# PURIFICATION OF A SUBTILISIN INHIBITOR FROM BLACK BEAN SEEDS

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## 1. Introduction

Common black beans contain two types of trypsin inhibitors: the Kunitz and the Bowman-Birk types. Of this last group we have detected a familiy of five molecular species in a genetically selected bean line [1]. In addition to these, the presence of a low molecular weight protein has been observed, which inhibits subtilisin, but in contrast with some other inhibitors, does not possess inhibitor activity against either trypsin or chymotrypsin. The purification of the subtilisin inhibitor (SI) to electrophoretic homogeneity, and some of its properties are described.

## 2. Materials and methods

Finely ground black bean seeds (*Phaseolus vulgaris*, var. 'Cubagua') 200 g, were extracted with 1 litre distilled water by stirring for 15 h at 4°C. After centrifugation at 3000 × *g* for 10 min, the supernatant was concentrated to final protein conc. 50–60 mg/ml. The solution was again centrifuged and 150 ml clear supernatant was precipitated by adding 200 ml ethanol at 4°C. The precipitate was discarded. The clear water alcohol mixture (315 ml) was brought to pH 5.3 with 1 M HCl, cooled to –4°C and precipitated by the addition of 2 vol. (630 ml) cold acetone [2]. The acetone precipitate was recovered and exhaustively dialyzed and freeze dried. The inhibitor preparation was further purified by chromatography on a DEAE–Sephadex A-25 column. Subtilisin and trypsin inhibition were determined in each fraction and appropriate fractions were pooled dialyzed until free of $Cl^-$ and lyophilized. The active SI fraction was submitted to a final chromatography on a Sephadex G-75 column. The active peak was pooled, dialyzed and lyophilized.

Protein content was determined by the Lowry method [3]. Subtilisin Carlsberg (proteinase type VIII, Sigma) activity was assayed with 2% casein as substrate in 0.1 M borate buffer, pH 7.6 [4]. Trypsin (type III, Sigma) activity was assayed with benzoyl-L-arginine-*p*-nitroanilide(BAPNA, Sigma) as the substrate [5].

The inhibition of the caseinolytic activity of the following enzymes was tested with the purified SI: chymotrypsin (type II, Sigma) [4]; papain (2 × cryst., Worthington) [4]; elastase (2 × cryst., Schwarz/Mann) [6]; pronase (protease type VI, Sigma) [4]; subtilisin BPN′ (protease type VII, Sigma) [4]. For pepsin inhibition pepsin (Worthington) and urea denatured hemoglobin were used [7]. Increasing amounts of inhibitor were added at the appropiate pH and incubated with the enzyme for 15 min before adding substrate.

Molecular weights were determined by chromatography on Sephadex G-75 [8] and by electrophoresis in SDS-containing polyacrylamide gels [9]. Cytochrome *c*, chymotrypsinogen and egg albumin (Schwarz/Mann) were used as reference proteins. Polyacrylamide gel electrophoresis [10] was routinely used as a criterion of purity throughout the purification process.

In experiments to modify the active site of the inhibitor, SI was incubated at room temperature with 1 mol% trypsin (TPCK-treated) or subtilisin, for 48 h in 0.05 M $CaCl_2$, pH 3.8. In some cases the pH was then adjusted to 8.2 by adding 1 M Tris buffer, pH 8.2 and incubated with 2.3 μg/nmol carboxypeptidase-B (CPB) for 6 h at room temperature.

Carbohydrates present in the inhibitor preparation were determined by the anthrone method [11] before

Table 1
Purification of the subtilisin inhibitor

| Purification step | Total protein (mg) | Spec. act. (IU/mg) | Purification (fold) | Total act. (IU) | Recovery (%) |
|---|---|---|---|---|---|
| Extract | 1187 | 0.19 | 1 | 226 | 100 |
| Acetone precipitate | 54 | 3.85 | 20 | 208 | 92 |
| DEAE–Sephadex chromatography | 3.8 | 50 | 263 | 190 | 84 |
| Sephadex G-25 chromatography | 1.06 | 167 | 879 | 178 | 79 |

IU is defined as mg protein required for total inhibition of 20 μg subtilisin under standard conditions

and after extraction with phenol [12]. Incubation of the inhibitor with α- and β-glucosidases (Sigma) was carried out in 0.1 M acetate buffer, pH 6.0, containing 1.35 M EDTA at 37°C for 5 h, the inhibitor–enzyme weight ratio being 2:1.

## 3. Results and discussion

The subtilisin inhibitor (SI) present in black bean extracts was purified over 800-fold to electrophoretic homogeneity (table 1, fig.1). The extractable inhibitor content of the bean represented ~ 0.006% of its dry weight (about 0.1% of the water soluble proteins). Extraction of maximal yield of SI was achieved with water within 4 h, whereas maximal yields of both SI and trypsin inhibitors (TI) required extraction with 1% NaCl. The water extraction method was found to be more suitable for the isolation of SI and the 15 h period was selected for convenience. The acetone precipitate contained 92% of the SI activity and part of the trypsin inhibitors. The two activities were separated from each other by DEAE–Sephadex

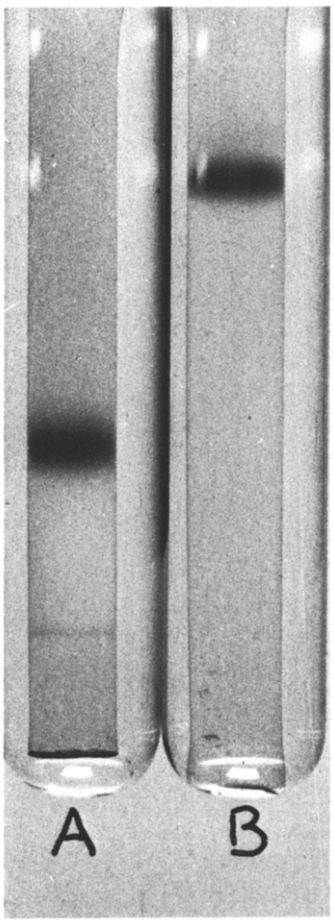


Fig.1. Polyacrylamide gel electrophoresis of SI. The procedure was essentially that in [10] except that the concentration gel was omitted. The gels (7%) were formed and run in 0.5 × 7 cm glass tubes. The inhibitor samples (30 μg) were dissolved in the electrophoresis buffer containing 20% sucrose and bromophenol blue as a tracer dye. The gels were stained with amido black. Pattern A was the result of electrophoresis at pH 8.3 whereas pattern B was run at pH 4.3.

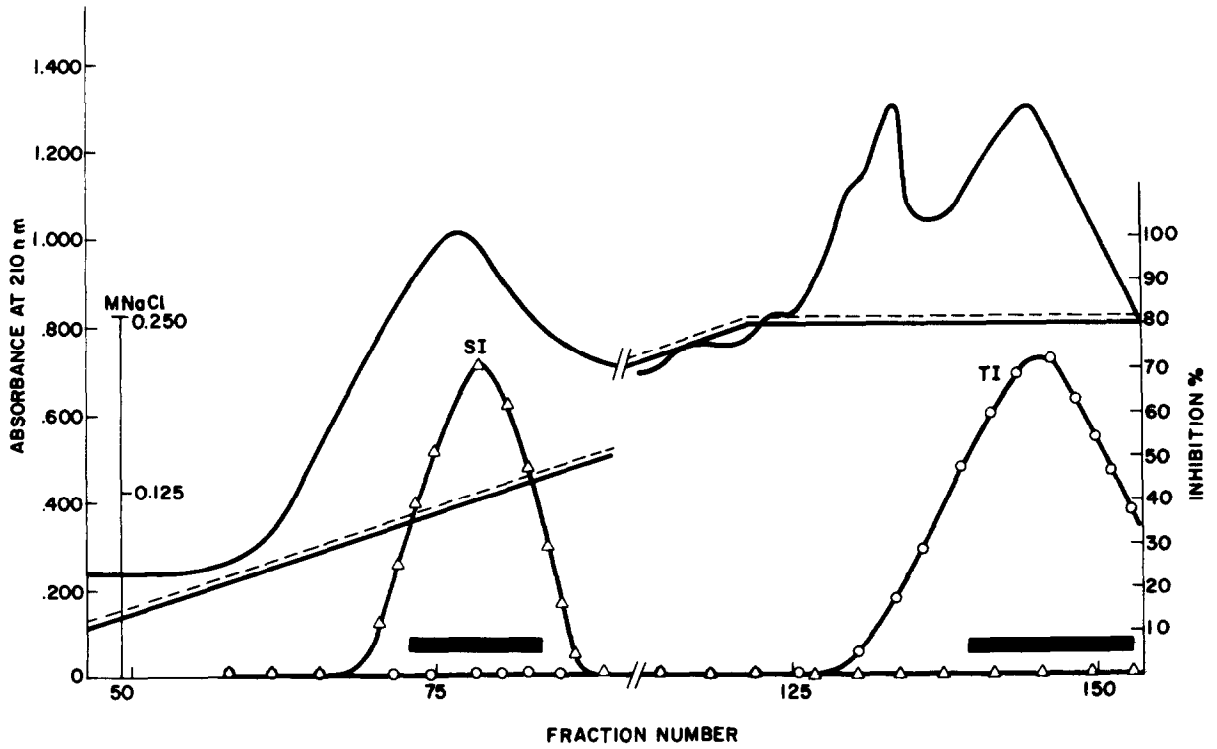


Fig.2. Purification of SI by DEAE–Sephadex chromatography. A column of DEAE–Sephadex A-25 (2.6 × 80 cm) was equilibrated with 0.05 M Tris–HCl buffer, pH 8.3. A sample of 100 mg acetone precipitate was dissolved in 3 ml equilibration buffer, applied to the column and eluted with 300 ml buffer untill no more protein was eluted (not depicted in figure). A linear gradient of 250 ml 0.05 M Tris–HCl, pH 8.3 and 0.25 M NaCl in the buffer was then applied to the column. At the end of the gradient 0.25 M NaCl–Tris solution was passed through the column. Flow rate was 48 ml/h and fraction vol. 4 ml. $A_{210}$ is denoted by solid line. Other curves are (- - -) NaCl gradient; (△-△-△) subtilisin inhibitor activity; (○-○-○) trypsin inhibitor activity; bars indicate pooled fractions.

chromatography (fig.2), SI eluting at 0.13 M NaCl concentration, and the trypsin inhibitors at 0.25 M NaCl. After a final chromatography on a Sephadex G-75 column (fig.3) the subtilisin inhibitor was homogeneous as judged by the single band after electrophoresis, at pH 8.3 and 4.3 (fig.1).

Figure 4 depicts the inhibition of subtilisin (Carlsberg) and pronase by SI. The shape of the pronase inhibition curve suggests that only its subtilisin-like component [13] was inhibited. In separate experiments it was demonstrated that subtilisin BPN′ is also strongly inhibited by SI. In contrast to some other inhibitors of subtilisin it does not possess inhibitor activity against either trypsin or chymotrypsin. It does not inhibit elastase, pepsin, papain or ficin. The known specificity spectrum of SI is therefore restricted to the subtilisins and pronase.

The pH optimun of the subtilisin inhibition is a broad plateau above pH 7 up to pH 11, but the inhibition rapidly decreases below pH 7. In the optimum pH range the complex formation time between enzyme and SI was $< 1$ min. When stored at pH 8.3 in 0.05 M Tris buffer containing 0.13 M NaCl, at 4°C, SI was rather stable, the activity loss after 45 days being only 17%.

A mol. wt 10 000 was estimated by molecular sieving on Sephadex G-75 and by electrophoresis in SDS-containing polyacrylamide gels. The SI preparation contained around 10% non-covalently bound carbohydrate which did not separate from the protein during electrophoresis. Separation of the carbohydrate was accomplished, however, by phenol extraction.

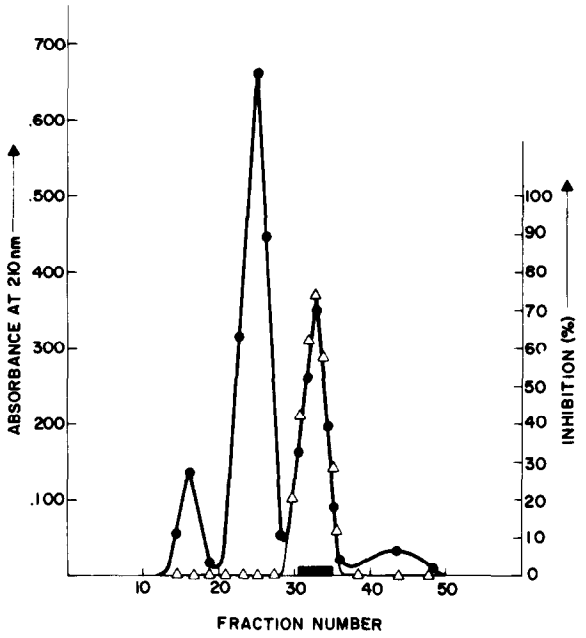


Fig.3. Purification of SI by Sephadex G-75 chromatography. The active protein fraction (1.2 mg) obtained by DEAE–Sephadex chromatography was dissolved in 0.5 ml 0.05 M Tris–HCl buffer, pH 7.2, applied to a Sephadex G-75 column (1.6 × 80 cm) and eluted with the same buffer. Flow rate was 24 ml/h, and fraction volume 2.6 ml. $A_{210}$ is denoted by solid line; (△-△-△) depicts subtilisin inhibitor activity; bar indicates pooled fractions.

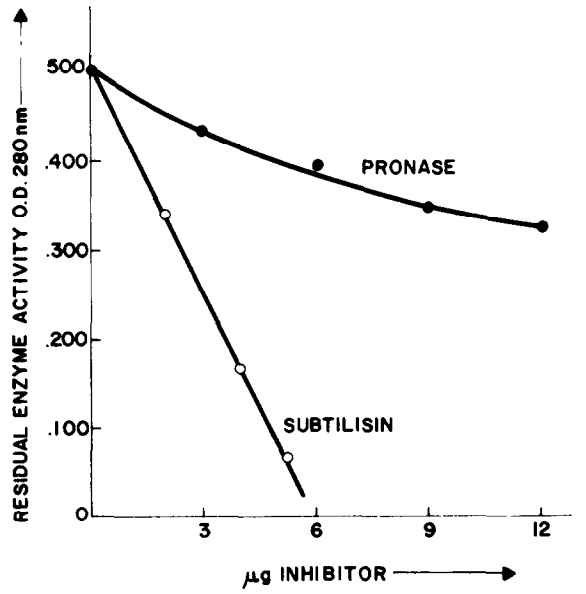


Table 2
Stability of SI

| Treatment | Time (h) | Decrease of inhibitor activity (%) |
|---|---|---|
| Heating 90°C pH 7 | 1 | 8 |
| Heating 90°C pH 4 | 1 | 37 |
| Heating 90°C pH 10 | 1 | 55 |
| Urea 8 M pH 7 | 48 | 0 |
| Urea 8 M pH 4 | 48 | 0 |
| Urea 8 M pH 10 | 48 | 0 |
| Heating 80°C Urea 8 M, pH 7 | 1 | 29 |
| Heating 80°C Urea 8 M, pH 4 | 1 | 44 |
| Heating 80°C Urea 8 M, pH 10 | 1 | 49 |
| Heating 90°C in 5% trichloroacetic acid | 0.25 | 100 |
| Pepsin hydrolysis E:SI = 1:2000 | 0.33 | 100 |

Inhibitor activity towards subtilisin was determined using casein as substrate [4]. The residual inhibitor activity was calculated related to a control solution of the inhibitor in Britton and Robinson's universal buffer pH 7.6. After acid treatment the pH was adjusted to 7.6 with 0.1 N NaOH. Inhibitor solutions treated with urea were 100-fold diluted before being assayed. An equal amount of urea added to the control assay did not alter its reading. The pepsin-SI ratio is expressed on weight basis

Incubation of the inhibitor with α- and β-glucosidases did not affect its activity toward subtilisin.

When subtilisin and an excess of SI were mixed at pH 7.6 and chromatographed on the same Sephadex column used for molecular weight determination, two peaks were obtained: the elution volume of the first corresponded to that expected for a complex having a 1:1 subtilisin: inhibitor molar ratio. The eluted protein had neither hydrolytic nor inhibitor activity (fig.5). After dissociating the enzyme–inhibitor

Fig.4. Inhibitory effect of SI toward 20 μg subtilisin Carlsberg and 80 μg pronase employing casein as substrate [4].

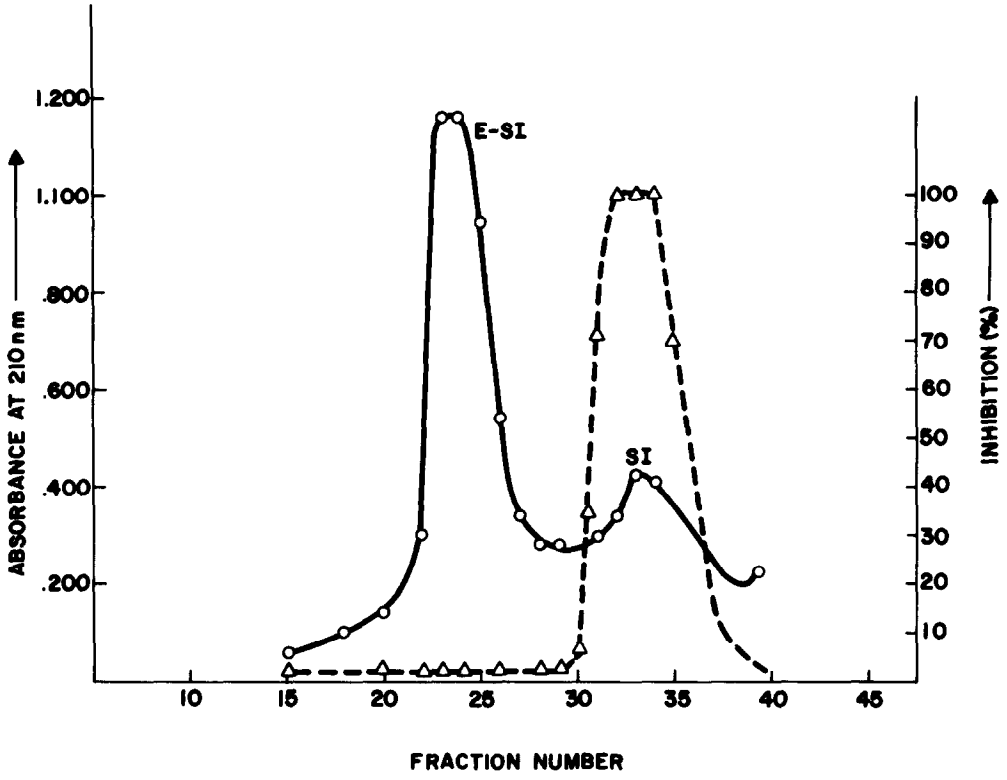


Fig.5. Isolation of the enzyme–inhibitor complex by gel filtration. A column (1.6 × 80 cm) of Sephadex G-75 was equilibrated and eluted with 0.05 M Tris–HCl buffer, pH 7.2. Flow rate was 24 ml/h and fraction vol. 2.6 ml. A mixture of 725 μg subtilisin inhibitor and 390 μg subtilisin were dissolved in 0.75 ml elution buffer, allowed to stand for 5 min, and then applied to the column. $A_{210}$ is denoted by solid curve; △-△-△ indicates subtilisin inhibitor activity.

complex by adjusting to pH 4 and inactivating the enzyme by heat treatment, full inhibitor activity was recovered. The second peak possessing inhibitor activity was eluted at the same position as the free inhibitor.

SI was hydrolyzed by pepsin when incubated at pH 3. It was, however, rather resistant against denaturing agents (table 2), a characteristic common to most plant protein-proteinase inhibitors.

When treated with catalytic amounts of subtilisin at acid pH, SI lost its inhibitor activity. The split was assumed to be at the active site between the $P_1$ and $P_1'$ residues [14] for the antichymotrypsin site in the Bowman-Birk soybean inhibitor. Similar treatment with TPCK-trypsin either at acid or at neutral pH resulted in only a slight decrease in its activity. However, when the newly formed cationic C-terminal was hydrolyzed by carboxypeptidase B, the inhibitor was rendered inactive against subtilisin.

When the Kunitz inhibitor from soybean is treated with subtilisin, a single peptide bond $Met^{84}$–Leu, and not the tryptic reactive site $Arg^{63}$–Ile, is specifically hydrolyzed [15]. Since the inhibitor does not inhibit subtilisin, the authors suggest that this site might be a vestigal reactive site for subtilisin or a secondary site for chymotrypsins. It is tempting to speculate that sometime in the past SI was able to inhibit trypsin-like enzymes. As a consequence of a mutation the molecule may have lost its antitrypsin activity but the 'trypsin susceptible bond' is still present. It is possible that a trypsin-like enzyme from a microorganism, insect or mammal other than bovine might be inhibited by this inhibitor.

Although subtilisin inhibitor activity has already

been detected in legumes [16] this is the first specific subtilisin inhibitor isolated in pure form. The only other one purified from plant material is the one from rice [15], and as far as we are aware its isolation and properties have not yet been published. In a separate paper we describe the detection of a subtilisin inhibitor in all 64 garden bean cultivars tested by a microelectrophoretic method [17].

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