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Research Article

Extraction of trypsin inhibitor from three legume seeds of the Royal Project Foundation

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Abstract

Trypsin inhibitor was isolated from navy bean (*Phaseoulus vulgaris)*, red kidney bean (*Phaseoulus vulgaris L*) and adzuki bean (*Vigna angularis*) from the Royal Project Foundation, Thailand. Extraction of navy bean and red kidney bean with 0.02 M NaOH showed the highest recovery of trypsin inhibitor, while water was the best extractant for adzuki bean. The extraction time significantly affected the trypsin inhibitor recovery (p<0.05). Two hours of extraction gave the highest yield of trypsin inhibitor from three legumes. The trypsin inhibitors were estimated to have molecular weight of 132, 118 and 13 kDa under non-reducing conditions for navy bean, red kidney bean and adzuki bean, respectively. The inhibitory activities of the inhibitor from these legumes were lost when they were treated with β-mercaptoethanol, indicating the subunit of polypeptide in its composition.

Keywords: trypsin inhibitor, legume, navy bean, red kidney bean, adzuki bean, seed

Introduction

Protease inhibitors are small protein molecules that have the ability to inhibit the action of target proteolytic enzymes. They are found in most organisms, mainly in plants where they are widely distributed among different families and are particularly abundant (1-10% of total proteins) in storage organs like seeds and tubers [1]. Trypsin inhibitors have been isolated and characterized from many legume seeds, e.g. soybean [2], tepary bean [3], navy bean [4], faba bean [5], cowpea [6], bambara groundnut and pigeon pea [7]. In most cases, trypsin inhibitors that have been purified from legumes consist of two types, the Kunitz trypsin inhibitor (20-24 kDa) and

Bowman-Birk inhibitor (8 kDa) [8]. Because of their ability to inhibit the enzymes involved in digestive processes of humans and animals, protease inhibitors have been referred to as an 'antinutritional factor'. This protease inhibitor will reduce the nutritive value of legumes. Therefore, a lot of studies were conducted to find an effective method to remove these factors. Protease inhibitors can be extracted by using alkaline extraction [9, 10, 11], aqueous salt solution with varying pH [12, 13] or with water. Each method has its own advantages and disadvantages with the different target protein components. However, investigations on the extraction of trypsin inhibitor for navy bean, red kidney and adzuki bean, especially those cultivated in the northern part of Thailand have not been reported. The objective of this work is to extract the trypsin inhibitor in navy, red kidney and adzuki beans obtained from the Royal Project Foundation in Chiang Mai, Thailand.

Materials and Methods

Chemicals & legume seeds

N-*α*-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), trypsin from bovine pancreas, *β*-mercaptoethanol (*β*ME), casein from bovine milk, bovine serum albumin (BSA), coomassie brilliant Blue R-250 and *N, N, N′, N′*-tetramethyl ethylene diamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulphate (SDS) was procured from Ajax Finechem (Auckland, NZ). Navy bean (*Phaseolus vulgaris*), red kidney bean (*Phaseolus vulgaris L*) and adzuki bean (*Vigna angularis*) were obtained from the Royal Project Foundation, Chiang Mai, Thailand.

Extraction of trypsin inhibitor

Sample preparation

The legume seeds were ground using a coffee mill to a particle size of 20 meshes. The seed flour was defatted by mixing with hexane at the ratio of 1:5 (w/v) for 10 min. The mixture was filtered through Whatman No.1 filter paper and the sediment was rinsed with hexane 3 times to remove the residual oil in the ground sample. The defatted sample was air-dried at ambient temperature (28-30°C) until dry and free of hexane odor.

Extraction media

The defatted samples were extracted with 0.01, 0.02 M NaOH, 0.15, 0.30 M NaCl and distilled water at the ratio of 1:5 (w/v) and then shaken at 180 rpm at ambient temperature for 1 hour. The supernatant was recovered by centrifuging the mixture at 8,000*xg* for 30 min at room temperature. The trypsin inhibitory activity and the protein content of extracted samples were determined. The specific trypsin inhibitory activity of the extracts using the different extractants was compared.

Extraction time

The best extractant that showed the highest specific inhibitory activity was used to study the effect of extraction time on the recovery of the trypsin inhibitor. The defatted samples were extracted with the best extractant obtained from 2.2.2 for 1, 2, 3, 4, and 5 h at room temperature. The inhibitory activity and the protein content were measured and compared in terms of specific inhibitory activity.

Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured according to the method of Benjakul *et al.* [7] using BAPNA as substrate. A solution containing 100 µL of extracted sample, 200 µL (20 µg/mL) trypsin and 100 µL of distilled water were pre-incubated at 37°C for 10 min. Then 500 µL (0.4 mg/mL) of BAPNA (pre-warmed to 37°C) was added to start reaction. After incubation at 37°C for 10 min, 100 µL 30% (v/v) acetic acid was added to terminate reaction and then subjected to centrifugation. Activity of trypsin was measured by the absorbance at 410 nm due to *p*-nitroaniline released. One unit of trypsin inhibitor was defined as 0.01 decreases in absorbance at 410 nm under assay conditions compared with the control (without inhibitor).

Protein determination

The protein concentration was determined by the Biuret method [14] using BSA as a standard.

Electrophoresis

Protein pattern

SDS-PAGE was carried out by the method of Laemmli [15] using 15% separating and 4% stacking gels. The samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, and bromophenol blue) with and without βME for reducing and non-reducing conditions, respectively. The mixture was boiled for 3 min to solubilize the protein. Twenty micrograms of protein were loaded and the proteins separated at 15 mA/gel using Mini Protean II unit (Bio-Rad Laboratories, Inc, Richmond, CA, USA). After separation, the protein was stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 20% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

Inhibitory activity staining

The protein separated by electrophoresis was subjected to incubation with 50 mL of a mixture of 0.2 mg/mL trypsin solution in Tris-HCl pH 8.3 for 60 min at 4°C to allow the trypsin to diffuse into the gel and was then washed with distilled water. The gel was incubated at 37°C for 90 min in 1% (w/v) casein in 0.1 M phosphate buffer, pH 6.0 and then rinsed with distilled water, fixed, and stained with Coomasie Brilliant Blue R-250. The inhibitory zone was detected as a dark band on a clear background. The apparent molecular weight of the trypsin inhibitor was estimated by comparing the Rf with those of the protein standard marker.

Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared. Analysis was performed using a SPSS package (SPSS 16.0 for Windows, SPSS Inc, Chicago, IL).

Results and Discussion

Effect of extractant on recovery of trypsin inhibitor

Trypsin inhibitor from three legumes was extracted with different media. The recovery in terms of protein content, trypsin inhibitory activity and specific activity was determined and is shown in Table 1. Among all extractants used, 0.02 NaOH had the highest specific inhibitory activity for navy bean and red kidney bean. When the pH of extraction is below or above the isoelectric

point (pI), the solubility of protein will increase. However, adzuki bean showed water as the best extractant for the highest specific activity. The specific activity of navy bean and red kidney bean extracted with NaCl was lower compared to other extractants. Navy bean extract that used 0.30 M NaCl showed higher protein content and trypsin inhibitory activity than 0.15 M NaCl. Red kidney bean extracted with the increasing concentration of NaCl showed a decrease in trypsin inhibitor and protein content. These results showed that 0.2 M NaOH is more selective to extract the trypsin inhibitor from navy bean and adzuki bean.

The aqueous alkali has been popular because it has high capability for protein solubilization. Protein solubility is a function of temperature, pH, presence of other ions and the values obtained for solubility are highly dependent on the method used to achieve the solubility [16]. Mild alkaline extraction might be effective because pH of the extractants is far from the pI of trypsin inhibitor from legumes (ranging from 4.6 to 7.6) [17]. This phenomenon possibly is caused by higher polar groups of protein contained in adzuki bean that is exposed to water. Harrison [18] reported that a higher ratio of polar residues in protein will increase the solute-solvent interations in aqueous solution. When legume proteins were extracted with media containing NaCl, they showed a significant decrease of protein content and activity. Schut [19] suggested that NaCl causes a shift in the pI to a more acidic pH as a result of specific ion binding effect. Thus with the addition of NaCl and selective binding of the chloride anions, the protein would have an excess of negative charges at the pH of original pI. Liu and Hung [20] also reported that high concentration of NaCl reduced chickpea protein solubility.

Table 1. **Effect of extractant on trypsin inhibitory activity of three legume seeds**.

Cultivars	Extractants	Trypsin Inhibitor (unit/ g seed)	Protein (mg/g seed)	Specific activity (units/mg protein)
Navy	Water	5845 ± 571a	109.92 ± 0.75a	53 ± 5b
	0.01 M NaOH	5751 ± 428a	150.12 ± 0.62b	38 ± 2a
	0.02 M NaOH	7894 ± 279b	143.58 ± 2.73b	54 ± 2b
	0.15 M NaCl	5811 ± 31a	147.71 ± 1.18b	39 ± 0a
	0.30 M NaCl	5944 ± 747a	149.40 ± 10.03b	40 ± 7a
Red Kidney	Water	1987 ± 427a	105.75 ± 2.43a	19 ± 4a
	0.01 M NaOH	2739 ± 396a	158.47 ± 1.36c	17 ± 2a
	0.02 M NaOH	4455 ± 1251b	156.36 ± 4.91c	28 ± 7b
	0.15 M NaCl	2418 ± 578a	142.11 ± 1.25b	17 ± 4a
	0.30 M NaCl	2260 ± 422a	138.91 ± 0.13b	16 ± 3 a
Adzuki	Water	8490 ± 91ab	83.33 ± 1.11a	102 ± 1c
	0.01 M NaOH	12175 ± 802c	151.29 ± 2.88d	80 ± 4b
	0.02 M NaOH	12354 ± 451c	184.04 ± 3.87e	67 ± 3a
	0.15 M NaCl	7794 ± 705a	93.97 ± 5.18b	83 ± 12b
	0.30 M NaCl	9245 ± 508b	138.12 ± 2.16c	67 ± 2a

*Mean ± SD of triplicate determinations

Different letters within the same column of each legume indicate significant difference ($p< 0.05$).

Effect of extractant on protein and inhibitory patterns of extracted inhibitor

The protein and inhibitory patterns of extracted inhibitors are depicted in **Figure 1.** Under non-reducing conditions, the major protein components in navy bean are 215, 132 and 34 kDa. For red kidney bean the main proteins are 118, 59, and 32 kDa. Interestingly, only one major band of

62 kDa was observed in adzuki bean. However, based on reducing conditions the changes of protein pattern were observed. Degradation of the 132 kDa into the 52 kDa was clearly observed in navy bean and the 118 kDa into the 50 kDa in red kidney bean. For adzuki bean the same pattern of proteins in both reducing and non reducing conditions was observed. From this result it can be stated that the major band of 132 kDa in navy bean and the 118 kDa in red kidney bean were stabilized by disulfide bonds, while this bond was absent in the extracted protein from adzuki bean.

The inhibitory activity staining revealed that the molecular weight of 132 kDa, 118 kDa and 13 kDa is the trypsin inhibitor in navy bean, red kidney bean and adzuki bean, respectively. However, interesting results showed that the reducing condition of navy bean and red kidney bean and adzuki bean can result in the loss of their inhibitory activity against trypsin. It demonstrates that the inhibitor bonds of these legumes are stabilized by an s-s bond. Moreover, the band with MW 59 in red kidney and 62 kDa in adzuki bean appeared only slightly on the inhibitor stained gel. Appearance of these bands is possibly due to the high amount of these components in the extracts, so it is hard to hydrolyze with limited trypsin.

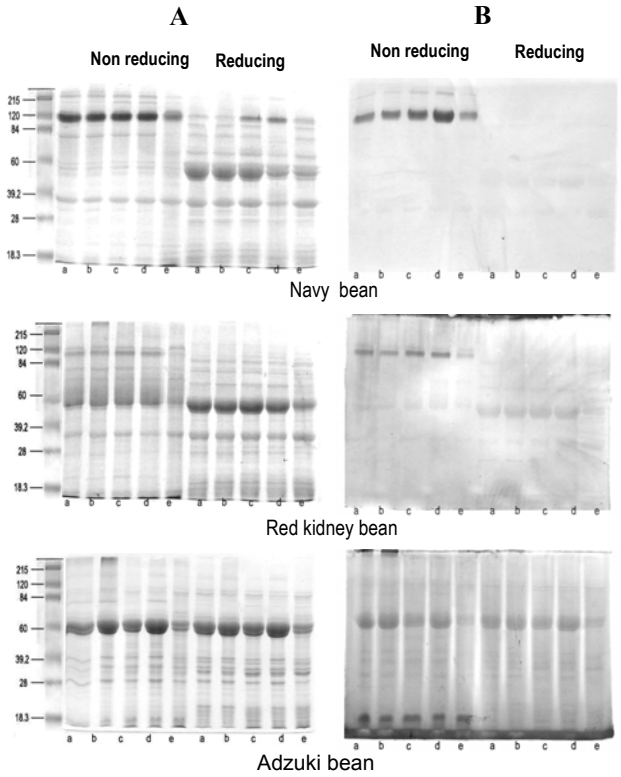

Figure 1. Effect of different extractants on the protein pattern (A) and inhibitory activity staining (B) of three legume seeds. Eighteen µg of protein were loaded. (a) 0.01 M NaOH; (b) 0.02 M (NaOH); (c) 0.15 M NaCl; (d) 0.30 NaCl; (e) water.

For inhibitory activity staining, trypsin inhibitors with different apparent molecular sizes were found in different seed extracts. The inhibitor band in navy bean, red kidney bean and adzuki bean was 132 kDa, 118 kDa and 13 kDa, respectively. Adzuki bean showed one inhibitory band with molecular weight of 13 kDa. Garcia-Carreno *et al*. [21] reported that the trypsin, chymotrypsin and papain inhibitors had molecular weight ranging from 14-66 kDa in non reducing condition, whereas the Bowman-Birk type inhibitor has molecular weight of 8-10 kDa and more than 20 kDa for Kunitz type. Dennison [22] reported SDS-PAGE might overestimate the molecular weight of some proteins. If the proteins are not reduced, then disulfide bridges may constrain the structure and prevent formation of the rod-like complexes. This can result in an incorrect apparent MW.

When the sample was exposed on reducing condition, a loss of inhibitory activity was observed. This condition showed the role of disulfide bond for stabilizing the inhibitor structure as well as the inhibitory activity. Godbole *et al.* [23] reported the inhibitor loses its three dimensional structure when the disulfide bonds are reduced and it also loses inhibitory activity towards trypsin. The intramolecular disulfide bridges may responsible for the functional stability of the protein molecules [24].

From these results, the highest specific inhibitory activity was achieved by using 0.02 M NaOH for navy bean, red kidney bean and water for adzuki bean. These conditions were chosen for the optimum extraction time.

Effect of extraction time on recovery of trypsin inhibitor

Sodium hydroxide (0.02 M) was used as the extractant for navy bean and red kidney bean and water for adzuki bean. The extraction times were varied from 1 to 5 h with gentle shaking. Table 2 shows that specific inhibitory activity of all seeds increased significantly up to 2 h and had some loss of activity when the extraction time was more than 2 h ($p<0.05$). Significantly decreased trypsin inhibitory activity was observed when the extraction process was longer than 2 h. With this result, it was presumed that trypsin inhibitor from navy bean, red kidney bean and adzuki bean underwent denaturation during the long time of extraction, especially in alkaline condition. The mechanical shaking during the extraction process can cause the denaturation of protein via thermal and/or physical denaturation. This phenomenon is due to the air-liquid interface spread over the surface of the bubble resulting in the unfolding of the molecule [25]. The highest specific activity was obtained at 2 h of extraction for three legumes.

Table 2. Effect of extraction time on trypsin inhibitory activity of three legume seeds.

Cultivars	Extraction Time (h)	Trypsin Inhibitor (unit/ g seed)	Protein (mg/g seed)	Specific activity (units/mg protein)
Navy	1	7560 ± 259b	144.039 ± 1.76c	52 ± 2a
	2	8679 ± 789 b	137.34 ± 0.670a	63 ± 6b
	3	6996 ± 708b	140.78 ± 0.60b	49 ± 5a
	4	6698 ± 395b	139.75 ± 1.36b	31 ± 1a
	5	6585 ± 713a	139.83 ± 1.32b	47 ± 5a
Red Kidney	1	5036 ± 510b	156.89 ± 1.11b	32 ± 3b
	2	6172 ± 149b	150.96 ± 0.85a	40 ± 1b
	3	4916 ± 981b	156.41 ± 1.85b	31 ± 6b
	4	5016 ± 1192b	155.21 ± 4.57ab	32 ± 8b
	5	2198 ± 798a	158.94 ± 1.73b	13 ± 5a
Adzuki	1	8352 ± 147bc	79.85 ± 0.29bc	104 ± 2b
	2	8503 ± 439c	76.91 ± 2.29ab	110 ± 4b
	3	7864 ± 238ab	73.62 ± 3.35a	107 ± 7b
	4	7638 ± 365a	82.43 ± 2.41c	92 ± 7a
	5	7508 ± 39a	73.11 ± 0.96a	102 ± 2b

*Mean ± SD of triplicate determinations
Different letters within the same column of each legume indicate significant difference ($p < 0.05$)

Effect of extraction time on protein and inhibitory patterns of extracted inhibitor

The protein pattern and inhibitory activity staining of extracted protein from three legume seeds extracted with different times (1-5 h) are depicted in Figure 2. The protein pattern of different extraction times showed the same pattern as mentioned before (3.2). Increasing the extraction time did not result in an increase in the major protein components of any of the legumes as observed in SDS-PAGE. These results are accord with the protein concentration as measured by using the test tube method. The major protein components in navy bean are 215, 132 and 34 kDa. Red kidney bean also gave the same pattern of 118, 59, and 32 kDa as the major protein bands. Only one major band with the molecular weight of 62 kDa was observed in adzuki bean. Based on reducing condition, the changes of protein pattern were observed as mentioned before in navy bean and red kidney bean, while they did not occur in adzuki bean.

The inhibitory activity staining also showed the molecular weight of 132 kDa, 118 kDa and 13 kDa is trypsin inhibitor in navy bean, red kidney bean and adzuki bean, respectively. The band presented on the substrate gel of SDS-PAGE confirmed that these protein bands are inhibitor bands that could not be hydrolyzed by trypsin in the assay condition. Under reducing condition, the inhibitory activity was not detected for navy bean and red kidney bean, while there was still some resistant band (13 kDa) on the bottom of the gel observed in adzuki bean. The major band of adzuki bean with molecular weight of 62 kDa was found to have no inhibitory activity based on reducing condition.

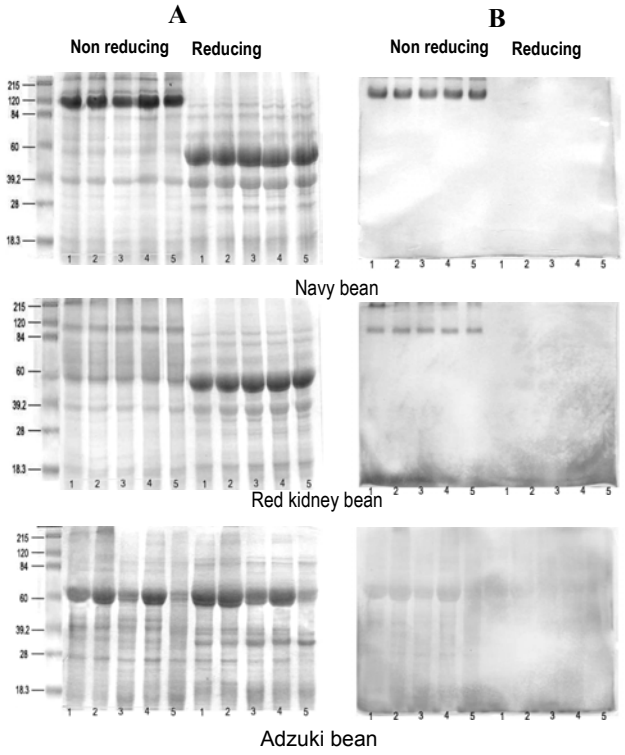

Figure 2. Effect of different extraction times on the protein pattern (A) and inhibitory activity staining (B) of three legume seeds. Eighteen µg of protein were applied. Numbers represent the extraction time (min).

Conclusion

Trypsin inhibitor from three legumes was successfully extracted using 0.02 M NaOH for navy bean, red kidney and water for adzuki bean. Two hours of extraction gave the trypsin inhibitor showing the highest specific inhibitory activity of all legumes with the different molecular weights.

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