COMPLETE AMINO ACID SEQUENCE OF MUNG BEAN TRYPSIN INHIBITOR

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ABSTRACT

The mung bean trypsin inhibitor has been found to be microheterogeneous at N-terminal region due to the presence of several isomers. After treatment with aminopeptidase M it becomes homogeneous and is suitable for sequence determination.

Based on the determination of the structures of two active fragments the complete amino acid sequence of mung bean trypsin inhibitor has been elucidated. It consists of 72 amino acid residues with 7 pairs of disulfide bonds. The results show that this inhibitor belongs to the Bowman-Birk inhibitor family.

I. INTRODUCTION

The cleavage and separation of the two active domains of the mung bean trypsin inhibitor have been achieved and the fact that Lys and Arg are respectively the active centers of the two domains have also been proved by chemical and tryptic modification in our laboratory. The Lys active fragment derived from the N-terminal region of the mung bean inhibitor consisted of two peptide chains with 26 and 9 amino acid residues respectively, these two peptide chains were held together through two pairs of disulfide bonds. The Arg active fragment derived from the C-terminal region of the inhibitor was a single peptide chain composed of 27 amino acid residues. Based on the determination of the structures of these two domains, the complete amino acid sequence of the mung bean inhibitor was deduced.

The primary structure of this inhibitor is similar to that of the soybean Bowman-Birk inhibitor[1]. So the mung bean inhibitor belongs to the Bowman-Birk inhibitor family[2]. However, this inhibitor is unique in its function of stoichiometrically inhibiting trypsin in the molar ratio of 1:2[3] Therefore, the elucidation of the primary structure is of value in the understanding of the structure-function relationship of this inhibitor.

II. MATERIALS AND METHODS

1. *Materials*

The mung bean inhibitor and its two active fragments were prepared according to

the methods described previously. Dimethylallylamide (DMAA) is a Pierce product, sequential grade; 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), Methylisothiocyanate (MITC), and aminopropyl glass (APG), from LKB ultropac; N-methylmorpholine from Koch-Light, redistilled; Dimethyl foramide (DMF), methanol, pyridine, acetonitrile, benzine, ethyl acetate and dichloroethane from Shanghai First Reagent Factory, redistilled; Phenylisothiocyanate (PITC) from Dungfeng Biochemical Reagent Factory attached to Shanghai Institute of Biochemistry; redistilled; Trifluoroacetic acid (TFA) from Shanghai Institute of Organic Fluoride, redistilled. Foil-backed silica plate, No. 5554, from E. Merck.

2. *Methods*

(1) *The attachment of peptides to the solid support (APG)*

This was performed essentially according to Beyreuther's method[4]. About 1—3 mg of the mung bean inhibitor or its active fragment were dissolved in 0.4 ml of 0.4 *M* DMAA buffer, pH 9.5, and 0.4 ml of 10% PITC in acetonitrile was added. After mixing and flushing with nitrogen stream, the reaction mixture was incubated at 50°C for half an hour and stirred at 5 min intervals. The excess reagent was extracted by benzene (5 × 1 ml). The aqueous phase was dried by nitrogen stream at 50°C and left overnight in a vaccum desiccator. Three mg of EDC in 0.5 ml of DMF were added. After standing at room temperature for 5 min, 180 mg of APG (previously washed three times with 1 *M* pyridine-HCl buffer, pH 5.0, five times with water and once with DMF) were added and incubated at 37°C for 3 hours with stirring, then left overnight at room temperature. About 0.25 ml of MITC and N-methylmorpholine were added, mixed and incubated at 45°C for 40 min to block the amino groups remaining on the support. The solid support with protein or peptide was washed with methanol thoroughly on a sinter glass funnel, then twice with ethyl acetate and after drying packed into the column for sequencing.

(2) *The attachment of peptides with C-terminal homoserine to the solid support (APG)*

This was carried out according to Horn and Laurson's methods[5]. About 1—2 mg of the peptide with C-terminal homoserine were dissolved in 0.5 ml of anhydrous trifluoroacetic acid and left at room temperature for 1 hr in the sealed tube. The solution was then dried with nitrogen stream. About 0.5 ml of methanol was added and the solution dried again. The peptide was redissolved in 300 μl of DMF (up to 25 μl H_2O were added to facilitate solution), then 180 mg of APG and 50 μl of triethylamine were added. This was stirred at 45°C for 2 hr and left overnight at room temperature, and the excess amino groups were blocked by adding MITC and N-methylmorpholine. The solid support was washed and dried as described above.

(3) *The operation of the solid-phase peptide sequencer*

LKB 4020 solid phase peptide sequencer was used. A punched card for single column was used to control the sequencer. The reaction of PITC with the amino group of the peptide chain was performed at pH 8.4, 50°C for 10 min. Each cycle of degradation took 80 min; the products of degradation, the anilinothiazolinone deriva-

tives of amino acids (ATZ-amino acid) were collected with the help of a fraction collector. The ATZ-amino acids were then converted by manual operation into their thiohydantoins (PTH amino acid), which were identified by means of thin layer chromatography.

(4) *The conversion of ATZ- and the identification of PTH- amino acids*

The solution of ATZ-amino acid was dried at 50°C with nitrogen stream and 0.5 ml of 1 *N* HCl added. After vigorous stirring, the reaction was allowed to proceed at 80°C for 10 min. The converted PTH-amino acids were extracted twice with ethyl acetate (2 × 1 ml). After centrifugation, the organic phase was dried with nitrogen stream. The residue was dissolved in 2 drops of ethyl acetate and applied on a sillica gel plate (10 × 10 cm). After developing with solvent 1[6] (chloroform: ethanol=98:2), PTH-Pro, -Leu, -Ile, -Val, -Phe, -Met, -Ala, -Gly, -Trp, -Tyr, -Lys, and Di-PTH-Cystine could be identified under UV light (254 nm). Using solvent system 2 (chloroform: ethanol: methanol = 88.2:1.8:10) PTH-Thr, -Ser, -Gln, -Asn, -Glu and -Asp could be identified under UV light. The aqueous phase was dried at 60°C with nitrogen stream, dissolved in acetone (containing 1% water) and applied on a silica gel plate. After developing with solvent 3 (butylacetate: isopropanol: pyridine: acetic acid: water = 10:4:4:1:1) and treated with iodine vapour, yellow spots of PTH-His, -Arg, and -Cysteic acid (Rf = 0.63, 0.42, and 0.33 respectively) could be identified.

During Edman degradation of the first half cystine residue, a gap appeared in the sequence on account of the fact that this first released half residue was still linked with the second half attached to the peptide chain through the disulfide bond. Di-PTH-Cys could be identified after a second degradation of this residue.

(5) *Manual sequence determination*

Manual Edman degradation was carried out essentially according to Edman[7], with some experimental modifications after Peterson et al.[8] A peptide or protein (100—300 n mole) was placed in a 10 ml stoppered glass centrifuge tube, 0.4 ml DMAA, pH 9.5 buffer solution and 0.3 ml of 10% PITC in acetonitrile were added. After flushing the tube with nitrogen stream and mixing the reagents thoroughly the coupling reaction was allowed to proceed for 30 min at 50°C. The excess reagent was removed by extraction with benzen (4 × 1.5 ml). The aqueous phase was subsequently dried with nitrogen stream at 55°C. Cleavage was performed by dissolving the dried peptide in 0.5 ml of trifluoroacetic acid and incubating it at 50°C under nitrogen. After 10 min the trifluoroacetic acid was removed in a stream of nitrogen, 0.4 ml of water was added, and the thiazolinone derivatives were extracted from the aqueous layer by extracting it with 1 ml of peroxide and aldehyde-free ethyl acetate. The ethyl acetate extract was dried in a stream of nitrogen and the conversion was carried out by heating the thiazolinone derivatives for 10 min at 80°C in 0.5 ml of 1 *N* HCl under nitrogen. The PTH-amino acids were extracted with ethyl acetate with the exception of PTH-Arg, -His, and -Cysteic acid which remained in the aqueous layer. The aqueous phase containing the shortened peptide was extracted twice with ethyl acetate and the organic phase was discarded. The next coupling was started after drying the peptide containing aqueous layer with nitrogen stream at 55°C. During the whole operation air was carefully kept

away, and the reactive solutions were kept under nitrogen all the time.

(6) *Acetylation of the mung bean inhibitor and its active fragments*

The acetylation of the inhibitor was performed essentially according to the methods of Riordan and Vallee[9]. Thirty mg of the mung bean inhibitor were dissolved in 3 ml of 0.4 M DMAA buffer, pH 9.5. The solution was well stirred and kept in an ice bath. Acetic anhydride was dropped into the mixture within half an hour and the pH maintained within the range of 8—9 by adding 5 N NaOH. After reaction, the solution was desalted on a Sephadex G-10 column.

The oxidation of disulfide bonds of the peptides and proteins: As described in the previous paper[10].

Tryptic modification of the mung bean inhibitor and its active fragments: As described in the previous paper.

III. Results

1. *Sequence of the N-terminal Region of the Mung Bean Inhibitor*

The preparation of the crystalline mung bean inhibitor showed four close bands in polyacrylamide gel electrophoresis. The main N-terminal residue was found to be Ser, and Asp also could not be neglected. In fact, they were the isoinhibitors with microheterogeneities, possessing the same inhibitory activity. This microheterogeneity has brought forth some difficulties in the determination of the primary structure of this inhibitor. When treated with aminopeptidase M in excess this inhibitor preparation released four amino acids, the amount of which decreasing in the following order: Ser, Asp, His, Lys (in the molar ratio 3.3 : 2.8 : 2 : 1). After enzymic digestion followed by purification of Sephadex G-50, a homogeneous preparation with Asp as the N-terminal residue could fortunately be obtained. This sample was suitable for manual Edman degradation involving 28 steps. When the original preparation was directly subjected to Edman degradation the main spots of PTH-Ser, -Asp and a small amount of PTH-Lys were found on the TLC plate in the first step, then PTH-His and -Asp in the second cycle. According to the molar ratio of amino acids released by aminopeptidase M the mung bean isoinhibitors were seen to have four components. Two isoinhibitors have a deletion of the first residue Ser, and start with Asp and Lys as the N-terminal residues at the second position respectively.

	5				10				15			
Ser.	His.	Asp.	Glu.	Pro.	Ser.	Glu.	Ser.	Ser.	Glu.	Pro.	Cys.	Cys.
Ser.	Asp.	···										
	Asp.	···										
	Lys.	···										

	20				25				30			
Asp.	Cys.	Thr.	Lys.	Ser.	Lys.	Pro.	Pro.	Gln.	Cys.	His.	Cys.	Ala.	Asn.

Amino acid sequence of the mung bean isoinhibitors at the N-terminal region.

When the same sample was coupled with aminopropylglass (APG) using the condensation reagent 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC), and the sequence determined with the aid of a LKB 4020 solid phase peptide sequencer, the result was identical to that obtained with manual operations.

2. *The Structure of the Lys Active Fragment*

In a previous paper[10] we reported that after oxidation of the Lys active fragment with performic acid, two peptide chains A_1 and A_2 could be separated on a column of Sephadex G-25. The sequence of the N-terminal region of the A_1 chain determined by Edman degradation was Ser·Ser·Glu·Pro·Cys. This sequence was in accordance with the residues from 8th to 12th in the intact inhibitor. This means that the N-terminal heptapeptide of this inhibitor was removed after peptic digestion. The C-terminal residue of the A_1 chain was identified as Leu by carboxypeptidase A, after further adding of carboxypeptidase B to the reaction solution Arg, Ile and Asn could subsequently be found. Thus, the sequence of C-terminal region of the A_1 chain could be deduced as -Asn-Ile-Arg-Leu. Comparing the amino acid composition of the A_1 chain with that of the N-terminal section of intact inhibitor (8—30), all the amino acid residues were the same except the extra residues Ile, Arg and Leu in the A_1 chain. The A_1 chain must therefore be the N-terminal fragment (8—33) of this inhibitor with the sequence:

10 15 20
Ser.Ser.Glu.Pro.Cys.Cys.Asp.Ser.Cys.Asp.Cys.Thr.Lys.Ser.Lys.Pro.
25 30
Pro.Gln.Cys.His.Cys.Ala.Asn.Ile.Arg.Leu.

The complete amino acid sequence of the A_2 chain determined on the solid phase sequencer is as follows:

Phe.Cys.Tyr.Lys.Pro.Cys.Glu.Ser.Met.

As the mung bean inhibitor belongs to the Bowman-Birk inhibitor family, there were a lot of resemblance in their structures. Moreover 50% of the total amino acid residues were identical, especially the sites of active centers, disulfide bonds and Pro were conservative[11]. The positions of the disulfide bonds in the mung bean inhibitor could

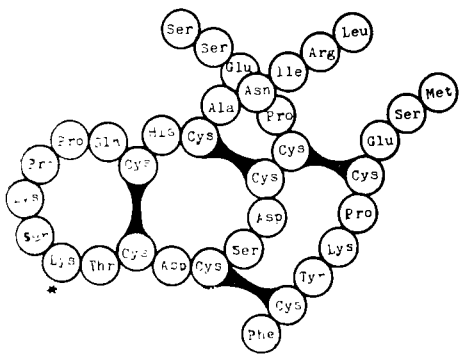

Fig. 1a. The primary structure of Lys active fragment.

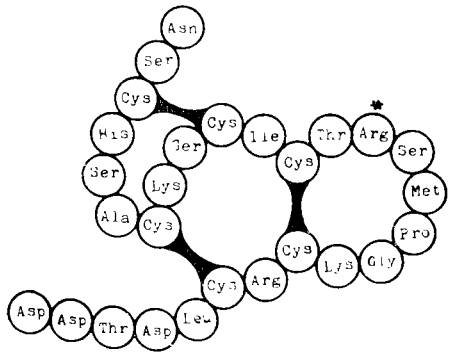

Fig. 1b. The primary structure of Arg active fragment.

be inferred by analogy with those in the Bowman-Birk inhibitor family[11]. Then the whole primary structure of the Lys active fragment was deduced as shown in Fig. 1a, the asterisk denoting the reactive site, Lys^{20}.

3. *The Structure of the Arg Active Fragment*

This active fragment consisted of a single peptide chain with Asn and Asp as N- and C-terminus. When subjected to manual Edman degradation the partial amino acid sequence is determined as follows:

Asn.Ser.Cys.His.Ser.Ala.Cys.Lys.Ser.Cys.Ile.Cys.Thr.Arg.Ser.Met.Pro.Gly.

There was only one Met residue in this peptide, hence it was amenable to special CNBr cleavage. In order to avoid the troubles involved in peptide separation, this Arg active fragment was first acetylated to block the N-terminus, followed by CNBr cleavage. The reaction mixture was then coupled with the solid support APG by means of the condensation reagent EDC for sequence determination on the solid phase peptide sequencer. Owing to the blockage of the N-terminus of the intact Arg fragment only the newly formed peptide after the Met residue could be submitted to Edman degradation. The result is as follows:

Pro.Gly.Lys.Cys.Arg.Cys.Leu.Asp.Thr.Asp.Asp.

It is well known that the peptide bond of the active site in the trypsin inhibitor could be specifically cleaved with trypsin at an acidic pH without any loss of activity, giving rise to the so called "modified inhibitor"[12]. Taking advantage of this feature the acetylated mung bean inhibitor was digested with trypsin at pH 3.5. Owing to the blockage of the Lys active site by acetylation at the ϵ-amino group, only the peptide bond of the Arg active site could be cleaved. This modified inhibitor was directly coupled with the solid support APG and applied to the solid phase peptide sequencer. The partial sequence after the Arg active site is determined as in the following:

Ser.Met.Pro.Gly.Lys.Cys.Arg.Cys.Leu.Asp.Thr.Asp.Asp.Phe.Cys.Tyr.

This sequence was the same as that at the C-terminal region of the Arg active fragment with the exception of the extension of Phe·Cys·Tyr. at its C-terminus. This tripeptide was none other than the N-terminal region of the A_2 chain of the Lys active fragment. This means that the site of peptic cleavage was at the peptide bond of Asp-Phe. The newly released residue of Asp turned to be the C-terminus of the Arg active fragment. By analogy with the positions of the disulfide bonds in Bowman-Birk inhibitors the whole primary structure of the Arg active fragment could be depicted as shown in Fig. 1b. The asterisk denotes the active site, Arg^{41}.

4. *The C-terminal Region of the Mung Bean Inhibitor*

After splitting the mung bean inhibitor with CNBr a small peptide could be separated on a Sephadex G-10 column. The sequence of this peptide was determined as Asp·Lys·Asp. by manual Edman degradation and amino acid composition analysis. When treated with carboxypeptidase Y, the intact inhibitor released Asp, Lys and Met in the molar ratio 2:1:1. Therefore this small peptide should be the C-terminal region of the intact inhibitor and joined to the C-terminal residue Met of the A_2

peptide chain of the Lys active fragment.

Summing up the above results, the complete primary structure of the mung bean inhibitor is shown in Fig. 2.

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                5          ↓       10                  15
Ser.His.Asp.Glu.Pro.Ser.Glu.Ser.Ser.Glu.Pro.Cys.Cys.Asp.Ser.
                20 *               25                  30
Cys.Asp.Cys.Thr.Lys.Ser.Lys.Pro.Pro.Gln.Cys.His.Cys.Ala.Asn.
          ↓     35                 40                  45
Ile.Arg.Leu.Asn.Ser.Cys.His.Ser.Ala.Cys.Lys.Ser.Cys.Ile.Cys.
        *       50                 55                  60  ↓
Thr.Arg.Ser.Met.Pro.Gly.Lys.Cys.Arg.Cys.Leu.Asp.Thr.Asp.Asp.
                65                 ↓70
Phe.Cys.Tyr.Lys.Pro.Cys.Glu.Ser.Met.Asp.Lys.Asp.
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* Active site

↓ Site of peptic cleavage

Fig. 2 Amino acid sequence of mung bean trypsin inhibitor.

The amino acid composition calculated from the primary structure was in close accord with that determined previously[3] by amino acid analysis except some slight differences in Arg and Lys residues (see Table 1).

IV. Discussion

The primary structure of the mung bean inhibitor showed that this inhibitor belonged to the soybean Bowman-Birk inhibitor family. They have some common characteristics[11] as follows; The molecular weights are lower than 10000. Owing to the presence of 7 pairs of disulfide bond the molecules are very rigid and stable. They are rich in polar amino acids which account for 60% of the total residues, and low in aromatic amino acids. In addition, all of them comprise two active domains. In general, these two domains inhibit trypsin, chymotrypsin or elastase with Lys (Arg) and Leu (Phe, Tyr) or Ala as their active centers respectively[2]. Besides the mung bean inhibitor, the adzuki inhibitor I[13] can also inhibit two molecules of trypsin. Another example is the soybean Bowman-Birk inhibitor D-II[14]. Though this inhibitor has two Arg residues as its active centers, it can merely inhibit trypsin in the molar ratio 1 : 1.4 instead of 1 : 2. Some inhibitors can inhibit trypsin and chymotrypsin simultaneously, such as the soybean Bowman-Birk inhibitor[15] and the lima bean inhibitor[16], but not in the case of the adzuki bean inhibitor II[17] even if it also pessesses two active centers responsible for inhibiting chymotrypsin and trypsin respectively. Thus, the functions of inhibitor are not only related to their active centers, but also to the amino acid sequences around them and to the conformation of the molecule. The amino acid sequences around two active centers of some Bowman-Birk inhibitors are listed in Table 1.

P_1 is responsible for the specificity of the active domain. P_2' and P_5' are variable and may exert some influence on the activity of the domain's. In the case of the mung bean inhibitor the P_2' in the first domain is Lys, obviously favourable to inhibiting trypsin. Whereas in the case of the soybean Bowman-Birk D-II inhibitor, P_5' is the hydrophobic amino acid Met, unfavourable to combining with trypsin. In the second domain the amino acid sequence around the active site of the mung bean inhibitor is identical with that of the garden bean inhibitor. Both of these two domains can com-

bine with trypsin in equal molar ratio. It is worth pointing out that all their P'_5 are Lys. On the other hand the P'_5 in the soybean Bowman-Birk D-II inhibitor is Gln. Perhaps such microheterogeneity in the amino acid sequence around the active centers may explain their functional difference. Though both the mung bean inhibitor and the soybean Bowman-Birk D-II inhibitor have the same specificity, their activities are different in inhibiting trypsin to some extent.

Table 1

The Amino Acid Sequence Around the Active Centers in the Bowman-Birk Inhibitor Family

Name of the Inhibitors	P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4 P'_5 P'_6	P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4 P'_5 P'_6
Bowman-Birk Inhibitor[18]	Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys	Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys
Bowman-Birk D-II[19]	Cys-Thr-Arg-Ser-Met-Pro-Pro-Gln-Cys	Cys-Thr-Arg-Ser-Gln-Pro-Gly-Gln-Cys
Garden Bean[20]	Cys-Thr-Ala-Ser-Ile-Pro-Pro-Gln-Cys	Cys-Thr-Arg-Ser-Met-Pro-Gly-Lys-Cys
Lima Bean[21]	Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys	Cys-Thr-Leu-Ser-Ile-Pro-Ala-Gln-Cys
Adzuki Bean[22]	Cys-Thr-Lys-Ser-Met-Pro-Pro-Lys-Cys	Cys-Thr-Tyr-Ser-Ile-Pro-Ala-Lys-Cys
Mung Bean	Cys-Thr-Lys-Ser-Lys-Pro-Pro-Gln-Cys	Cys-Thr-Arg-Ser-Met-Pro-Gly-Lys-Cys

The successful cleavage and separation of the two active domains also were achieved in two other Bowman-Birk inhibitors; the soybean Bowman-Birk inhibitor by Odani[24] and Ikenaka in 1974, and the adzuki bean inhibitor II by Yoshikawa[25] in 1980. The

Table 2

Amino Acid Compositons of Mung Bean Inhibitor and Its Two Active Fragments

Amino Acid	Lys Active Fragment				Arg Active Fragment	Mung Bean Inhibitor	
	A_1	A_2	A_1	A_2		a	b
Asp	3		3		4	10	10
Thr	1		1		2	3	3
Ser	4	1	5		4	11	11
Glu	2	1	3			5	5
Gly					1	1	1
Ala	1		1		1	2	2
½Cys	6	2	8		6	14	14
Met		1	1		1	2	2
Ile	1		1		1	2	2
Leu	1		1		1	2	2
Tyr		1	1			1	1
Phe		1	1			1	1
Lys	2	1	3		2	5	6
His	1		1		1	4	3
Arg	1		1		2	4	3
Pro	3	1	4		1	5	6
Total	26	9	35		27	72	72
N-terminal	Ser	Phe	Ser	Phe	Asn	Ser	Ser
C-terminal	Leu	Met	Leu	Met	Asp	Asp	Asp

a. Determined by amino acid composition analysis.
b. Calculated from amino acid sequence.

separated active fragments were responsible for inhibiting trypsin or chymotrypsin respectively. However, in the above two examples one of these two fragments partially lost its activities, so the mung bean inhibitor seemed to be a unique instance in which both the active fragments retained all the activities for inhibiting trypsin in equal molar ratio. Recently Yoshikawa's group[26] showed that the partial loss in the activity of the fragment inhibiting trypsin could be explained by the so-called "temporary inhibition" as a result of the cleavage of the Arg peptide bond in the Cys·Arg·Cys. sequence by trypsin. The corresponding tripeptide in the mung bean inhibitor, residues 26—28, Cys·His·Cys., was obviously resistant to tryptic digestion so it would not be surprised that the Lys fragment kept in the form of the crystalline compound with trypsin for one month or more still retained inhibitory activity after dissociation of this compound at an acidic pH.

The cleavage sites of pepsin digestion in both the mung bean inhibitor and the adzuki bean inhibitor II were similar, i.e. the peptide bonds Glu^{7}-Ser^{8}, near the N-terminus and Leu^{33}-Asn^{34} (Leu-Asp in the case of adzuki bean inhibitor), Asp^{60}-Phe^{61} of the connecting peptides. In the C-terminal region the peptide bond split was Met^{69}-Asp^{70} in the mung bean inhibitor and Asp-Trp in the adzuki bean inhibitor II. The successful cleavage and the elucidation of the structure of the two domains of the mung bean inhibitor opens the scope for further studying the structure-function relationship of this inhibitor.

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