Two Kunitz-Type Proteinase Inhibitors from Potato Tubers

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ABSTRACT

Two proteinase inhibitors have been isolated from tubers of potato (Solanum tuberosum). Based on N-terminal amino acid sequence homologies, they are members of the Kunitz family of proteinase inhibitors. Potato Kunitz inhibitor-1 (molecular weight 19,500, isoelectric point 6.9) is a potent inhibitor of the animal pancreatic proteinase trypsin, and its amino terminus has significant homology to a recently characterized cathepsin D Kunitz inhibitor from potato tubers (Mares et al. [1989] FEBS Lett 251:94–98). Potato Kunitz inhibitor-2 (molecular weight 20,500, isoelectric point 8.6) is an inhibitor of the microbial proteinase subtilisin Carlsberg; its amino terminus is almost identical to an abundant 22 kilodalton protein from potato tubers (Suh et al. [1990] Plant Physiol 94:40–45) and has significant homology to other Kunitz-type subtilisin inhibitors from small grains. Both Kunitz inhibitors are abundant proteins of the cortex of potato tubers.

Potato tubers have long been recognized as an abundant source of a wide range of inhibitors of several different classes of proteinase, e.g. inhibitors of serine proteinases (2, 11), thiol proteinases (17, 18), metalloproteinases (14), etc. These inhibitors may play a significant role in the natural defense mechanisms of the tuber against insect and pathogen attack. However, it is only recently that Kunitz-type inhibitors from potato tubers have been described in detail. For example, a novel inhibitor of cathepsin D, an acid proteinase, has been isolated and characterized from potato tubers (10), and the amino acid sequence of this protein (and another closely related inhibitor, 16) is homologous to the Kunitz family of proteinase inhibitors. Also, the sequence of an abundant 22 kD protein from potato tubers has homology to Kunitz trypsin inhibitors (19, 20). In this paper, we describe two serine proteinase inhibitors from potato tubers that have differing proteinase inhibitory profiles but are both Kunitz-type inhibitors based on amino-terminal sequence homologies. One closely resembles the previously described cathepsin D inhibitor (10). The other is an inhibitor of the microbial proteinase subtilisin and appears to be the same as the abundant 22 kD protein isolated by Suh et al. (19). This is the first Kunitz-type inhibitor of a microbial proteinase to be identified in a source other than from small grains.

MATERIALS AND METHODS

Potato (Solanum tuberosum) tubers (var Russet Burbank) were purchased from a local market. Bovine trypsin, bovine α-chymotrypsin, subtilisin Carlsberg, papain, azocasein, and soybean Kunitz trypsin inhibitor were purchased from Sigma Chemical Co.

Purification of Proteinase Inhibitors

Protein extracts of potato tubers were prepared by a method based on that of Bryant et al. (2) for the initial purification of potato inhibitors I and II. Protein concentrations were determined by the method of Bradford (1). Potato tubers (4.5 kg) were homogenized in a Waring blender with 2 L of 0.7% sodium metabisulfite. After filtration through cheesecloth, the pH was adjusted to 3 with 6 N HCl and the extract was left to stand at 4°C for 30 min. After centrifugation, the supernatant was adjusted to 70% ammonium sulfate. The precipitate was collected by centrifugation, dissolved in water, and incubated at 80°C for 10 min. The resulting precipitate was removed by centrifugation and the supernatant dialyzed for 48 h against several changes of water. The solution was then lyophilized and stored at −20°C. Protein extracts prepared with this methodology consistently contained polypeptides with Mr approximately 20,000 as the principal components (>100 mg from 5 kg tubers).

The results reported here are from a single preparation of 240 mg that contained two major components with Mr approximately 20,000. Other preparations contained 2 to 5 different polypeptides with Mr approximately 20,000 that we have not characterized further, in addition to potato inhibitors I and II (subunit Mr approximately 12,000 and 5,000, respectively) as expected. A crude preparation of most of the Mr, 20,000 proteins free of patatin (the predominant protein in tuber extracts) may be directly obtained by passing a clarified homogenate of potato tubers in 25 mM sodium phosphate (pH 6.8) over a DEAE-cellulose column equilibrated in the same buffer (13). Patatin adheres to the column and the majority of the Mr, 20,000 proteins pass through.

HPLC

Anion exchange HPLC was performed with a Pharmacia Mono S 5/5 column equilibrated in 25 mM sodium acetate (pH 5.0) and using a 0 to 0.5 M NaCl gradient over 30 min with a flow rate of 1 mL/min. Reverse phase HPLC was performed with a Vydac C4 4.5 × 300 mm column equilibrated in 0.1% TFA and eluted with a gradient of 0 to 35% acetonitrile over 8 min, then 35 to 65% acetonitrile over 30 min at a flow rate of 1 mL/min. Peaks were collected and lyophilized prior to SDS-PAGE analysis and amino terminal sequencing.
Proteinase inhibition was assayed using azocasein as a substrate for selected proteinases. Assays were performed in Eppendorf tubes containing 10 μg proteinase with 10 or 50 μg inhibitor in 0.1 mL buffer. Buffers were 50 mM Tris-Cl (pH 7.5), 20 mM CaCl₂ for trypsin, 50 mM Tris-Cl (pH 7.5), 5 mM CaCl₂ for α-chymotrypsin and subtilisin, and 50 mM sodium acetate (pH 6.0), 2 mM DTT for papain. Control samples contained no inhibitor. Samples were preincubated for 10 min prior to the addition of 0.1 mL 2% azocasein. After 1 h (2.5 h for α-chymotrypsin), 0.4 mL 12% TCA was added and the precipitate removed by centrifugation after standing for 30 min. Supernatants were added to 0.4 mL 4 N NaOH and the absorbance measured at 440 nm.

Electrophoresis and Isoelectric Focusing

SDS-PAGE and isoelectric focusing were performed with a Pharmacia PhastSystem using 20% Phastgels for SDS-PAGE analyses and IEF 3-9 Phastgels for isoelectric focusing. Gels were stained with Coomassie blue.

Amino-Terminal Sequencing

Amino-terminal sequencing was performed on proteins purified by reverse phase HPLC by Dr. William S. Lane at the Harvard Microchemistry Facility (Cambridge, MA) using an Applied Biosystems 477 liquid phase sequencer with an on-line 120A PTH analyzer. Sequence analysis was performed using MacVector software from IBI.

RESULTS

Protein extracts from potato tubers prepared by the method of Bryant et al. (2) consistently produced preparations that contained large amounts of proteins with Mr approximately 20,000 by SDS-PAGE analysis. SDS-PAGE of a crude extract of potato tubers shows a family of bands at Mr approximately 20,000, in addition to the major band at Mr 40,000 that corresponds to patatin, the storage protein of potato tubers (Fig. 1, lane 2). In order to determine the nature of these predominant proteins, one preparation of 240 mg material obtained from 4.5 kg tubers was characterized further (Fig. 1, lane 3). Analysis of this preparation by cation exchange HPLC at pH 5.0 gave two well resolved major peaks, denoted PKI-1 and PKI-2 in Figure 2, which were collected and analyzed by SDS-PAGE (Fig. 1, lanes 4 and 5). The first peak contained a single polypeptide of Mr 19,500 and the second peak a slightly larger one of 20,500. The pl value of the polypeptide

Abbreviations: PKI, potato Kunitz inhibitor; pl, isoelectric point.
in peak 1 was 6.9 and that in peak 2 was 8.6, as determined by isoelectric focusing. These values are consistent with their relative elution sequence from the Mono S column.

The proteins were also well resolved by reverse phase HPLC (Fig. 2B), but in this case the more basic polypeptide with a slightly larger Mr eluted first (PKI-2 in Fig. 2A). The two peaks were collected, lyophilized, and subjected to amino-terminal sequencing. A total of 40 of the first 42 amino-terminal residues of PKI-1, and 25 amino-terminal residues of PKI-2 were determined. These sequences were used to search the National Biomedical Research Foundation Protein Information Resource database (release 22.0). Both sequences had significant homology to several Kunitz-type inhibitors from a variety of plant sources. Figure 3A and B show comparisons of the amino-terminal sequences of PKI-1 and PKI-2 with each other and with other members of the Kunitz inhibitor family (5, 8–10, 15, 19, 22, 23). Both potato proteins show conservation of residues that are characteristic of Kunitz inhibitors; Gly-13 of PKI-1 (Gly-11 of PKI-2), Tyr-22 of PKI-1 (Tyr-20 of PKI-2), and Gly-33 and 34 of PKI-1.

Although both PKI-1 and 2 are clearly members of the Kunitz family, they bear more similarity to other members of this family than to each other. PKI-1 is homologous to a cathespain D inhibitor isolated from potato tubers (10), being identical in residues 21 to 42, but it differs in 12 of the first 20 residues (Fig. 3A). The amino-terminal sequence of PKI-2 is quite different from PKI-1 but is almost identical to that of a 22 kD protein also isolated from potato tubers (19), with only 2 of 21 residues being different. When compared to Kunitz-type inhibitors from other sources, PKI-2 bears more homology to the Kunitz-type subtilisin inhibitors from various grains than to PKI-1 (Fig. 3B).

To further define the differences between PKI-1 and 2, the purified proteins were tested for inhibitory activity against trypsin, chymotrypsin, subtilisin Carlsberg, and papain in an azocasein digestion assay in comparison with soybean Kunitz trypsin inhibitor. The effect of 50 μg inhibitor on the activity of 10 μg proteinase is shown in Figure 4. The spectrum of inhibition by PKI-1 was similar to that of soybean Kunitz trypsin inhibitor; it was a potent inhibitor of trypsin, giving complete inhibition at the 10 (data not shown) and 50 μg level. It exhibited little inhibition of chymotrypsin or subtilisin and none against papain. In contrast, PKI-2 was only a moderate inhibitor of trypsin and chymotrypsin, but gave complete inhibition of subtilisin at the 50 μg level. However, at the 10 μg level, the inhibition of subtilisin dropped to 25% (data not shown). Thus, PKI-2 has a relatively broad spectrum of inhibition relative to PKI-1 and soybean Kunitz trypsin inhibitor, with some significant inhibition of all four proteinases tested.

**DISCUSSION**

We have characterized two Kunitz-type proteinase inhibitors from a single preparation of potato tubers that are quite distinct from each other in amino acid sequence, pl and inhibitory profile. We obtained over 80 mg of each inhibitor from 4.5 kg tubers, indicating that the proteins are relatively abundant. Other extracts prepared in a similar manner show at least two to five more polypeptides of Mr, with approximately 20,000 that can be readily separated from PKI-1 and 2 by cation exchange HPLC and which we have not yet characterized. Siekema et al. (20) showed that the sequence of an abundant cDNA clone prepared from tuber mRNA was related to the Kunitz inhibitor family and the deduced amino acid sequence of this clone is identical to that of several members of the 22 kD complex of proteins characterized by Suh et al. (19). The inhibitory properties of these proteins have not been described, but a cathespain D inhibitor recently characterized from tubers is a Kunitz-type inhibitor and is also quite abundant in tubers (7, 10). This suggests that there may be several other Kunitz inhibitors with differing properties produced in potato tubers. For example, some members of this group of tuber proteins are inhibitors of cysteine proteinases (18; T. A. Walsh, unpublished data).

![Figure 4](https://example.com/figure4.png)
The amino-terminal sequence of PKI-1 has strong homology to the sequences of two closely related cathepsin-D inhibitors (10, 16), with identity between residues 21 to 42. These cathepsin D inhibitors, like PKI-1, possess significant inhibitory activity against trypsin. However, PKI-1 is distinct from these proteins, as 12 of the first 20 residues are different, including loss of the glycosylation site at Asn-19 of the cathepsin-D inhibitor. Also, the pl of the cathepsin D inhibitor is 8.5 (16), significantly higher than the value of 6.9 for PKI-1. The extent of sequence variation in the Kunitz family between tubers from different potato varieties is unknown, and so further data on amino acid sequences and activity profiles are required to ascertain the true diversity of Kunitz inhibitors in potato tubers.

PKI-2 is clearly equivalent to several members of the 22 kD complex of tuber proteins described by Suh et al., but the inhibitory profile of these proteins has not been described (19). We have shown that PKI-2 is an inhibitor of subtilisin, a microbial protease. Although there have been numerous descriptions of the various protease inhibitory activities contained in potato tubers, PKI-2 is one of the relatively few examples of a microbial protease inhibitor from this source. It is also the first example of a Kunitz-type inhibitor of a microbial protease from a dicot. Other well characterized subtilisin inhibitors from dicots are members of the protease inhibitor I and II families, e.g. the inhibitors from adzuki beans (12) and broad beans (21), and an inducible microbial protease inhibitor from tobacco (4). In contrast to these inhibitors, PKI-2 appears to have a somewhat broad specificity, with limited but significant activity against both trypsin and chymotrypsin. Three Kunitz-type subtilisin inhibitors from monocot seeds, barley (22), wheat (9), and rice (22), have been characterized and sequenced. The inhibitors from barley and wheat are bifunctional and possess activity against insect amylases (3). It will be of interest to further characterize the inhibitory spectrum and potency of PKI-2 and other potato Kunitz inhibitors against a variety of microbial proteases, as they may contribute to the natural defense mechanisms of the tuber against pathogen attack.

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LITERATURE CITED

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