

One-step purification of Kunitz soybean trypsin inhibitor

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Received 3 December 2002, and in revised form 21 March 2003

Abstract

A fast and simple method for the extraction and purification of Kunitz trypsin inhibitor from soybean seeds is described. The first step consisted in the heat treatment of whole soybean seeds in water at 60 °C for 90 min. It was found that 8.4% of total trypsin inhibitory activity of the seeds was secreted during heat treatment. The aqueous medium was loaded onto an affinity chromatography column with immobilized trypsin. The retained fraction, eluted with 0.01 N HCl, contained the purified Kunitz trypsin inhibitor, which was subsequently stabilized by freeze-drying without loss of activity. From 1 g soybean seeds, 0.7 mg inhibitor with a specific trypsin inhibitory (TI) activity of 11,430 TIU/mg was obtained. The yield was greater than that obtained with established procedures. Due to the ease of the procedure proposed, the method is readily scalable to pilot plant or industrial preparations. © 2003 Elsevier Science (USA). All rights reserved.

Keywords: Affinity chromatography; Hydrolase inhibitor; Heat treatment; Seed proteins; Water extraction

The seeds of all plants contain several biologically active proteins, which play various specialized functions, including defense mechanisms against pathogens. Among these molecules, hydrolytic enzymes, enzyme inhibitors, lectins, and also cell killing factors, such as the ribosome inactivating proteins, are the most represented [1]. The class of hydrolase protein inhibitors comprises various protein types, including amylase and protease inhibitors. These molecules have been referred to so far as “antinutritional compounds,” because of their ability to inhibit the enzymes involved in the digestive processes of humans and animals. Most of these compounds are currently being reconsidered with a view to the potential exploitation of their biological activities in several pharmacological, medical, and cosmetic applications. In particular, the present interest for trypsin inhibitors (TIs)¹ arises from their ability to prevent or

suppress carcinogen-induced transformation, as detected in various in vitro and in vivo model systems [2].

In mature soybean (*Glycine max*) seeds, two major proteins showing trypsin inhibitory activity have been identified since the 1960s: the Bowman-Birk TI and the Kunitz TI (KI) [1]. The latter has recently been taken into consideration as a chemopreventive agent and for other biomedical applications [3], including stimulation of hair growth, size, and pigmentation [4]. It is a protein of 20.1 kDa, the amino acid sequence of which was determined by Koide and co-worker [5]; it contains 4 cysteine residues forming 2 disulfide bridges. Three closely related isoforms have been described [6]. The 3D structure of KI has also been determined [7]. KI was first described as an alcohol-insoluble factor [8].

Various long and often tedious procedures for the purification of the soybean KI have been proposed, including the one based on a combination of different chromatographic approaches [9], one making use of preparative electrophoresis [10], and another using immobilized metal affinity chromatography [11]. A quick, effective and scalable procedure for the purification of the TIs would help both studies on the biological

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¹ Abbreviations used: TI, trypsin inhibitor; KI: Kunitz trypsin inhibitor; BAPA, *N*-benzoyl-L-arginine-4-nitroanilide; TIU, trypsin inhibitory unit.

properties of these proteins and their applications in various fields. This paper describes such a procedure for KI. The novelty consists in the exploitation of the legume seed property to selectively release specific proteins in the medium when incubated in heated water [12,13].

Materials and methods

Materials

Plant material consisted of commercially available soybean seeds. Although a careful selection of TI-rich cultivars may increase the purification yields, no specific investigations in this respect have been carried out in this study, since all the catalogued soybean varieties are positive for TIs [14].

The affinity chromatography matrix with immobilized trypsin (cat. no. T1763), *N*-benzoyl-L-arginine-4-nitroanilide (BAPA), bovine trypsin, soybean TI type I-S (cat. no. T9003), and all other reagent grade chemicals were from Sigma.

Extraction and purification

A weighed amount of whole soybean seeds was washed with 5 volumes of diluted sodium hypochlorite (0.1%) to remove dust and reduce microbial contamination. Seeds were soaked in milliQ water in the ratio 1:5 (w/v) at 25, 40, and 60 °C for 90 and 180 min with occasional mild stirring. After heat treatment, the seeds and the small seed debris generated by the treatment itself were removed by filtration with Miracloth (Calbiochem) and 0.45 µm filters to obtain a clear yellowish solution. An aliquot was taken for trypsin inhibitory unit (TIU) assessments, while the rest of the solution was immediately loaded on to an affinity column with immobilized trypsin. The ratio between inhibitory activity loaded and bed volume of the resin was 250 TIU per immobilized trypsin unit, although overloading helped decrease aspecific binding of other proteins. The resin was then washed with 5 volumes of 20 mM Tris-HCl, pH 8.5, containing 0.5 M KCl, to remove unbound or aspecifically bound proteins. A further washing with 2 volumes of milliQ water was carried out to reduce the ionic strength of the subsequent eluate. The KI inhibitor was eluted with 0.01 N HCl. Aliquots of the eluted fraction were kept for further analyses before and after freeze-drying.

Trypsin inhibitory activity measurement

TI activity of the various fractions was measured following the AACC method N. 71-10 [15] with minor modifications. Total TI activity in soybean seeds was measured on 0.01 N NaOH-extracted soybean flour [15].

Electrophoretic techniques

SDS-PAGE was carried out essentially as described by Laemmli [16] on 12 and 15% polyacrylamide gels. Electrophoretic separations were performed by using a Minigel Protean II system (BioRad). Gels were stained with Coomassie brilliant blue R250.

The relative molecular masses (M_r) of the polypeptide bands were determined by comparison with a standard protein solution (Pharmacia) containing phosphorylase *b* (90 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

Gels were digitized with a Studio Scan II scanner (Agfa) and quantitatively processed with Cream (Kem-En-Tec, Copenhagen, Denmark) software.

Amino acid sequencing

Amino acid sequencing was carried out on immobilized proteins. N-terminal amino acid sequence was determined by Edman degradation using a Procise automated liquid phase sequencer (Perkin-Elmer).

Polypeptide blotting onto PVDF membranes was performed according to Matsudaira [17] in a Bio-Rad Trans Blot Cell at a constant current of 100 mA for 1.5 h at 4 °C.

Results and discussion

Already in the 1990s Hirano et al. [12] showed that soybean seeds and other legume seeds incubated in water at 60 °C release into the medium various polypeptides, including those responsible for the enzyme's inhibitory activity. However, these Authors did not perform any purification of single protein components.

For the purification of KI, we tested three different temperatures for protein extraction from whole soybean seeds and two incubation times (Table 1). The release of trypsin inhibitory activity was most effective at the highest temperature of incubation, namely 60 °C. Increasing incubation time did not improve the extraction yields. In the subsequent experiments, the extraction was thus performed at 60 °C for 1.5 h. The pH of the suspension at the beginning and at the end of the extraction treatment was 5.8–6.3, indicating that no buffering system was needed.

Table 2 shows the purification scheme of KI. The major feature of the approach is its simplicity. The final activity recovered is a small but significant percentage of the total inhibitory activity present in the protein extract from soybean seeds. The protein yield was 0.07% of seed weight. This yield is about 1.4 times greater than that obtainable following already established procedures [9,10]. The specific activity of the purified KI was about

Table 1
TIUs extracted from soybean seeds at different incubation times and temperatures

Time (h)	25 °C		40 °C		60 °C	
	TIU	Activity yield (%) ^a	TIU	Activity yield (%) ^a	TIU	Activity yield (%) ^a
1.5	1,812	1.9	2,910	3.1	8,000	8.4
3.0	1,282	1.4	3,270	3.4	7,586	8.0

^a Expressed as a fraction of the total inhibitory activity present in the seed (95,000 TIU/g).

Table 2
Purification table of KI from whole soybean seeds

	mg protein/g seed	TIU/g seed	TIU/mg protein	Purification fold	Protein yield (%)	Activity yield (%)
Soybean seed	400 ^a	95,000	238	1	100	100
Aqueous extract	n.d.	8,000	n.d.	n.d.	n.d.	8.4
Affinity purified trypsin inhibitor	0.7	8,000	11,430	48	0.2	8.4

n.d., not determined.

^a From [8].

2.7 times higher than that of the commercially available product (4180 TIU/mg, see Materials and methods).

When the aqueous extract was analyzed by SDS-PAGE (Fig. 1), a number of polypeptide bands with M_r s ranging from more than 95 kDa to less than 12 kDa were visible. Most of them coincided with those shown previously by Hirano et al. [12]. When the incubation times were extended to more than 2 h, the electrophoresis analysis evidenced a smeared pattern, possibly because of the partial degradation of some polypeptides (not shown).

After clarification of the aqueous medium by filtration, the solution was loaded onto a trypsin-conjugated chromatographic resin. In this step, various ratios between inhibitory activity loaded and the trypsin activity

present on the matrix were tested. In our conditions 250 TIU per immobilized trypsin unit was found to be a convenient ratio, although overloading did slightly improve the homogeneity of the purified TI fraction. After washing, the specifically bound polypeptides were eluted by 0.01 N HCl. The choice of this eluant was due to the need to keep the composition of the solutions as simple as possible, in view of the scaling up of the procedure and the final stabilization of the preparation by freeze-drying. The fraction eluted with diluted HCl was shown to have tryptic inhibitory activity. The yield of protein release from the matrix was quantitative. The fraction was submitted to SDS-PAGE (Fig. 1) and was found to consist of a single polypeptide band around 20 kDa. The identification of this polypeptide was carried out by N-terminal amino acid sequencing as detailed in Materials and methods. The N-terminal sequence of the first 11 amino acids was the following:

Asp-Phe-Val-Leu-Asp-Asn-Glu-Gly-Asn-Pro-Leu

This sequence corresponded to the N-terminal sequence of the mature chain of Kunitz TI reported in the SWISSPROT database (Accession No. P01070).

Since it was outside of the scope of this work, we did not investigate whether the secretion of Kunitz TI from heated soybean seeds has some physiological meaning, such as the seed response to the heat-stress conditions or whether it is simply a passive release of the protein. Although the soybean seeds were no longer viable after the extraction, they could still be used for bulk protein wet extraction in industrial applications.

Due to the simplicity of the described procedure, its scaling-up is only limited by the amount of immobilized trypsin matrix used, since none of the other procedures require expensive apparatus or any special skill. The

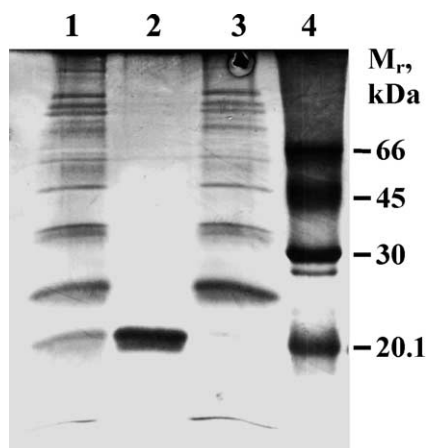


Fig. 1. SDS-PAGE of the polypeptides separated by trypsin affinity chromatography. One μ g of protein was loaded in each lane. Lane 1, aqueous extract; lane 2, bound fraction; lane 3, unbound fraction; and lane 4, molecular weight markers.

trypsin-conjugated chromatographic matrix, which is the only expensive step in our procedure, has been repeatedly used tens of times over at least 6 months without any detectable loss of TI binding capacity.

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