have other physiological functions [8].

The Kazal type trypsin inhibitors from the pancreas of birds have not been so extensively studied. Only from chicken [9] and turkey pancreas [10] trypsin inhibitors were isolated and their amino-acid sequences were established. PSTI from the chicken pancreas consists of 69 amino-acid residues, whereas the inhibitors

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Abbreviations: AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; BAPNA, Nα-benzoyl-DL-arginine-*p*-nitroanilide; ChPTI, chicken pancreatic trypsin inhibitor; GPTI, goose pancreatic trypsin inhibitor; HPLC, high pressure liquid chromatography; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PSTI, pancreatic secretory trypsin inhibitor; RP-HPLC, reverse phase high performance liquid chromatography; TPTI, turkey pancreatic trypsin inhibitor.

from turkey consist of 72 and 70 amino-acid residues and they differ slightly in sequence. In contrast, all mammalian pancreatic trypsin inhibitors have only 56 amino acids and a very high degree of homology.

The purpose of our research was to isolate the goose pancreatic trypsin inhibitor (GPTI) and compare its sequence to that of other species.

MATERIALS AND METHODS

Materials. Goose pancreas was removed from freshly killed animals, placed on ice and stored frozen at –18°C until used. Trypsin from bovine pancreas was prepared according to Wilimowska-Pelc & Mejbaum-Katzenellenbogen [11], β-trypsin was isolated from this preparation as described by Liepniecks & Light [12], anhydrotrypsin was obtained from trypsin by the method of Ako *et al.* [13] and immobilized on Sepharose 4B according to March *et al.* [14]. Cathepsin G from human leukocytes was isolated by the method of Wątorek *et al.* [15]. Sepharose 4B was from Pharmacia Fine Chemicals (Sweden). Trifluoroacetic acid (TFA) and acetonitrile were from Pierce Chemical Company (U.S.A.). Chymotrypsin Aα, BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide), Suc-Ala-Ala-Pro-Phe-pNA, pNPGB (*p*-nitrophenyl *p*-guanidinobenzoate), CNBr were from Sigma Chemical Company (U.S.A.). Reverse phase Delta PAK C18 and DEAE -5 PW columns were from Waters Division of Millipore (U.S.A.).

All other chemicals used were of the highest purity commercially available.

Protein assay. Protein was determined at 280 nm or by the method of Goa [16]. Trypsin concentration was calculated by titration of the enzyme active center with pNPGB according to Chase & Shaw [17].

Proteinase and inhibitor activities measurement. Enzyme activities were measured spectrophotometrically at 25°C in a final volume of 1 ml, using 1 mM chromogenic substrates as follows: BAPNA for trypsin [18], and *N*-Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin and cathepsin G [19]. The following buffers were used for enzyme assays: 0.05 M Tris/HCl, pH 8.0, 5 mM $CaCl_2$ for trypsin and chymotrypsin, 0.1 M Tris/HCl, pH 7.5, 0.5 M NaCl for cathepsin G. Enzymatic reactions were terminated by addition of 0.5 ml of 30% acetic acid. One unit of antiproteolytic activity was defined as the amount of an inhibitor which reduced by half the activity of 2 μg of the enzyme tested.

Assay for stability of the trypsin-trypsin inhibitor complex. This was studied by incubating trypsin with different amounts of the inhibitor in 0.05 M Tris/HCl buffer, pH 8, containing 20 mM $CaCl_2$. Samples of the incubation mixture were periodically withdrawn, trypsin activity was determined and compared with the activity of trypsin which had been incubated under the same condition with no inhibitor added.

The equilibrium association constant (K_a) for β-trypsin-GPTI complex measurement. This was measured in 0.1 M Tris/HCl, pH 8.3, 20 mM $CaCl_2$, at 25°C, by the method developed in M. Laskowski's laboratory [20] in conditions described by Otlewski *et al.* [21]. The β-trypsin concentration was standardized by the active sites titration with pNPGB. For K_a measurement, β-trypsin concentration was 2.5×10^{-8} M and 3.33×10^{-4} M BAPNA was used to measure free enzyme concentration.

Electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE) was performed in 15% gel by the method of Laemmli [22]. The gel was stained with 0.25% Coomassie Brillant Blue R-250 in the mixture of 60% methanol in 10% acetic acid and destained with 60% methanol in 10% acetic acid. The marker proteins were: egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), α-lactalbumin (14.2 kDa) and basic trypsin inhibitor from bovine pancreas (6.5 kDa). For detection of antitryptic activity proteins were subjected to PAGE using the method of Davis [23] at pH 8.3, in 7.5% gel containing 0.1% edestin [24]. After electrophoresis the gels were incubated in 0.05 M Tris/HCl buffer, pH 8.0, containing about 1 mg percent of trypsin at room temperature until white zones became visible. Then the gels were stained with 0.05% Amido Black 10B in 7% acetic acid.

Free amino groups modification. Free amino groups in GPTI were acetylated with acetic acid anhydride according to Frankel-Conrat [25].

Sequence determination

–1. Cleavage of the methionyl bonds in GPTI was carried out in 85% formic acid by adding

one small crystal of CNBr to protein (1 mg/ml). After 24 h of the reaction at room temperature (in the dark) the solution was lyophilized and submitted to sequence determination.

–2. Sequence analyses were performed with amino-acids sequencer Model 6600/6625 Milli Gen/Biosearch. The phenylthiohydantoin derivatives of amino acids were identified by reverse phase high performance liquid chromatography (RP-HPLC). First, the inhibitor was introduced into the sequencer without any treatment. Automated NH_2-terminal sequencing yielded 28 residues from the NH_2 terminus. The N-terminal sequencing of GPTI after CNBr treatment resulted in two amino acids being released for each degradation cycle. No signals were detected at 1/2 Cys residues and the 1/2 Cys assignment in the inhibitor is based on: 1) the absence of any other signals, 2) signal at 313 nm assigned to dehydroserine, and 3) by homology to the turkey and chicken inhibitors.

–3. Amino-acids analysis was performed by the precolumn derivatization procedure. Hydrolysis (6 M HCl with 0.1% phenol, 8 h, 120°C) was followed by manual derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [26]. The AQC-derivatives of amino acids were identified using RP-HPLC. Cysteine was determined as cysteic acid after oxidation of the protein with a mixture of H_2O_2 and 88% performic acid (1:9, v/v), followed by 20 h hydrolysis at 105°C with 6 M HCl containing 0.1% of phenol.

Purification of the trypsin inhibitor from goose pancreas. All procedures except HPLC were performed at 0–5°C. Pancreas were partially thawed at room temperature, and after removal of fat homogenized in a Warning-Blendor homogenizer with 3 volumes of 80% methanol in 0.3 M HCl. The homogenate was left standing for about 20 h. After centrifugation, the supernatant was decanted and the sediment was re-extracted with 1.5 volumes of the same solvent for 2 h under constant mechanical stirring. The two extracts were pooled and 6–7 volumes of cold acetone was added. After 20 h the precipitate was collected by centrifugation and dried under vacuum. The powder was solubilized in 0.1 M HCl (150 ml/kg of tissue) and 1.8 M $HClO_4$ was added to a final concentration of 0.45 M. The precipitate was removed by centrifugation and the pH of the supernatant adjusted to 7.0 with 10 M KOH. The formed precipitate of $KClO_4$ was centrifuged off and the supernatant was applied to a column of anhydrotrypsin-Sepharose 4B (2 cm × 5 cm), equilibrated with 50 mM Tris/HCl, pH 7.0. The column was washed with about 500 ml of the same buffer containing 0.5 M NaCl, then with 300 ml of water. The adsorbed inhibitors were eluted with 10 mM HCl. Fractions with antitryptic activity were pooled and lyophilized. The inhibitor preparation was further subjected to HPLC on DEAE-5PW column (8 mm × 75 mm), equilibrated with 50 mM Tris/HCl, pH 8.4. Proteins were eluted with a linear NaCl gradient (0–55 mM) at a flow rate of 0.7 ml/min. The fractions of the main peak were pooled, lyophilized and additionally purified by RP-HPLC on a Delta PAK C18 column (3.9 mm × 300 mm) using a linear gradient of acetonitrile (0–30%) in 0.1% TFA, over 40 min at a flow rate of 1.5 ml/min.

RESULTS AND DISCUSSION

Purification and inhibitory properties

The trypsin inhibitor preparation from goose pancreas obtained after affinity chromatography was found to be heterogeneous. PAGE at pH 8.3 in the presence of edestin revealed four protein bands with antitryptic activity. The next step of purification of the inhibitor involved a HPLC anion exchange column (DEAE). The basic trypsin and cathepsin G inhibitor(s) remained unbound and were eluted from the column. The adsorbed proteins were resolved into several peaks with antitrypsin activity (Fig. 1). In this paper we have focused only on the main peak eluted at 53 mM NaCl with the highest inhibitory activity. The fractions of this peak, pooled, desalted and additionally purified by reverse phase HPLC (Fig. 2) displayed a single band of antitrypsin activity which coincided with a single protein band of 8.1 kDa as judged from SDS/PAGE. The consecutive steps of inhibitor purification are summarized in Table 1. From 1 kg of pancreas about 1.5 mg of GPTI was obtained.

As shown in Fig. 3, the isolated protein inhibited the amidase activity of bovine β-tryp-

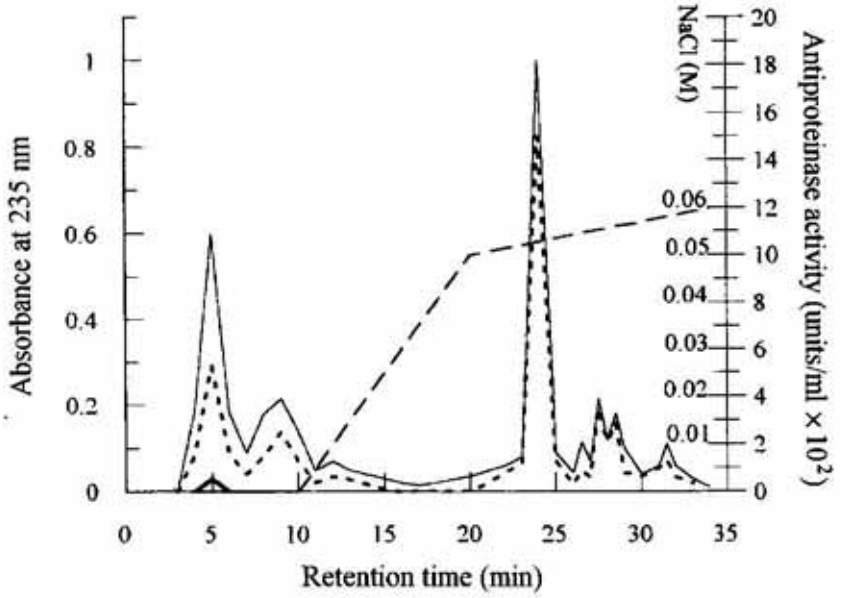

Fig. 1. HPLC-pattern of GPTI anion exchange chromatography.
The lyophilized inhibitor (1 mg) after affinity chromatography was applied on DEAE-5PW column (8 mm × 75 mm) and eluted with a NaCl gradient (0–0.055 M) in 0.05 M Tris/HCl buffer, pH 8.4. —— A_{235}, - - - NaCl [M], · · · antitryptic activity, ——— anticathepsin G activity.

sin. The association constant (K_a) was determined to be $5.99 \times 10^8\ M^{-1}$.

Like other Kazal type trypsin inhibitors, GPTI inhibits trypsin activity temporarily. As incubation proceeds the enzymatic activity of trypsin reappears from its complexes with inhibitor. In the presence of an excess of inhibitor in the incubation mixture the complexes are stable even for a few days.

The inhibitor does not inhibit either cathepsin G or chymotrypsin Aα activity and, in contrast to PSTI from mammalian pancreas, is digested by the latter enzyme.

Structural studies

Trypsin inhibitor from goose pancreas, like trypsin inhibitor from chicken pancreas (ChPTI), consists of 69 amino acids. The amino-acid sequence of GPTI is shown in Fig. 4. For comparison, the sequences of PSTI from chicken and from turkey pancreas (TPTI) are also presented [9, 10]. The sequences of these three peptides are highly homologous, differing only at Asp13, Met26, Ser46 Leu49, Tyr59, Asn65. Both in ChPTI and TPTI Ala, Asn, Val, Met, Asn and Glu are at the same positions. The differences in amide groups exist between GPTI and TPTI only at position 7 but between GPTI and ChPTI at positions 35, 50, 54.

GPTI, like TPTI, contains one methionine residue but it is located at a different position (26 in GPTI, 49 in TPTI).

The reactive site of GPTI was found to be formed by Lys25 at P_1 position and Met26 at

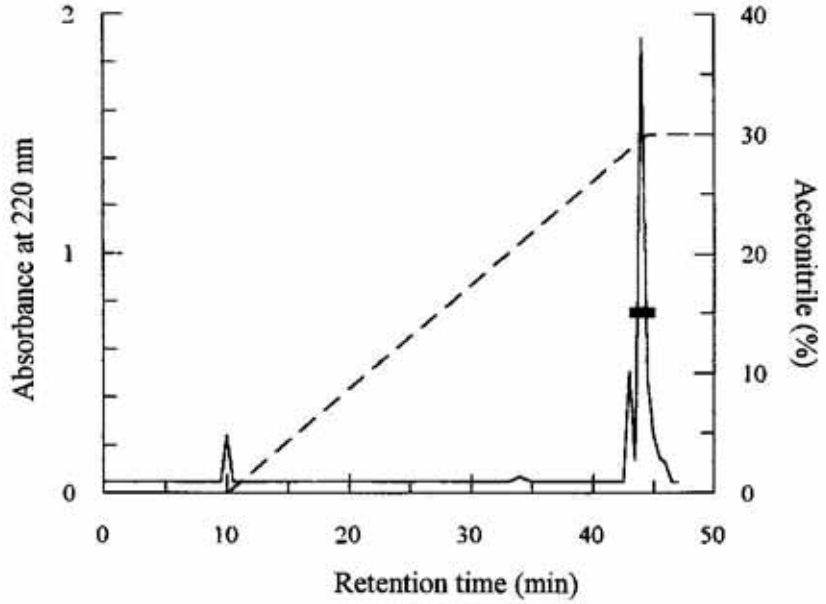

Fig. 2. Reverse phase HPLC purification of GPTI.
The inhibitor was separated on a Delta Pak C 18 column (3.9 mm × 300 mm), using a linear gradient of acetonitrile (0–30%) in 0.1% TFA, over 43 min, at a flow rate of 1.5 ml/min. ——— A_{220}, - - - acetonitrile (%). The bar indicates the pooled fractions.

Table 1
Isolation of trypsin inhibitor from goose pancreas

Purification step	Protein (mg/kg fresh weight)	Total activity (units)	Specific activity (units · mg^{-1})	Purification (fold)	Yield (%)
1. Extraction to 80% methanol in 0.3 M HCl	ND	ND	ND	ND	ND
2. Acetone powder solubilized in 0.1 M HCl	6060.0	57120.0	9.4	1	100
3. Perchloric acid precipitation-supernatant	1160.0	34510.0	29.7	3.2	60.4
4. Affinity chromatography on anhydrotrypsin Sepharose 4B[a]	8.02	23399.4	2917.6	310.4	40.9
5. Ion exchange chromatography on DEAE-5PW column (HPLC)[a]	2.5	10692.8	4277.1	455.0	18.7
6. Reverse phase chromatography (HPLC)[a]	1.4	5503.9	3931.3	418.2	9.63

[a]Protein was determined spectrophotometrically assuming that the absorption of a 0.1% solution of inhibitor at 280 nm is 1.0.

position P'_1 (Fig 4). Thus, the residue at P_1 position of GPTI is the same as in Kazal type inhibitors from chicken and turkey pancreas. This was confirmed by chemical modification of free amino groups. In contrast to ChPTI, TPTI, PSTI isolated from mammals and other inhibitors belonging to the Kazal's family of inhibitors, in GPTI there is a methionine residue at P'_1 position. The presence of a single methionine residue in the isolated trypsin inhibitor enabled us to determine the complete amino-acid sequence just after cyanogen bromide cleavage. As expected, the amino-terminal sequencing of GPTI after CNBr treatment resulted in two amino acids being released for each degradation cycle. The first sequence started from Glu1 and ended at Lys25, the second sequence started from Phe27 and ended at Arg69. NH_2-terminal sequencing of native GPTI yielded 28 residues from NH_2-terminus and was in a good agreement with the NH_2-terminal sequence after CNBr fragmentation.

The sequence data are consistent with the amino-acid composition reported in Table 2, with the exception of 1/2 Cys residues. Even after performic acid oxidation we are able to

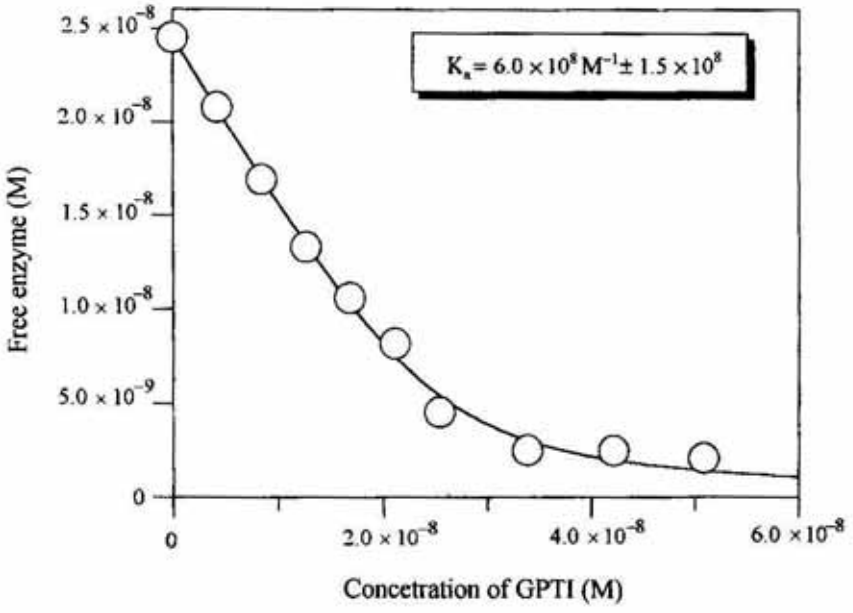

Fig. 3. Inhibition curve of bovine β-trypsin by goose pancreatic trypsin inhibitor in 0.1 M Tris/HCl buffer, pH 8.3, 20 mM $CaCl_2$, 0.005% Triton X-100 at 25°C.
β-Trypsin concentration was 2.5×10^{-8} M, 3.33×10^{-4} M BAPNA was used to measure free enzyme concentration.

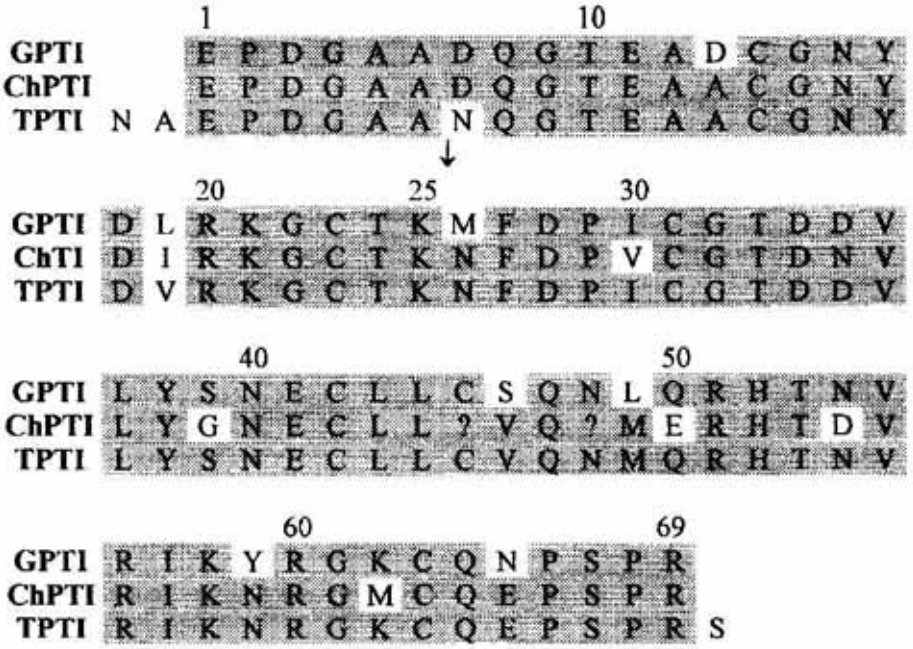

Fig. 4. The primary structure of goose pancreatic trypsin inhibitor (GPTI).
For comparison the amino-acid sequences of the trypsin inhibitors from chicken (ChPTI) and turkey (TPTI) pancreas are shown. The reactive site is indicated by the arrow. Identical residues are shadowed.

estimate not more than 4 to 4.4 residues of this amino acid per mol of GPTI.

The molecular mass of 7.712 kDa calculated from sequence analysis is in agreement with the value determined by SDS/PAGE (8.1 kDa).

Goose pancreatic trypsin inhibitor is the third Kazal type trypsin inhibitor isolated from a bird's pancreas, the primary structure of which has been elucidated. There are small differences in the amino-acid sequences between inhibitors from goose, turkey and chicken. All of them consist of a greater number of amino acids than Kazal type trypsin inhibitors isolated from pancreas of mammals.

The differences in primary structure between them concern mainly the N-terminal and C-terminal part of these proteins. On the other hand, the amino-acid sequences surrounding the reactive site bonds are very similar to those of other known Kazal type trypsin inhibitors (Fig. 5). The main feature that distinguishes GPTI from all trypsin inhibitors belonging to the Kazal type inhibitors family is the presence of the methionine residue at P'_1 position in its reactive site bond.

				↓					
Goose	Gly	Cys^{23}	Thr	*Lys*	*Met*	Phe	Asp	Pro	Val
Chicken	Gly	Cys^{23}	Thr	*Lys*	*Asn*	Phe	Asp	Pro	Val
Turkey	Gly	Cys^{25}	Thr	*Lys*	*Asn*	Phe	Asp	Pro	Val
Human	Gly	Cys^{16}	Thr	*Lys*	*Ile*	Tyr	Asn	Pro	Val
Porcine	Gly	Cys^{16}	Pro	*Lys*	*Ile*	Tyr	Asn	Pro	Val
Bovine	Gly	Cys^{16}	Pro	*Arg*	*Ile*	Tyr	Asn	Pro	Val
Ovine	Gly	Cys^{16}	Pro	*Arg*	*Ile*	Tyr	Asn	Pro	Val
Canine	Gly	Cys^{17}	Asn	*Lys*	*Ile*	Tyr	Asn	Pro	Ile

Fig. 5. The amino-acid sequence near the reactive site bond of the secretory trypsin inhibitors from the pancreas of several species. The reactive site for each species is underlined.

Table 2
Amino-acid composition of GPTI

Amino acid	Mol residue/mol protein	Sequence data
Asx	11.8 (12)	12
Ser	3.3 (3)	3
Glx	6.6 (7)	7
Gly	6.5 (6)	6
His	1.13 (1)	1
Arg	5.3 (5)	5
Thr	3.9 (4)	4
Ala	3.2 (3)	3
Pro	4.4 (4)	4
Tyr	3.0 (3)	3
1/2Cys[a]	4.4 (4)	6
Val	2.3 (2)	2
Met	0.9 (1)	1
Lys	3.9 (4)	4
Ile	2.0 (2)	2
Leu	5.0 (5)	5
Phe	1.02 (1)	1
Total	67	69

[a]Determined as cysteic acid

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