Partial Characterization of Trypsin Inhibitors and N-Terminal Sequences of Five Trypsin Isoinhibitors of Great Northern Beans (*Phaseolus vulgaris*)†

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A purified pool of Great Northern (GN) bean trypsin inhibitors (TIs), subjected to SDS–PAGE containing 7 M urea, produced four broad bands when stained for protein. Reduction and S-carboxymethylation of the purified GN bean TIs followed by SDS–PAGE resulted in the production of several sharp bands with estimated molecular weights ranging from approximately 8000 to 17 000. Plasma desorption mass spectrometry of the collective pool of purified GN bean TIs resulted in two peaks of molecular weight 8406 and 8957. One isolated GN bean TI, subjected to amino acid compositional analysis, contained neither Val nor Met, was low in Tyr, Leu, Phe, and Arg, and contained high amounts of Cys, Asp, Ser, and Glu. The p*I* values of seven of the GN bean TIs were estimated to be pH 5.14, 4.97, 4.88, 4.76, 4.69, 4.64, and 4.56. N-Terminal sequence analysis of five GN bean TIs revealed that four of the TIs contained Ser at the N terminus, while a fifth contained Lys at position 1 and Ser at position 2. Three of the TIs exhibited a high degree of sequence homology among themselves and with other plant source TIs, while two of the TIs exhibited little sequence homology among themselves or with the other GN bean TIs.

INTRODUCTION

The common bean, *Phaseolus vulgaris*, is an important leguminous seed crop that originated in the geographic area of southern Mexico and Central America (Sinha, 1977) and is now cultivated throughout the world. Dry beans are low in the sulfur-containing amino acids Met and Cys but contain high concentrations of Lys, to thus complement the amino acid profile of cereal grains (Rockland and Radke, 1981). Although dry beans are a good source of protein (18.3–23.2% protein on a dry weight basis; Deshpande and Nielsen, 1987), they contain several nutrient-limiting factors, one of which is trypsin inhibitors (TIs). Dietary TIs reduce growth and cause pancreatic enlargement in laboratory animals (Rackis, 1965; Kakade et al., 1970, 1973). Short-term dietary intake of low levels of TIs causes no pancreatic hypertrophy in rats (Rackis et al., 1975). However, a major TI chronic feeding study revealed that long-term feeding of low levels of TIs causes adverse physological effects in rats (Gumbmann et al., 1985; Liener et al., 1985; Rackis et al., 1985; Sprangler et al., 1985). Studies have examined the incidence of pancreatic cancer in rats fed trypsin inhibitors over extended periods (McGuinnes et al., 1980; McGuinness and Wormsley, 1986). However, there are no studies in the literature to date on long-term effects of low-level dietary exposure to TIs in humans. Short-term exposure to Bowman–Birk soybean TI causes increased pancreatic secretion in humans (Liener et al., 1988).

Trypsin inhibitors have been purified and characterized from several cultivars of dry beans over a period of years (1944 to date) by many investigators. The number of trypsin isoinhibitors present in dry beans has been in question, along with the reported physical and chemical characteristics of the isolated inhibitors. It is often difficult to compare results among literature reports because it is unclear which specific TI is being characterized. It is also unclear as to whether these inhibitors are identical to each other except for some limited N-terminal cleavages or whether the inhibitors are truly isoinhibitors. This question of identifying specific isoinhibitors in Great Northern beans was the subject of this investigation, which is a continuation of previous work (Rayas-Duarte et al., 1992) in which up to 11 isoinhibitors were identified in Great Northern beans.

MATERIALS AND METHODS

Materials and Reagents. Great Northern beans (*P. vulgaris*) were a gift from Roger Brothers Seed Co., Boise, ID. All chemicals were of reagent grade or electrophoresis grade where applicable.

Purification of Trypsin Inhibitors. Great Northern bean TIs were purified as a collective pool following the purification protocol of Rayas-Duarte et al. (1992). Purification of the TIs from a bean albumin extract involved anion exchange, hydrophobic interaction, and affinity chromatography on anhydrotrypsin–Sepharose.

SDS–Polyacrylamide Gel Electrophoresis. Discontinuous SDS–PAGE was performed using the method of Hames (1990) as described by Rayas-Duarte et al. (1992).

Urea SDS–PAGE. The general method of Hashimoto et al. (1983) for SDS–PAGE containing urea was used. A 7 M urea gradient was prepared with 10–18% acrylamide, 0.5–0.9% bis(acrylamide), and 0–10% sucrose gradient containing 0.45 M Tris-HCl (pH 8.8) and 0.1% SDS (180 × 160 × 1.5 mm slab, Protean 16 cm electrophoresis unit, Bio-Rad Laboratories, Richmond, CA). The stacking gel was composed of 5% acrylamide, 0.13% bis(acrylamide), 0.067 M Tris-HCl (pH 6.8), 0.1% SDS, and 7 M urea.

Samples and a mixture of protein standards (Pharmacia-LKB, Piscataway, NJ) were prepared in 7 M urea, 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 1% β-mercaptoethanol, and 0.0025% bromophenol blue, heated for 10 min at 60 °C, and then stored overnight before electrophoresis. Samples were loaded onto the gel and electrophoresed with a pH 8.3 electrode buffer at 120-V constant voltage until the tracking dye had run off the gel (15 h). The gel was then run for an additional 2.5 h. Following electrophoresis, the gels were stained with Coomassie Blue R-250 (0.1% in 5:5:2 H_2O/MeOH/HOAc) and destained with a solution of 5% MeOH/7.5% HOAc.

Reduction and S-Carboxymethylation of Trypsin Inhibitors. The method of Pospisilova et al. (1967) was used to prepare

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reduced and S-carboxymethylated TIs for molecular weight estimation by SDS–PAGE. This procedure involved dissolving 10 mg of the purified pool of TIs in 800 μL of 9 M urea and 1.4 M Tris-HCl buffer (pH 8.6). To this mixture was added 60 μL of β-mercaptoethanol, then the mixture was incubated at 25 °C under N_2 for 4 h. Iodoacetic acid (162 mg) in 1 mL of 1 M NaOH was added and the mixture incubated for an additional 20 min. Following the incubation period, the reduced and S-carboxymethylated TIs were dialyzed (molecular weight cutoff 3500) against deionized distilled water (DDW) in a cold room and then freeze-dried in preparation for SDS–PAGE.

Molecular Weight Determination of Trypsin Isoinhibitors Using Mass Spectrometry (MS). Molecular weight determination of the pool of purified Great Northern bean trypsin isoinhibitors by MS was performed in the Department of Biochemistry at Purdue University. The sample was prepared for introduction into the mass spectrometer by absorbing the solubilized protein onto an electrosprayed nitrocellulose-coated aluminized Mylar sample target disk (15 min) and then washing with a 0.1% trifluoroacetic acid solution. Mass spectrometry was performed on the prepared sample using a Bioion 20R plasma desorption mass spectrometer (PDMS) (Applied Biosystems) for 2 h at an accelerating potential of 17 000 V, using ^{252}Cf as the ionizing source. This instrument operates with a mass accuracy of 0.1% of the mass obtained.

IEF of Sample for Amino Acid Sequence Analysis. Individual trypsin isoinhibitors subjected to amino acid sequence analysis (described later) were obtained by IEF of pooled purified TIs from the anhydrotrypsin–Sepharose affinity column. The IEF procedure was performed as described previously (Rayas-Duarte et al., 1992) with the following changes. The anode solution was composed of 1 M H_3PO_4, and the cathode was composed of a 2% solution of ampholines, pH 4–6 (Pharmacia-LKB). The gel size was approximately 9 × 11 cm. Prior to sample application, the gel was prefocused for 20 min (with water cooling) at 1000 V, 15 mA, and 15 mW using an LKB 2197 power supply (Pharmacia-LKB). Samples were applied to precut filter paper applicator strips (Pharmacia-LKB) and focused for 90 min at 1200 V, 15 mA, and 15 mW. The applicator strips were then removed, and the samples were focused for an additional 30 min at the same power supply setting.

Preparation of Trypsin Isoinhibitors for Amino Acid Sequence Analysis. *Protein Electroblotting.* Individual TIs and standard proteins with known isoelectric points (Sigma Chemical Co., St. Louis, MO) were resolved using IEF, with the gel cast on plastic support backing film (GelBond PAG film, Pharmacia-LKB). After IEF, the gel on the support film was rinsed with DDW, placed in a glass tray containing 0.7% HOAc, carefully removed from the support film, and then equilibrated in 0.7 HOAc for 5 min.

Protein electroblotting was performed using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The proteins were electroblotted onto Problott poly(vinylidene difluoride) (PVDF) membrane (Applied Biosystems, Inc., Foster City, CA). Prior to assembling the gel sandwich within the gel holder cassette, the PVDF membrane was wetted with 100% MeOH and then equilibrated in electroblotting buffer (Applied Biosystems, 1991). The gel sandwich was assembled according to the instructions of the manufacturer (Bio-Rad, 1991, 1992). An electroblotting buffer of 0.7% HOAc (Bio-Rad, 1992) was employed. The proteins were electroblotted at 100 V for 60 min using the Bio-Ice cooling unit (Bio-Rad).

Protein Detection on PVDF Membrane. After the proteins were electroblotted onto PVDF membrane, the membrane was removed from the gel sandwich, rinsed with DDW, and stained with 0.1% Coomassie Blue R-250 in 1% HOAc/40% MeOH for 1 min (Applied Biosystems, 1991). The PVDF membrane was then destained with 50% MeOH, with gentle swirling and several changes of destain solution. The destained membrane was rinsed with DDW and air-dried.

Amino Acid Sequence Analysis of Trypsin Isoinhibitors. Amino acid sequence analysis of the Great Northern bean trypsin isoinhibitors was performed in the Laboratory for Molecular Structure, Department of Biochemistry, Purdue University. The trypsin isoinhibitors separated by IEF were excised from an electroblot on PVDF membrane and subjected to sequence analysis (single sample) in a Model 470A gas-phase sequenator (Applied Biosystems).

Amino Acid Compositional Analysis of Trypsin Isoinhibitors. Amino acid compositional analysis of a Great Northern bean trypsin isoinhibitor was performed in the Department of Chemistry at Purdue University. The isoinhibitor was excised from a protein electroblot of an IEF gel on PVDF membrane. The protein was subjected to hydrolysis on a Savant amino prep AP100 hydrolyzer (Savant Instruments Inc., Farmingdale, NY) with 6 N HCl for 2 h (165 °C) under vacuum. Amino acid analysis was then conducted on the sample using a Beckman System 7300 high-performance amino acid analyzer (Beckman, Allendale, NJ).

RESULTS AND DISCUSSION

Molecular Weight Estimation. A wide range of molecular weights have been reported for dry bean TIs depending on the method of molecular weight estimation, cultivar of bean investigated, and specific investigator. Reports in the literature of dry bean TI molecular weights range from 10 000 to 20 000 by ultracentrifugation (Pusztai, 1968; Wagner and Riehm, 1967; Sgarbieri and Whitaker, 1981), from 13 000 to 20 000 by size exclusion chromatography (Wagner and Riehm, 1967; Mosolov et al., 1976; Wang, 1975; Miyoshi et al., 1978; Whitaker and Sgarbieri, 1981; Sgarbieri and Whitaker, 1981; Jacob and Pattabiraman, 1986; Wu and Whitaker, 1990), from 13 000 to 20 000 by SDS–PAGE (Miyoshi et al., 1978; Gomes et al., 1979; Sgarbieri and Whitaker, 1981; Jacob and Pattabiraman, 1986; Wu and Whitaker, 1990), and from 8000 to 23 000 by amino acid composition (Wagner and Riehm, 1967; Wilson and Laskowski, 1973; Wang, 1975; Mosolov et al., 1976, 1977; Miyoshi et al., 1978; Gomes et al., 1979; Wu and Whitaker, 1990).

Reports of self-association of TIs resulting in a large overestimation of molecular weight by size exclusion chromatography and SDS–PAGE are frequent in the literature (Haynes and Feeney, 1967; Millar et al., 1969; Gennis and Cantor, 1976b; Whitley and Bowman, 1975). In solution, trypsin inhibitors associate reversibly in a monomer–dimer–trimer system (Millar et al., 1969; Gennis and Cantor, 1976b). We previously estimated the apparent molecular weight of the pool of Great Northern bean trypsin isoinhibitors to be approximately 18 000 and 16 000 by SDS–PAGE and size exclusion chromatography, respectively (Rayas-Duarte et al., 1992). These values were within the range of molecular weights reported in the literature for dry bean TIs as determined by these methods.

In an attempt to dissociate the TIs, the pool of purified TIs from Great Northern beans was subjected to SDS–PAGE containing 7 M urea. The TIs produced four broad protein bands, unsuitable for molecular weight estimation (gel not shown).

Because SDS–PAGE and size exclusion chromatography are known to greatly overestimate the molecular weights of TIs (Wu and Whitaker, 1991), two other methods were investigated to estimate molecular weight. First, the collective pool of Great Northern bean trypsin isoinhibitors was subjected to reduction in 9 M urea followed by S-carboxymethylation with iodoacetic acid and then dialyzed and subjected to SDS–PAGE (Figure 1). The reduced and S-carboxymethylated TIs produced several sharp bands on SDS–PAGE with molecular weights ranging from approximately 8000 to 17 000 (Figure 1, lanes 3 and 4). The presence of several bands was most probably the result of incomplete reduction and S-carboxymethylation of the TIs resulting in different three-dimensional shapes of the TIs. Gennis and Cantor (1976a) reported similar results for Bowman–Birk-like black-eyed pea TIs. A black-eyed pea TI that exhibited a molecular weight of

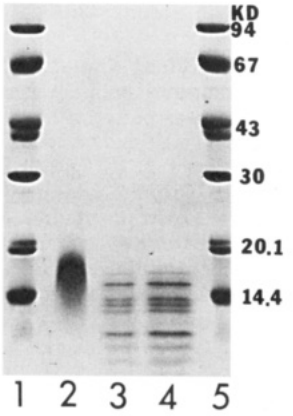

Figure 1. SDS–PAGE of reduced and S-carboxymethylated Great Northern bean trypsin inhibitors. (Lanes 1 and 5) Molecular weight standard proteins (5 μg of each component); (lane 2) purified trypsin isoinhibitors (10 μg); (lanes 3 and 4) reduced and S-carboxymethylated TIs (10 and 20 μg, respectively).

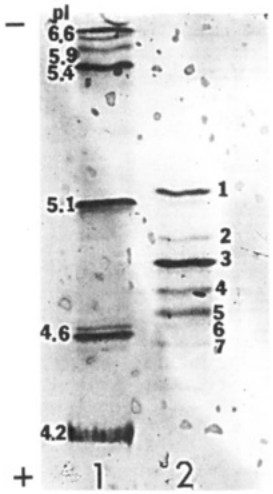

Figure 2. Protein electroblot on poly(vinylidene difluoride) (PVDF) membrane of an isoelectric focusing (IEF) gel. (Lane 1) IEF protein standard mix containing carbonic anhydrase I (p*I* 6.6), carbonic anhydrase II (p*I* 5.9), carbonic anhydrase II (p*I* 5.4), β-lactoglobulin A (p*I* 5.1), soybean trypsin inhibitor (p*I* 4.6), and glucose oxidase (p*I* 4.2); (lane 2) purified Great Northern bean trypsin isoinhibitors.

17 000 by SDS–PAGE exhibited the more accurate molecular weight of 8000 when reduced and S-carboxymethylated followed by SDS–PAGE (Gennis and Cantor, 1976a).

The collective pool of Great Northern bean trypsin isoinhibitors was also subjected to molecular weight determination by plasma desorption mass spectrometry. The trypsin isoinhibitors were resolved into two peaks of molecular weight 8406 and 8957. Assuming that the mean molecular weight of an amino acid residue is 110 (Stryer, 1988), the TI inhibitor chain lengths were 76 and 81 residues, respectively.

Isoelectric Point Estimation of Trypsin Isoinhibitors. A purified pool of Great Northern bean TIs was previously resolved into a group of 11 isoinhibitors by IEF (Rayas-Duarte et al., 1992). These isoinhibitors exhibited p*I*s of approximately pH 4–5. The Great Northern bean isoinhibitors separated by IEF were electroblotted onto PVDF membrane and stained with Coomassie Blue R-250 (Figure 2). Seven of the 11 trypsin isoinhibitors previously visualized by IEF and trypsin inhibitor staining were visible after electroblotting and staining of the PVDF membrane (Figure 2, lane 2). Either the protein did not transfer sufficiently to the blotting membrane for four of the trypsin isoinhibitors or, more likely, there was not enough total protein present for these inhibitors to be visualized by Coomassie Blue staining of the PVDF membrane. The p*I*s of the seven trypsin isoinhibitors were estimated to be as follows: band 1, p*I* 5.14; band 2, p*I* 4.97; band 3, p*I* 4.88; band 4, p*I* 4.76; band 5, p*I* 4.69; band 6, p*I* 4.64; band 7, p*I* 4.56. These p*I*s are in agreement with literature reports of *P. vulgaris* TI p*I* values of 5.0 (Pusztai, 1968), 4.3, 4.5, 4.7, and 4.9 (Mosolov et al., 1976), and 4.46, 4.82, 4.84, and 5.09 (Wu and Whitaker, 1990) for kidney beans, 4.4 and 4.45 for navy beans (Gomes et al., 1979), and 4.7 for kintoki beans (Miyoshi et al., 1978).

Amino Acid Compositional Analysis. Great Northern bean trypsin isoinhibitor band 3 (Figure 2) was excised from an electroblot (on PVDF membrane) of an IEF gel and subjected to amino acid compositional analysis. Band 3 was selected for analysis because it stained darkest on the PVDF membrane after electroblotting, thus indicating the largest amount of protein present. Compositional analysis was not attempted on the other bands due to lower protein levels. The number of residues per protein molecule was estimated on the basis of mass spectrometry analysis of the collective pool of purified TIs (discussed previously). Great Northern bean trypsin isoinhibitor band 3 contained neither Val nor Met, was low in Tyr, Leu, Phe, and Arg, and was high in Cys, Asp, Ser, and Glu (data not shown). This is consistent with studies in the literature for TIs from *P. vulgaris* (Wilson and Laskowski, 1973, 1975; Whitley and Bowman, 1975; Mosolov et al., 1976, 1982; Gomes et al., 1979; Wu and Whitaker, 1990). The amino acid profiles of Great Northern bean ground flour, albumin, and globulin fractions (Sathe et al., 1981) are quite different from that of the trypsin isoinhibitor band 3, with the TI being substantially higher in Ser, Pro, Gly, and Cys and substantially lower in Ala, Val, Arg, and Trp.

Amino Acid Sequence Analysis. Amino acid N-terminal sequence analysis was performed on five of the Great Northern bean trypsin isoinhibitors (Figure 2, lane 2, bands 1–5) (Table I). Some N-terminal sequences of other trypsin inhibitors (all plant source) listed in the literature are included in Table I for comparison.

Four of the Great Northern bean trypsin isoinhibitors (bands 2–5) contained Ser at the N terminus, while isoinhibitor band 1 contained a Lys residue. However, isoinhibitor band 1 contained a Ser at the second position. Serine has been reported to occupy the N-terminal position of several trypsin inhibitors, including Brazilian pink bean inhibitors B(B) and B(C) (Wu and Whitaker, 1991), kidney bean inhibitors IIIB (Mosolov et al., 1977) and R(C) (Wu and Whitaker, 1991), lima bean inhibitor IV (Stevens et al., 1974), adzuki bean inhibitor IA (Kiyohara et al., 1981), mung bean inhibitor F (Wilson and Chen, 1983), and chickpea inhibitor 5 (Belew and Eaker, 1976). Some of the other N-terminal positions for trypsin inhibitors are Asx an Glx for Great Northern bean inhibitors II and II′, respectively (Wilson and Laskowski, 1975), Gly for kidney bean inhibitor II (Mosolov et al., 1977), Asp for Bowman–Birk inhibitor (Odani and Ikenaka, 1973), and Gly for faba bean inhibitor (Asao et al., 1991).

Great Northern bean inhibitor bands 1–3 exhibited a high degree of homology among themselves with only an occasional substitution. Homology was highest between isoinhibitor bands 1 and 3. Isoinhibitor bands 4 and 5 exhibited little homology between themselves or with the other three isoinhibitors.

Great Northern bean isoinhibitor bands 1–3 were

Table I. Comparison of Amino Acid N-Terminal Sequence Analysis of Great Northern Bean Trypsin Isoinhibitors with Sequences of Trypsin Inhibitors from Other Plant Sources[a]

Great Northern band 1 1	Great Northern band 2 2	Great Northern band 3 3	Great Northern band 4 4	Great Northern band 5 5	Great Northern inhibitor II 6	Great Northern inhibitor II 7	Brazilian pink inhibitor B (B) 8	kidney bean inhibitor II 9	kidney bean inhibitor IIIb 10	lima bean inhibitor IV 11	Bowman–Birk inhibitor 12	adzuki bean inhibitor IA 13	mung bean inhibitor F 14	peanut inhibitor BIII 15	chick pea inhibitor 5 16	faba bean inhibitor 17
Lys																
Ser	Ser	Ser	Ser	Ser			Ser	Gly	Ser	Ser		Ser	Ser			
Gly	Asp	Gly	Ile	His	Asx		Gly	Gly	Gly	Gly		Val	Ser			
His	His/Tyr	Ala	Pro	Asp	His		His	Leu	Leu	His		His	His			
Tyr/Arg	Glu	Tyr	Ala	Ser	Asx		Arg	Pro	Pro	His		His	His			
His	Pro	Ala	Gln	Ser	Glx		His	Asp	Asp	Glu		Gln	His			
Glu	Glu	Glu	Ser	Ile/Asp	His		Glu	Ser	Ser	His		Asp	Asp			
Ser	Trp	Ser	Ser	Glu/Ile	Ser		Ser	Glx	Glx	Ser		Ser	Ser			
Thr	Thr	Thr	Ser	Ile/Gln	Ser		Thr			Thr		Ser	Ser			
Asp	Asp	Asp	Thr	Ser	Asx		Asp			Asp		Asp	Asp			
Glu		Glu	Pro/Asp	Glu/Ser	Glx	Glx	Glu			Glx		Glu	Glu			
Pro		Pro	Ile	Thr/Ser	Pro	Pro	Pro			Pro	Asp	Pro	Pro			Gly
Arg		Ser	Arg	Ser	Ser	Ser	Ser			Ser	Asp	Ser	Ser			Asp
Glu		Ser	Leu	Ser	Glx	Glx	Glu			Glx	Glu	Glu	Glu		Ser	Asp
		?	Phe	Asp	Ser	Ser	Ser			Ser	Ser	Ser	Ser		Thr	Val
		?	?		Ser	Ser	Ser			Ser	Ser	Ser	Ser		Thr	Lys
		Lys	Tyr		Pro	Pro	Lys			Lys	Lys	His	Glu		Thr	Ser
		Ala			Pro	Pro				Pro	Pro	Pro	Pro	Val	Ala	Ala
					Cys	Cys				Cys	Cys	Cys	Cys	Cys	Cys	Cys
					Cys	Cys				Cys	Cys	Cys	Cys	Cys	Cys	Cys

[a] Columns 1–5, Great Northern bean trypsin isoinhibitors (Figure 2); columns 6 and 7, Wilson and Laskowski (1975); column 8, Wu and Whitaker (1991); columns 9 and 10, Mosolov et al. (1977); column 11, Stevens et al. (1974); column 12, Odani and Ikenaka, (1973); column 13, Kiyohara et al. (1981); column 14, Wilson and Chen (1983); column 15, Ikenaka and Norioka (1983); column 16, Belew and Eaker (1976); column 17, Asao et al. (1991).

homologous, although not identical, to the Great Northern beans TIs II and II′ of Wilson and Laskowski (1975). Inhibitor II′ of Wilson and Laskowski (1975) was an identical (however truncated) version of their inhibitor II with eight amino acid residues cleaved from the N terminus. In contrast, the Great Northern bean isoinhibitor bands 1–5 do not appear to contain an identical N-terminal sequence with varying degrees of N-terminal cleavage.

The N-terminal sequence of Great Northern bean isoinhibitor band 1 was identical to that of the Brazilian pink bean inhibitor B(B) (Wu and Whitaker, 1991) except that the Great Northern isoinhibitor had an N-terminal Lys in place of an N-terminal Ser, and an Arg_{13} in place of a Ser_{12} for the Brazilian pink bean inhibitor. Isoinhibitor bands 1–3 from Great Northern bean also exhibited a high degree of sequence homology with TIs of non *P. vulgaris* sources such as lima bean inhibitor IV (Stevens et al., 1974), adzuki bean inhibitor IA (Kiyohara et al., 1981), and mung bean inhibitor F (Wilson and Chen, 1983). This is consistent with literature reports of a high degree of sequence homology for navy bean inhibitors II and II′ (Wilson and Laskowski, 1975) and Brazilian pink bean inhibitor B(B) (Wu and Whitaker, 1991), as compared to the non *P. vulgaris* TIs of lima bean (inhibitor IV), soybean (Bowman–Birk inhibitor), and adzuki bean. The N-terminal sequence, molecular weight, and isoelectric point information obtained in this study of Great Northern bean TIs supports and clarifies information on TIs in other cultivars of dry beans.

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