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Formation of Bowman–Birk inhibitors during the germination of horsegram (*Dolichos biflorus*)

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Abstract

Three Bowman–Birk type inhibitors (HGGI-I, II and III), which appear in the cotyledons of 120 h germinated horsegram (*Dolichos biflorus*) seeds have been purified to homogeneity by size-exclusion chromatography and ion-exchange chromatography. HGGI-I, HGGI-II and HGGI-III differ from each other and from the dormant seed inhibitors in amino acid composition, molecular size and charge. The amino-terminal sequence analyses indicate that these inhibitors are derived from the isoinhibitors of the dormant seed by a limited proteolysis and not by de novo synthesis. These inhibitors differ from each other at their amino-terminus. HGGI-II identical to HGGI-I except for the loss of a single amino-terminal aspartyl residue, where as HGGI-III shows the loss of a pentapeptide. All the three inhibitors are potent competitive inhibitors of trypsin and chymotrypsin. The dissociation constants (K_is) for trypsin inhibition indicate that amino-terminal tail of the inhibitors play a role in trypsin binding probably through electrostatic interaction.

Keywords: *Dolichos biflorus*; Horsegram seeds; Germination; Bowman–Birk inhibitors; Amino-terminal proteolysis

1. Introduction

Legume seeds are noted for the large amount of reserve protein in their cotyledons and also for their levels of protein-proteinase inhibitors active against mammalian pancreatic proteases (Ryan, 1973). These inhibitors impair the nutritional quality of the seeds by reducing the protein digestibility and absorption by inducing pancreatic hypertrophy (Liener and Kakade, 1980). Two types of proteinase inhibitors, Kunitz and Bowman–Birk types widely distributed in legumes have been studied extensively (Ikenaka and Norioka, 1986; Belitze and Weder, 1990; Richardson, 1991). The Bowman–Birk type inhibitors (BBI) are small molecules of M_r 7–9 kDa and contain 14 half cystines linked by seven disulfide bridges in a single chain (Birk, 1987). These double-headed inhibitors interact simultaneously with two molecules of proteinases (Hary and Steiner, 1970) without any conformational change (Sierra et al., 1999).

Some of the suggested functions of the proteinase inhibitors include their role as storage proteins, regulation of endogenous proteinases particularly during the dormancy of the seeds and acting as protective agents against insect or microbial predators (Ryan and Green, 1974). BBIs have been implicated as potential cancer chemopreventive agents (Kennedy, 1993; Flecker, 1993; Ware et al., 1999; Koepke et al., 2000) and inhibit the serine proteases, chymase and tryptase stored in the mast cell granules, which are released upon degranulation (Ware et al., 1997). Definite experimental evidence for the in vivo role of these proteinase inhibitors is sparse. A possible storage function is likely as observed by the decline in the inhibitory activity during germination of several legumes (Pusztai, 1972; Wilson, 1981; Freed and Ryan, 1978; Sreerama and Gowda, 1998). However Godbole et al. (1994b) demonstrated that in pigeon pea (*Cajanus cajan*) these inhibitors have neither a storage role nor do they control endogenous protease activity. The bulk of the decline in the inhibitory activity is generally attributed to the proteolysis of the inhibitor by seed proteinases (Lorenson et al., 1981). During the germination of horsegram (*Dolichos biflorus*) the BBIs present in the resting seed rapidly disappeared with the

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concomitant appearance of new active species (Sreerama and Gowda, 1998). These inhibitor species were electrophoretically similar to the BBIs found in the flower, leaf and early stages of seed development. In this paper we report the purification and characterization of three BBIs present in cotyledons of horsegram (*D. biflorus*) after 120 h of germination. We further provide evidence that these inhibitors are posttranslational products originating from the limited proteolysis of the resting seed inhibitors and not via de novo synthesis.

2. Results and discussion

2.1. Purification

Germination considered as a simple, inexpensive and effective technology induces qualitative and quantitative changes in the levels of proteinase inhibitors of legumes (Wilson, 1988; Yoshikawa et al., 1979; Tan-Wilson et al., 1982; Pusztai, 1972; Lorenson et al., 1981; Godbole et al., 1994b; Ambekar et al., 1996). In a previous paper (Sreerama and Gowda, 1998), we reported the rapid disappearance of the major isoinhibitors from germinating horsegram seeds (*D. biflorus*), accompanying their loss was the sequential appearance of new active inhibitor species. Three isoinhibitors (HGGI-I, HGGI-II and HGGI-III) obtained after 120 h of germination were purified and characterized. The results of the purification are summarized in Table 1. Size-exclusion chromatography of the Sephadex G-50 (Fig. 1) yielded a single trypsin/chymotrypsin inhibitor peak. Ion-exchange chromatography on CM-Sephadex C-25 resulted in the elution of HGGI-I and HGGI-II in the buffer wash as unbound protein peaks. The electrophoretic mobility on PAGE at pH 8.8 (results not shown) of these inhibitors corresponded to the two inhibitors detected previously (Sreerama and Gowda, 1998). Upon washing the column with 0.3 M NaCl in the equilibrating buffer for column regeneration a major protein peak eluted that showed both trypsin and chymotrypsin inhibitory activity. Native PAGE of this fraction at pH

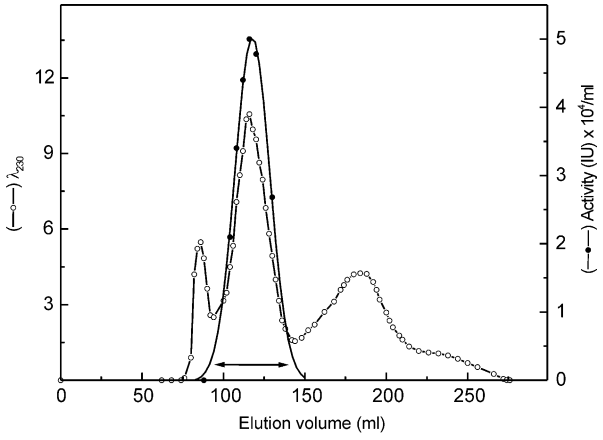

Fig. 1. Sephadex G-50 chromatography elution profile of germinated horsegram isoinhibitors. Elution buffer, 0.025 M Tris–HCl pH 7.5; flow rate, 12 ml/h. Inhibitory activity peak pooled as indicated.

Table 1
Purification of germinated horsegram isoinhibitors[a]

Step	Total protein (mg)	Total inhibitory units (IU)×10^5	Specific activity (units/mg)×10^3	Fold purification	Yield (%)	CIU/TIU
Crude extract	39.21	T 6.64	16.94	–	100	2.3
		C 15.33	39.10	–	100	
$(NH_4)_2SO_4$ precipitation	8.55	T 4.02	47.02	2.8	60.5	2.2
		C 9.05	105.85	2.7	59.0	
Sephadex G-50 chromatography	3.20	T 3.72	116.25	6.9	56.1	2.3
		C 8.74	273.13	7.0	57.0	
CM-Sephadex C-25 ion-exchange chromatography						
HGGI-I	0.35	T 0.38	108.57	6.4	5.7	2.3
		C 0.86	245.70	6.3	5.6	
HGGI-II	0.66	T 0.70	106.06	6.2	10.5	2.3
		C 1.59	240.90	6.2	10.4	
HGGI-III	1.87	T 1.66	88.77	5.2	25.0	2.1
		C 3.48	186.10	4.8	22.7	
DEAE-Sephadex A-25 ion-exchange chromatogaphy	0.21	T 0.28	133.33	7.9	4.2	2.3
HGGI-I		C 0.64	304.76	7.8	4.2	
	0.41	T 0.55	134.15	7.9	8.3	2.4
HGGI-II		C 1.32	321.95	8.2	8.6	

[a] These are the results of a typical purification starting from 200 g (85 g cotyledons) of horsegram seeds. These values were reproduced in two separate purifications.

IU = Inhibitory units (T: trypsin; C: chymotrypsin) are expressed as 1 unit = 0.01 decrease in absorbance at 410 nm under assay conditions. TIU: trypsin inhibitory unit; CIU: chymotrypsin inhibitory unit.

8.8 showed no visible band by either protein or activity staining. However PAGE and gelatin-embedded PAGE at pH 4.5 indicated that the protein was a BBI, HGGI-III (Fig. 3, lane 1). Re-examination of gelatin-PAGE gels (pH 8.8) indicated a visible activity band at the origin, previously considered as aggregated protein unable to enter the gel. Contaminating traces of HGGI-II in the HGGI-I fraction (Fig. 2) and vice versa was removed by anion-exchange chromatography on DEAE-Sephadex A-25 equilibrated in 0.1 M NH_4HCO_3 buffer pH 8.2.

DEAE-Sephadex chromatography was successfully used for the purification of the four inhibitors found in the dormant seed of horsegram (Sreerama et al., 1997). All the four inhibitors bound strongly to DEAE-Sephadex and could be resolved using a NH_4HCO_3 gradient. However the inhibitors of germinated seed did not bind to DEAE-Sephadex at pH 8.2, indicating a decrease in the net negative charge. During the purification, the ratio of chymotrypsin inhibitory units (CIU) to trypsin inhibitory units (TIU) remained nearly constant, indicating that the same protein was responsible for the both the activities (Table 1). The recovery of HGGI-I, HGGI-II and HGGI-III were 4.2, 8.2 and 25%, respectively. Among the three isoinhibitors, HGGI-III is the major isoinhibitor (Table 1). The specific activity of these new inhibitor species towards the trypsin amidase activity is five fold higher than that earlier reported for the dormant seed inhibitors (Sreerama et al., 1997).

2.2. Criteria of homogeneity

The homogeneity of the three isoinhibitors was examined by native PAGE and gelatin-PAGE. The three inhibitors were homogenous migrating as single species both by protein staining (Fig. 3a) and by specific staining for trypsin inhibitory activity (Fig. 3b) and chymotrypsin inhibitory activity (Fig. 3c) using Gelatin-PAGE. RP-HPLC analysis (results not shown) and the release of the single amino terminal residues Asp, Glu and Ser for HGGI-I, II and III, respectively indicate that these isoinhibitors are homogenous.

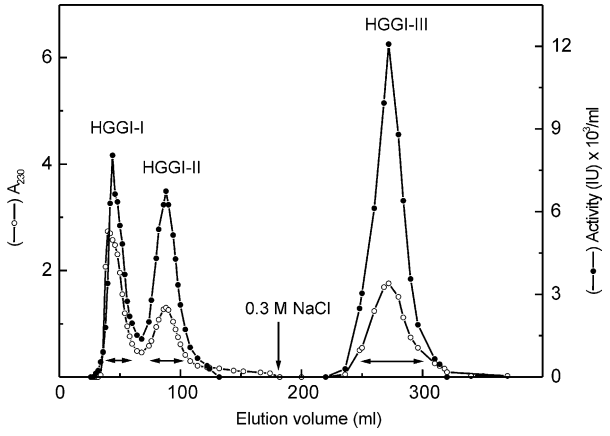

Fig. 2. CM-Sephadex C-25 chromatography of germinated horsegram isoinhibitors. The column was washed with 0.05 M NaOAc pH 5.0 and inhibitors eluted with 0.05 M. NaOAc buffer containing 0.3 M NaCl; flow rate 20 ml/h. The active inhibitor fractions were pooled as shown (⟷).

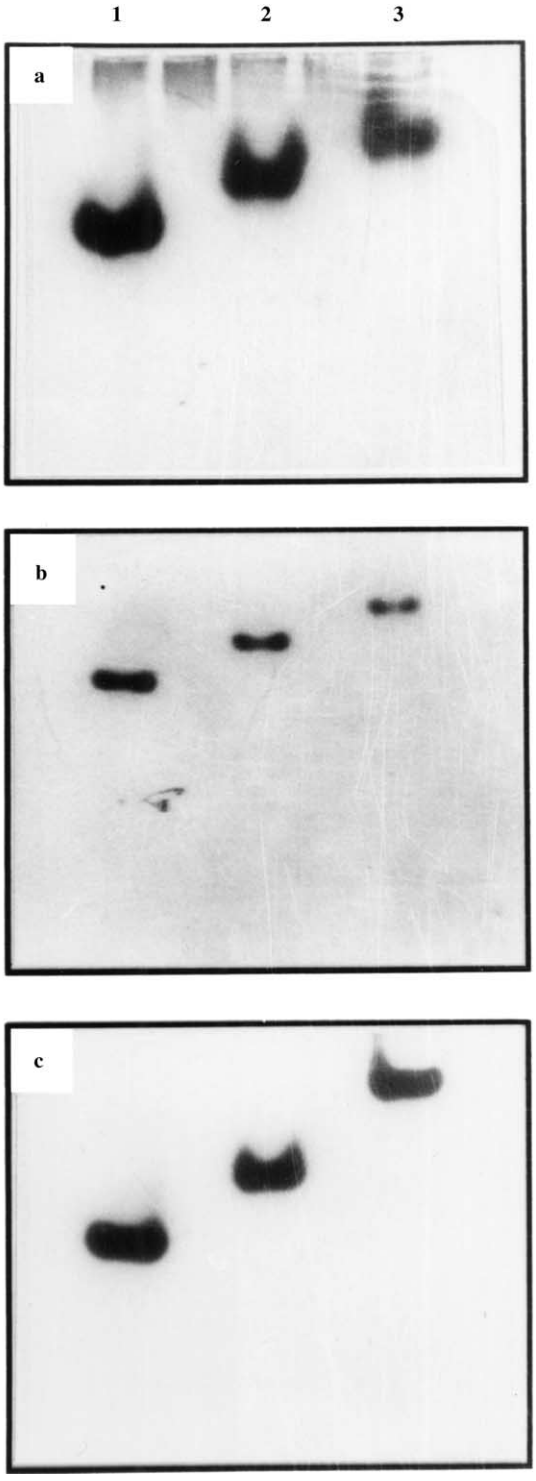

Fig. 3. Native PAGE (10% T, 2.7% C) of the purified isoinhibitors at pH 4.5. HGGI-III (lane 1); HGGI-II (lane 2) and: HGGI-I (lane 3). The gels were stained for (a) protein, (b) trypsin inhibitory activity and (c) chymotrypsin inhibitory activity.

2.3. Molecular weight

The apparent molecular weights of the isoinhibitors estimated by both SDS-PAGE (Fig. 4) and by analytical gel filtration on a Superdex-75 column were 7000 ± 500 Da, indicating them to be single polypeptides. The molecular weights of these inhibitors by mass spectrometry analysis are shown in Table 2. The inhibitors of the dormant seed exhibited molecular masses of ≈l6,000 Da by SDS–PAGE and analytical gel filtration, although ESMS analysis showed them to have a mass of ≈8000 Da (Sreerama et al., 1997). This large overestimation of molecular weights has been attributed to the legume BBIs existing in a state of equilibrium between monomer–dimer–trimer forms (Wu and Whitaker, 1990; Terada et al., 1994b; Godbole et al., 1994a). However such an overestimation of the molecular mass of HGGI-I, -II and -III was not observed by SDS–PAGE or size-exclusion chromatography suggesting that they exist only as monomers.

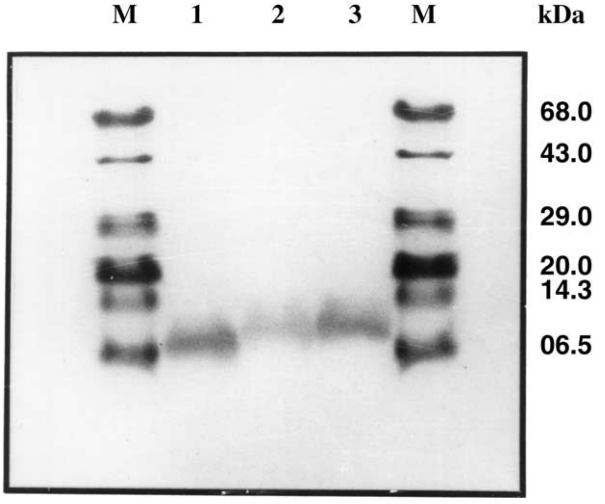

Fig. 4. SDS-PAGE (15% T, 2.7% C) of purified isoinhibitors from germinated horsegram. Molecular weight standards (lane M): bovine serum albumin (68 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa). HGGI-III (lane 1); HGGI-II (lane 2) and HGGI-I (lane 3).

2.4. Amino-terminal sequence and amino acid composition

The pyridylethylated isoinhibitors were subjected to amino-terminal sequence analysis on an automated gas phase sequenator PSQ-1. The three isoinhibitors vary at the amino-terminii but have identical core sequences. The amino-terminal sequence of the germinated horsegram isoinhibitors shows a very high degree of homology to the four isoinhibitors of the dormant seed (Table 4). The BBIs of horsegram seeds also show a high degree of homology to the other legume BBIs (Balaji et al., 1996). Comparison of the determined sequences of the isoinhibitors of the germinated seed with those of the dormant seed show that, the HGGIs are derived from the dormant seed inhibitors. The amino-terminal sequence analysis of the HGGIs, which appear upon germination, indicated that they are in situ products of limited amino-terminal proteolysis of the isoinhibitors present in the dormant seed. Wilson and Chen (1983) demonstrated that the electrophoretically distinct inhibitors (MBI-E and MBI-C) present in the germinated mung bean are the products of carboxy-terminal proteolysis of the major inhibitor MBI-F. The initial event in this degradation of the mung bean (*Vigna radiata*) trypsin inhibitor during germination was the removal of the carboxy-terminal tetrapeptide Lys^{77}-Asp-Asp-Asp^{80}. Degradation then proceeded through a number of specific cleavages including an internal cleavage and the removal of eight residues of the amino-terminus (Wilson and Chen, 1983). A similar modification by limited proteolysis has been observed during the germination of adzuki bean (*Vigna angularis*) (Yoshikawa et al., 1979). The reduced electrophoretic mobility of the HGGIs at pH 8.8 observed earlier (Sreerama and Gowda, 1998) can now be attributed to the loss of the aspartyl and glutamyl residues from the amino-terminus of the dormant seed inhibitors, HGI-I, -II, -III and -IV (Table 4). The initial proteolytic event that occurs during germination to generate HGGI-I and II appears to be catalyzed by the action of an endoproteinase that is specific for the amino-terminal side of an acidic residue. Recently we have isolated a metalloendoproteinase from germinating horsegram, which causes proteolysis of dormant seed

Table 2
Kinetic and molecular properties of Bowman–Birk isoinhibitors of germinated horsegram seeds

Inhibitor	Dissociation constant (K_i, M)		Molecular mass (Da) by MALDI-MS	Isoelectric point (pI)
	Trypsin	Chymotrypsin		
HGGI-I	1.98×10^{-7}	4.77×10^{-7}	7216.7	4.99
HGGI-II	1.89×10^{-7}	3.64×10^{-7}	7074.6	5.09
HGGI-III	0.98×10^{-7}	7.73×10^{-7}	6493.5	5.28

HGGI-I, HGGI-II and HGGI-III: Horsegram germinated inhibitors.

Table 3
Amino acid composition of the Bowman–Birk isoinhibitors of germinated horsegram seeds

Amino acid	Relative amino acid composition (mol%)[a]			
	HGGI-I[b]	HGGI-II[b]	HGGI-III[b]	HGI-III[c]
Asp[d]	6.4	4.2	4.8	11.4
Glu[e]	7.5	5.1	4.4	10.5
Ser	8.2	8.5	7.5	15.2
Gly	0	0	0	0
His	0.5	0.6	0.7	5.0
Arg	3.6	2.4	2.5	2.9
Thr	3.3	3.1	3.2	4.0
Ala	4.6	4.6	4.5	5.9
Pro	6.3	7.3	6.5	8.5
Tyr	1.4	0.7	0.6	1.6
Val	1.2	2.8	3.1	5.4
Met	0.2	0.3	0.2	0.7
1/2Cys[f]	12.5	16.2	14.2	17.7
Ile	2.1	2.0	2.0	2.8
Leu	1.9	1.0	1.0	1.3
Phe	3.2	1.9	1.7	2.4
Lys	2.4	2.6	2.7	4.5

[a] Average of duplicates.
[b] Horsegram germinated inhibitors.
[c] Horsegram inhibitors from the dormant seed (Sreerama et al., 1997).
[d] Determined as aspartate and aspargine.
[e] Determined as glutamate and glutamine.
[f] Determined as pyridylethylated cysteine.

inhibitor in vitro (unpublished results). In both the BBIs and the Kunitz type inhibitors of soybean, the initial proteolysis occurs at the amino-terminal side of an acidic residue i.e. between Lys-Glu in BBSTI-E and Leu-Asp of Kunitz soybean trypsin inhibitor (Orf and Hymowitz, 1979). The amino acid composition of the isoinhibitors HGGI-I, II and III are shown in Table 3 and compared with the major isoinhibitor (HGI-III) of the dormant seed (Sreerama et al., 1997). The substantially high cysteine content of all the three isoinhibitors is characteristic of all BBIs (Liener and Kakade, 1980). A notable feature is the drastic reduction of the aspartate, serine and histidine content of these inhibitors when compared to HGI-III. A part of this loss can be accounted for by the observed amino-terminal proteolysis that occurs during germination (Table 4). In addition, proteolysis of the carboxy-terminal tail of isoinhibitors that are also rich in serine, histidine and aspartate (Balaji et al., 1996; Sreerama and Gowda, 1997) could add to the drastic loss observed. The results of complete primary structure determination of the HGGIs that are in progress would confirm if any carboxy-terminal proteolysis occurs upon germination.

2.5. Isoelectric point

The isoelectric focusing of the isoinhibitors HGGI-I, -II and -III on pre-cast Ampholine® PAGplate (3.5–9.5) showed a single protein band at pI's corresponding to 4.99, 5.09 and 5.28, respectively. HGGI-I and II do not bind to the cation-exchanger CM Sephadex C-25 at pH 5.0 (Fig. 2) as at this pH their charge is near neutral. This increasing order of pI is also reflected in the electrophoretic mobility of the isoinhibitors at pH 4.5, where HGGI-III with the highest pI also has the highest R_f (Fig. 3).

2.6. Inhibitory properties

The three isoinhibitors were tested for their inhibition individually against trypsin and chymotrypsin using specific substrates BAPNA and BTPNA respectively. All the three isoinhibitors were competitive in nature and inhibited both trypsin and chymotrypsin independently. Using the specific substrates the inhibition of trypsin was measured in the presence of excess chymotrypsin and vice versa. The HGGIs were shown to inhibit both trypsin and chymotrypsin independently and simultaneously. Stoichiometric inhibition studies revealed that these isoinhibitors bind trypsin and chymotrypsin in a 1:1 molar ratio (results not shown). The K_is of the inhibitor complexes with trypsin at pH 8.2 and chymotrypsin at pH 7.8 were determined (Table 2). All the three inhibitors show a higher binding affinity towards trypsin when compared to chymotrypsin. These values are comparable to the values of the HGIs (Sreerama et al., 1997) and other BBIs (Terada et al., 1994a). The binding affinity for trypsin are of the order HGGI-III > HGGI-II > HGGI-I (Table 2). HGGI-III displays a higher binding affinity to trypsin compared to HGGI-I

Table 4
Comparison of amino-terminal sequences of Bowman–Birk isoinhibitors of horsegram seeds

[a]HGGI-I							D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[a]HGGI-II								E	P	S	E	S	S	K	P	C	C	D	Q	C
[a]HGGI-III													S	K	P	C	C	D	Q	C
[b]HGI-I					S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI-II		H	H	E	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI-III	D	H	H	Q	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI-IV		H	H	E	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C

[a] Horsegram germinated inhibitors.
[b] Horsegram inhibitors from the dormant seed (Sreerama et al., 1997).

and II despite its missing amino-terminal tail (Table 4). This implicates the contribution of the amino-terminal of the inhibitor in trypsin binding possibly via electrostatic interaction. As the negative charge of the amino-terminal from HGGI-I to III decreases (Fig. 3), the binding affinity of the inhibitor to trypsin increases (Table 2). In the dimeric crystal structure of BBI inhibitor from pea seeds, the carboxy-terminus tail of the inhibitor is shown to make contact with the trypsin molecule (Sierra et al., 1999). However in the X-ray structure of the adzuki bean inhibitor–trypsin complex, no interaction of the carboxy-terminal tail with trypsin was detected (Tsunogae et al., 1986). The involvement of amino-terminus in trypsin needs to be investigated more deeply.

3. Conclusions

The amino-terminal sequence, M_r and amino acid composition are consistent with the HGGIs being derived from HGIs of the dormant seed by proteolysis during germination and not by de novo synthesis as distinct gene products. The relatively high M_r of BBIs (8–10 kDa) is a limitation for use as cancer chemopreventive agents. A smaller version of soybean BBI obtained by chemical and enzymatic cleavage (Birk, 1975; Odani and Ikenaka, 1978) inhibited radiation transformation as effectively as whole BBI (Kennedy, 1993). Therefore HGGI-III smaller in size; a potent inhibitor of chymotrypsin and devoid of the highly immunogenic amino-terminus of HGI-III (Sreerama and Gowda, 1997) may serve as an effective cancer chemopreventive agent.

4. Experimental

4.1. Materials

Horsegram (*D. biflorus*) seeds were obtained locally. Bovine serum albumin (BSA), bovine pancreatic trypsin, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) and Guanidine hydrochloride (GuHCl) were obtained from Sigma (St. Louis, MO, USA). Bovine pancreatic α-chymotrypsin was obtained from Boehringer Mannhiem. Sephadex G-50, CM-Sephadex C-25 and DEAE-Sephadex A-25 were from Pharmacia (Uppsala, Sweden). All other chemicals and reagents were of analytical grade.

4.2. Purification of isoinhibitors

4.2.1. Germination of horsegram seeds

Horsegram (*D. bifiorus*) seeds in 200 g batches were imbibed in distilled water for 24 h at room temperature. These seeds were germinated on moist filter paper in the dark for 120 h at room temperature. After 120 h of germination, the cotyledons were dissected free of the seed coats and axes and frozen at −20 °C until used.

4.2.2. Extraction and purification

The cotyledons were homogenized with 5 volumes of (w/v) 0.1 M Gly–HCl buffer, pH 2.5 in a Waring blender. The meal was then extracted by stirring overnight at 4 °C. The extract was filtered through cheese cloth and then centrifuged at 10,000 *g* for 30 min. The pH of the supernatant was adjusted to 7.5 with liquid ammonia. To the crude extract, solid $(NH_4)_2S0_4$ (36.1 g/100 ml) was added to obtain 60% saturation at 4 °C. The precipitate obtained by centrifugation at 10,000 *g* for 30 min, was re-dissolved in a minimal quantity of distilled water and dialyzed against water (5×500 ml) and freeze-dried. The inhibitor fraction was further purified by size-exclusion chromatography on Sephadex G-50 column (100×2.1 cm) pre-equilibrated with 0.025 M Tris–HCl buffer, pH 7.5 at a flow rate of 12 ml/h at room temperature (25±3 °C). The fractions having trypsin/chymotrypsin inhibitory activity were pooled (Fig. 1), and dialyzed against water and freeze-dried. A final step of ion-exchange chromatography on CM-Sephadex C-25 at room temperature (25±3 °C) was used to resolve the three isoinhibitors.

The freeze-dried isoinhibitor fraction from the size-exclusion chromatography step was dissolved in 0.05 M sodium acetate buffer pH 5.0 and loaded on to CM-Sephadex C-25 column (14×3.4 cm) pre-equilibrated with same buffer at a flow rate of 20 ml/h at room temperature (25±3 °C). Two isoinhibitors were recovered as the unbound protein fraction in the buffer wash (Fig. 2). Subsequently elution of bound protein with buffer containing 0.3 M NaCl afforded a third inhibitor. These isoinhibitors were designated as HGGI-I, HGGI-II and HGGI-III in order of their elution from the CM-Sephadex column. The fractions corresponding to each inhibitory activity were dialyzed and freeze-dried. Further HGGI-I and HGGI-II inhibitor fractions were repurified individually using DEAE-Sephadex A-25 column (7.2×2.7 cm) equilibrated with 0.1 M ammonium bicarbonate buffer, pH 8.2 at a flow rate 20 ml/h. HGGI-I and HGGI-II were obtained in the buffer wash as the unbound protein fraction. The isoinhibitors fractions dialyzed against water, freeze-dried and stored at 4 °C, were used for further studies.

4.3. Trypsin and chymotrypsin inhibitory assay

The amidase activity of trypsin and its inhibition was assayed using the chromogenic substrate BAPNA at pH 8.2 in 0.05 M Tris–HCl containing 20 mM $CaCl_2$ according to the method of Kakade et al. (1969). The amidase activity of chymotrypsin and its inhibition was assayed using the chromogenic substrate BTPNA in

0.08 M Tris–HCl buffer pH 7.8 containing 20% DMSO and 20 mM $CaC1_2$. One unit of trypsin/chymotrypsin enzyme activity is defined as the increase in the absorbance of 0.01 at 410 nm under assay conditions. One inhibitory unit is defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

4.4. Protein estimation

The protein concentration was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

4.5. Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing page (10% T, 2.7% C) was performed at pH 4.5 according to the procedure of Reisfeld et al. (1962). SDS–PAGE (15% T, 2.7% C) was performed according to the procedure of Laemmli (1970). The gels were stained for protein with 0.1% Coomassie brilliant blue and destained.

4.6. Gelatin-embedded polyacrylamide gel electrophoresis (gelatin–PAGE)

Gelatin–PAGE (Felicioli et al., 1997) was performed by adding gelatin (1% w/v final concentration) to the polyacrylamide (10% T, 2.7% C) prepared according to the method of Reisfeld et al. (1962). Following electrophoresis at pH 4.5, the gel was washed three times with distilled water and then incubated at 37 °C in a 0.1 M Tris–HCl bath (pH 8.0 for trypsin and pH 7.8 for chymotrypsin) containing either trypsin or chymotrypsin (40 μg/ml) for 60 min. After the gelatin hydrolysis, the gel was washed with distilled water and stained with Coomassie brilliant blue and destained. The presence of the proteinase inhibitor is detected as a dark blue band in a clear background due to the complex of the unhydrolyzed gelatin with the stain.

4.7. Matrix assisted laser desorption ionization-mass spectroscopy (MALDI-MS)

The exact molecular mass of purified inhibitors HGGI-I, HGGI-II and HGGI-III were obtained from MALDI-MS, Ketos Analytical (UK) Kompact Seq model which uses a 337 nm nitrogen laser desorption and 1.7 nm linear flight path. The instrument was calibrated over the low mass range of 399–5000 Da.

4.8. Amino-terminal sequence analysis

The protein was first pyridylethylated by the method of Hermodson et al. (1973). The alkylated protein was separated from the reagents and native protein by RP-HPLC using an Aquapore C-8 column (220×2.11 mm Pierce) with a linear gradient of 0–70%, 0.1% TFA and 70% acetonitrile containing 0.05% TFA. The protein was further repurified using a Phenomenex C-18 (250×4.6 mm) column with the same solvent system. The amino-terminal sequences of the pyridylethylated inhibitors were determined by Edman degradation using Shimadzu PSQ-l protein sequenator.

4.9. Amino acid composition of isoinhibitors

The pyridylethylated isoinhibitors were hydrolyzed in vacuum at 110 °C in 5.8 M HCl for 24 h. Amino acid analysis was performed by pre-column derivatization with phenylisothiocynate. The phenylthiocarbomoyl amino acids were analyzed by RP-HPLC as described by Bidlingmeyer et al. (1984).

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