# A PROTEASE INHIBITOR IN BEE VENOM
## Identification, Partial Purification and some Properties

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## 1. Introduction

During studies on the pharmaco-biochemistry of bee venom it was found that it exhibited an inhibitory effect on trypsin. There are no data on the presence of proteinase inhibitors in bee venom and poisonous extracts from insects and reptiles in general. The biologically and biochemically active components of bee venom are the peptides: melittin, apamin, mast degranulating peptide, minimine and the enzymes: hyaluronidase and phospholipase A [1, 2].

This paper present data on the isolation and characterization of the protease inhibitor in bee venom.

## 2. Materials and methods

Freeze-dried bee venom obtained in Bulgaria, USSR, Czechoslovakia, and USA was used.

The Bulgarian bee venom was obtained from CCU "Nektarkoop" Sofia. The Czechoslovakian venom was obtained from "Slovakofarma", Bratislava; the American in two grades — first and ordinary — from "Sigma", and the Soviet was courteously supplied by Sh. Omarov-Mahatshkala.

The bee venom was isolated as described by Benton et al. [3].

The assays were performed with 2 X crystallized salt-free trypsin from "Boehringer", benzoylarginine-*p*-nitroanilide-HCl (BAPNA) and casein Hammarsten from BDH, agarose from "Serva" crystalline hemoglobin and cytochrome *c* were products of "Fluka" soya bean trypsin inhibitor from "Koch Light", "Trasylol Bayer", 5 X crystallized ribonuclease "Reanal", melit-

tin-peptide from bee venom was separated and purified by us by the method of Habermann [4], apaminpeptide from bee venom was a generous gift from R. Shipolini.

Sephadex was obtained from Pharmacia, Ltd., U.K.

### 2.1. Gel Filtration on Sephadex G-50

Bulgarian bee venom was utilized for gel filtration. Prior to fractionation the bee venom was subjected to vacuum dialysis in "Visking" tubing (8/32) against 0.1 M ammonium formate buffer, pH 4.8. The solution of the substances not retained by the membrane were freeze dried and chromatographed on Sephadex G-50 column (250 X 5.5 cm) equilibrated with 0.1 M ammonium formate buffer, pH 4.8. Elution was carried out with the same buffer. The fractions were examined for trypsin inhibitors activity using BAPNA as a substrate.

### 2.2. Chromatography on Sephadex CM 25

A portion of the melittin peak carrying trypsin inhibitory activity was chromatographed on CM-Sephadex column (45 X 2.5 cm). Elution was performed with a linear gradient of ammonium formate buffer, pH 5.2 ranging from 0.1 to 2.5 M. The electrical conductivity of the fractions was measured with an LKB conductolyser.

Melittin in the eluted fractions was determined as detergent by the hemolytic test. The solution containing 0.5 ml of the solution examined + 0.5 ml 0.067 M isotonic phosphate buffer, pH 7.2 + 3 ml of buffered human red cell suspension ($A_{600}^{1cm} = 2$) was incubated for 30 min at $37°C$.

## 2.3. *Determination of the trypsin inhibitory activity*

The trypsin inhibitory activity of the bee venom and the eluates was determined using BAPNA substrate following the procedure of Markwardt and Richter [5]. The inhibitor was added in a concentration producing 40–60% inhibition. The inhibitory activity decreasing the rate of hydrolysis of the substrate by 1 $\mu$mole per min was defined as one protease inhibitory unit (PIU). One PIU equals 1880 kalikrein units (KIU) [5].

The inhibition of the proteolytic activity of trypsin was determined by the method of Kunitz employing casein Hammarsten as substrate [6]. The inhibitory activity causing a reduction of the extinction of the sample, measured at 280 nm, by one per min in the trichloroacetic acid filtrate was defined as one inhibitory unit. The inhibitor concentration was chosen so as to cause 40–60% inhibition. The enzyme–buffer solution was reincubated for 5 min with the inhibitor prior to the assay.

## 2.4. *Molecular weight estimation*

The molecular weight of the partially purified inhibitor was determined by thin-layer chromatography on Sephadex G-50 superfine [7]. The plate was equilibrated and developed with 0.1 M ammonium formate buffer, pH 4.8. Cytochrome *c*, hemoglobin, soya bean trypsin inhibitor, ribonuclease, trasylol, apamine (mol. wt. on gel filtration 3,100) serving as standards.

The protein spots were transferred on Whatman-3 filter paper and stained with Bromphenol Blue. The location of the inhibitor spot was determined enzymatically using 1.2% agarose plate containing 2 mg per cent trypsin, buffered with 0.1 M Tris-CaCl$_2$, pH 7.8. To this end the material was transferred from the thin layer on "Whatman-3" filter paper and was allowed to diffuse into the agarose plate for 2 hr at 2°C. A strip of filter paper soaked in 40 mg per cent BAPNA was then spread over the plate. Following diffusion for 2 hr at 2°C and incubation for 3 hr at 37°C the inhibitor spot was developed.

## 2.5. *Electrophoresis*

The partially purified inhibitor was subjected to vertical plate electrophoresis in 2 X 20 X 120 mm cuvette loaded with 15% polyacrylamide gel, at pH 2.6, for 90 min (U = 8 V/cm, i = 6.8 mA/cm$^2$ [8]. The gel

was stained with Amido Black 10B and scanned with a Carl-Zeiss Jena densitometer. The location of the protease inhibitor on the electrophoretogram was determined enzymatically placing the polyacrylamide plate on a buffered (0.1 M Tris-CaCl$_2$, pH 4.8), 1.2% agarose plate containing trypsin (2 mg per cent). Diffusion, incubation and development were the same as previously described for Sephadex thin-layer chromatography.

Performate oxidation was performed by the method of Hirs [9].

Peptic digestion of the inhibitor was carried out with 10 mg per cent solution of pepsin (1:10 000), in 0.05 N HCl for 20 hr at 37°C.

The inhibitory activity following temperature, chemical and enzymatic treatment was estimated by the method of Markwardt and Richter [5]. Spectrophotometric measurements were made using an "Opton" MPQ$_2$II Spectrophotometer.

# 3. Results and discussion

Bee venoms of different origins, using BAPNA as a substrate, showed the following trypsin inhibitory activity per g:

| Bulgarian | Soviet | Czecoslovakian | American, grade I | American, ordinary |
|---|---|---|---|---|
| 7.9 PIU | 8.2 PIU | 8.0 PIU | 12.6 PIU | 11.5 PIU |

The manifestation of protease inhibition by all samples examined and particularly the stronger inhibitor by the purer bee venom of Sigma indicated that the inhibitory principle is a natural component of the venom and is not an extraneous product. The Bulgarian bee venom was subjected to vacuum ultrafiltration against 0.05 M ammonium formate buffer, pH 4.8.

It was established that the inhibitor passes through "Visking" dialysis tubing (8/32). The dialysable portion of the venom was lyophilized and fractionated on Sephadex G-50. Only the melittin peak (fig. 1) exhibited trypsin inhibitory activity. The latter was most strongly manifested in the descending slope of the curve. The asymmetry of the melittin peak, noted by other authors as well is probably due to aggregation
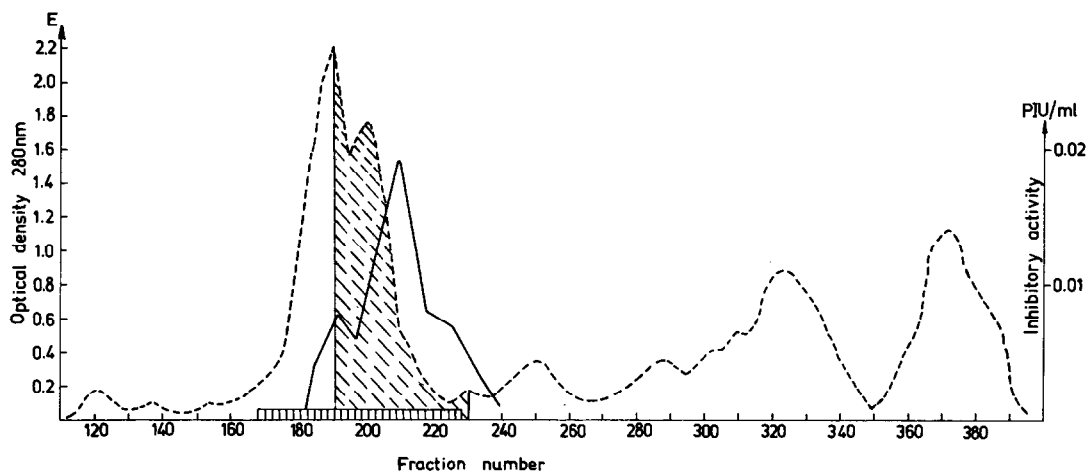
Fig. 1. Gel filtration of 4 g freeze-dried dialysate on Sephadex G-50 column (230 X 5.5 cm). Elution with 0.1 M ammonium formate buffer, pH 4.8. Fractions of 14 ml were collected. The dotted line represents the optical density at 280 nm. The continuous line, the inhibitory activity (PIU). The portion hatched with sloped lines are the fractions pooled. The fractions denoted with vertical lines showed hemolytic activity (melittin).

phenomena [10] . Purified melittin however has no effect on trypsin activity. Autoclaving of the melittin fraction for 30 min at 120°C resulted in nearly complete disappearance of trypsin inhibitory activity. Under these conditions melittin retained its biological and chemical properties.

These findings suggest that the melittin fraction contains another component with trypsin inhibitory properties.
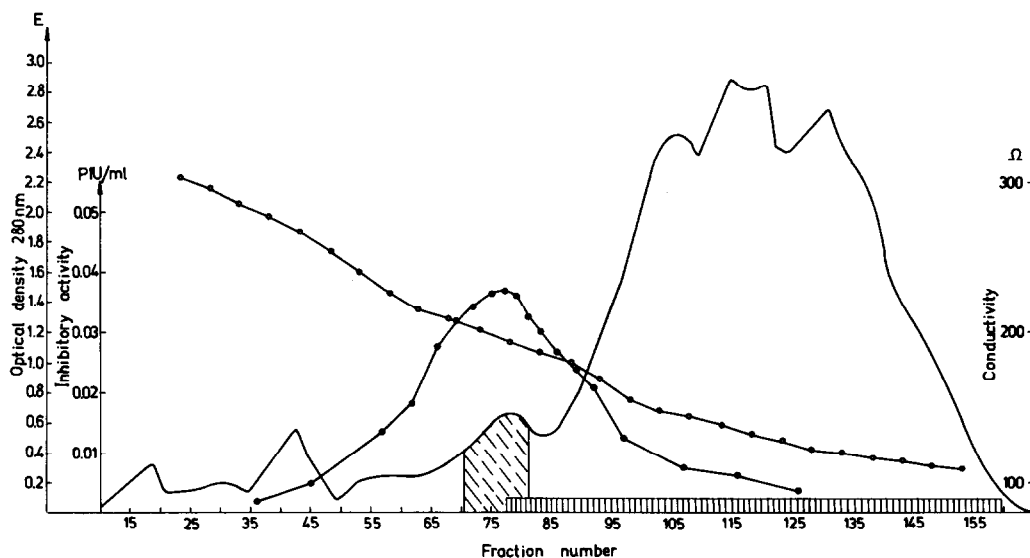


Fig. 2. Ion exchange chromatography of 1.1 g freeze-dried eluate from Sephadex G-50 on CM-Sephadex C-25 column (45 X 2.5 cm). Linear gradient of ammonium formate buffer, pH 5.2 ranging from 0.1 M to 2.5 M was applied. (——) Optical density at 280 nm, (●—●—●) trypsin inhibitory activity in PIU, (o—o—o) conductivity in ohms. The portion hatched with sloped lines are the fractions pooled. The fractions denoted with vertical lines showed hemolytic activity.
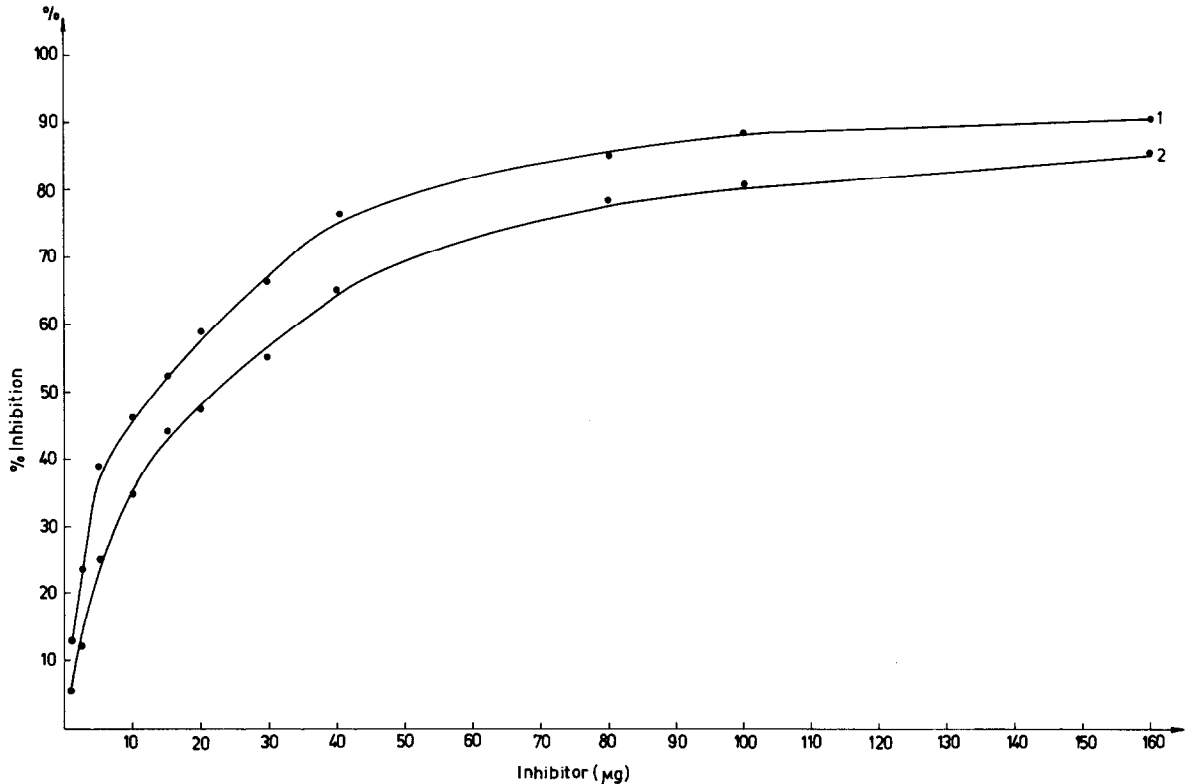
Fig. 3. Activity of the partially purified protease inhibitor at two enzyme concentrations – 10 g trypsin/4 ml (1) and 20 g trypsin/ 4 ml (2). Substrate – BAPNA (2 g/4 ml), 0.1 M Tris-HCl buffer containing 0.025 M $CaCl_2$, pH 7.8.

Rechromatography of the freeze-dried inhibitory portion of the melittin peak on Sephadex G-25 column (2.5 × 120 cm) and its elution with 0.1 M ammonium formate buffer, pH 4.8, did not separate the inhibitor from melittin. This was achieved by chroma-



Fig. 4. Densitogram of the proteins of the partially purified protease inhibitor. Migrations: pH 2.6; 90 min; 8 V/cm; 6.8 mA/cm$^2$. The arrows indicate the location of melittin (M), protease inhibitor (I) and apamin (A).

tography on CM–Sephadex (fig. 2).

The freeze-dried inhibitory peak showed an activity of 470 PIU/g, i.e. it was purified 60-fold with respect to the crude venom. Its proteolytic inhibitory activity was 210 units according to Kunitz. The partially purified product inhibited a considerable number of other proteinases. Studies on its inhibitory spectrum are now in progress and will be published elsewhere.

The trypsin inhibitory activity of the partially purified inhibitor at two enzyme concentrations is presented in fig. 3. On account of the insufficient purity of the preparation as evidenced from the densitogram (fig. 4), these findings cannot provide conclusive data about the stoichiometry of the inhibitor–enzyme reaction.

Its electrophoretic mobility in 15% acrylamide gel characterizes it as a low molecular basic protein. Its cathode mobility is intermediate between the mobility of melittin and apamin. Thin-layer chromatogra-
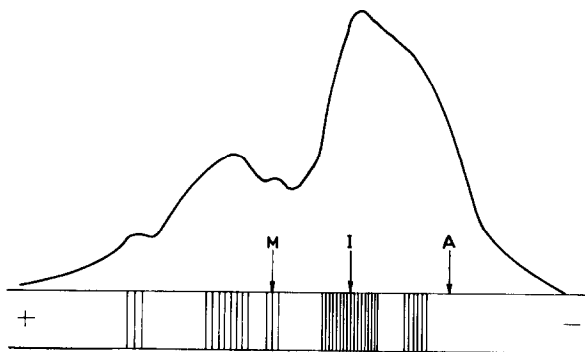
Table 1
Loss of inhibitory activity at various temperatures and pH.

| T | pH | (hr) | Loss in percentage |
|---|---|---|---|
| 20° | 1.5 | 48 | 6 |
| 20° | 10 | 48 | 10 |
| 97° | 3.5 | 1 | 12 |
| 97° | 6 | 1 | 30 |
| 97° | 10 | 1 | 65 |
| 120° | 6 | 0.5 | 85 |

1 mg/ml solutions of the inhibitor in the following media
were treated: pH 1.5 — 0.05 M HCl; pH 3.5 — 0.05 M
$HCOOH-HCOONa$; pH 6 — 0.05 M $NaH_2PO_4-Na_2HPO_4$;
pH 10 — 0.05 M glycine-NaOH.

Table 2
Loss of inhibitory activity caused by various reagents.

| Reagents | Loss in percentage |
|---|---|
| 0.01 M Iodoacetamide | 0 |
| 0.01 M p-Chloromercury benzoate | 0 |
| Performic acid — conc. | 100 |
| 0.05 M Cysteine | 0 |
| 0.05 M Mercaptoethanol | 8 |
| 8 M Urea | 0 |
| 8 M Urea in 0.05 M mercaptoethanol | 90 |

phy on Sephadex G-50 showed that the molecular
weight of the inhibitor in 9000 ± 1000. These proper-
ties classify it as a low molecular protease inhibitor
[11] . The inhibitor is not precipitated by a saturated
solution of ammonium sulfate, 10% trichloroacetic
acid, 8% perchloric acid and 95% ethanol (table 2).
Pepsin does not degrade it. The effect of temperature
and pH is presented in table 1. The inhibitor mani-
fests relative thermostability. The higher stability at
acid pH is probably related to the preponderance of
basic amino acids. The thermostability and the insen-
sitivity to precipitating agent may be ascribed to the
low molecular weight. The absence of SH groups
(table 2) is also important for the stability. The de-
struction of the inhibitory activity on oxidation with
performic acid is presumably due to the splitting of
the disulfide bonds which are essential for the inhibi-
tory activity.

The bee venom contains no proteolytic enzymes.
According to Laskowski, however, the biological role
of the natural inhibitors consists of the regulation and
balancing of the activity of the corresponding en-
zymes in the biological materials [12] . The discovery
of a proteinase inhibitor in bee venom supports the
view of Vogel and Werle [13] contesting the concept
of Laskowsky. As for the biological role of the pro-

teinase inhibitor in bee venom it protects probably the
aggressive (hyaluronidase) and toxic (phospholipase,
melittin and apamin) components of the venom
against the proteases of the stung man or animal.

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