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ISOLATION AND CHARACHTERIZATION OF CRUDE PROTEASE INHIBITORS FROM *RICINUS COMMUNIS* (Castor Beans)

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ABSTRACT:

Protease inhibitors are frequently found in different sources such as animals, plants and microorganisms and they inhibit the activity of proteases. In our studies protease inhibitors (PIs) were isolated and characterized from plants tissues such as seeds. *Ricinus communis* has shown highest inhibition activity as compared to other seeds (*Glycin max, Triticum astevium, Zey mays, Helianthus annus, Pennisetium typheides, Avena sativa, Brassica compestris* etc.) at optimized conditions. Crude protease inhibitor sample (extracted from castor beans) incubated for 60 minutes at 35 ºC, using sodium phosphate buffer pH =7.5, soluble casein as a substrate and trypsin enzyme were used for maximum inhibition activity. The crude protease inhibitors were characterized at different parameters such as effect of time period, substrate concentration, enzyme and sample concentration, variable temperature and pH control.

INTRODUCTION:

The protease inhibitor (PI) proteins are natural antagonists of protease, which are quite common in nature and also present in all living forms (Fritz, 2000). Most protease inhibitor interacts with their target proteases by contact with the active site, resulting in to the formation of a stable protease inhibitor complex that is incapable of enzymatic activity (Norton, 1991). Protease inhibitors have enormous diversity of function by regulating the protease activity of their target proteases (Leung et al., 2000). Protease inhibitors are divided into different groups according to homology of amino acids sequences, active centre structure, position of disulfides bond, and mechanism of Inhibition (Laskowki, 1980).

Serine protease inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the numbers of known and partially characterized inhibitors of serine proteinases are enormous (Haq et. al., 2004). Serine protease inhibitors have been reported from vast variety of plant sources and they are highly studied branch of protease inhibitors (Mello et. al., 2002; Haq and Khan, 2003). Chiche et.al., in 2004 first time introduced the squash inhibitor, a well-established family of highly potent canonical serine protease inhibitors isolated from *Cucurbitaceae*. Plant cystatins or phytocystatins are the second most studied class of inhibitors and have been identified and characterized from several plants such as cowpea, potato, cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, and Job's tears**.** Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants includes sunflower, rice, wheat, maize, soybean, and sugarcane (Kuroda et.al., 2001, Yoza et. al., 2002, Connors et. al, 2002). Aspartic proteinase inhibitors are

relatively less-studied class partly due to their rarity, and the metallo-proteinase inhibitors in plants has represented by the metallo-carboxypeptidase inhibitor family in tomato and potato plants (Hass et. al., 1975, Graham and Ryan, 1981).

Twelve families of inhibitors has been recognized on the basis of their amino acid sequences and target proteases (Shewry, 1999). However, the studies have been focused on crop plants (cereals, legumes, and *solanaceous* species) with their economically unimportant species, which were previously ignored (Konarev et. al., 2004). Soybean trypsin inhibitor was the first Protease inhibitor isolated and characterized. Since then many Protease inhibitors have been characterized from the *Gramineae (Poaceae), Leguminosae (Fabaceae), and Solanaceae* families (Brzin and Kidric, 1995). Currently plant-protease inhibitors contain information for 495 inhibitors and several isoinhibitors, which are identified in 129 different plants (De Leo et. al., 2002). Protease inhibitors are usually found in storage organs, such as seeds and tubers but their occurrence in the aerial part of plants as a consequence of several stimuli has also been widely documented (De Leo et. al., 2002). Protease inhibitors may accumulate to about 1 to 10% of the total proteins in these storage tissues. An increasing number of Protease inhibitors are found in non-storage tissues such as leaves, flowers and roots (Brzin and Kidric, 1995, Xu et. al., 2001, Sin and Chye, 2004). Some Protease inhibitors are also isolated from yeast (Matern et. al., 1979) and other fungi (Richardson, 1977). The wound-induced inhibitors accumulated in vacuoles of tomato, wild tomato and potato leaves. Xe et. al., (2004) described the expression of a Protease inhibitor II protein from *S.americanum* Mill. in phloem of stems, roots and leaves suggesting a novel endogenous role of Protease inhibitor II in phloem. Further investigation showed that both soybean protease inhibitor IIa and soybean protease inhibitor IIb are expressed in floral tissues (Sin and Chye, 2004).

The aim of present work is to isolate and characterize protease inhibitors from low cost available indigenous sources for academic and medicinal purpose.

MATERIAL AND METHODS

Plant material: Different types of seeds *Ricinus communis, Glycin max, Triticum astevium, Zey mays, Helianthus annus, Pennisetium typheides, Avena sativa, Brassica compestris* and *Moringga olifera* were purchased from the local market. The chemicals used in this study were purchased from E. Merck and Fluka chemicals.

Preparation of sample: The seeds were defatted with diethyl ether and were dried at room temperature. 10.0g of defatted seeds were crucified in pestle and mortar in 30ml cold de-ionized water. After centrifugation, the supernatant was transferred to 100ml volumetric flask. The extraction process was repeated twice and volume was make up to 100ml with distilled water.

Protease Inhibitor Activity: Protease inhibitor activity assay was carried out as reported by Walsh and Twitch et al., (1991) method with slight modification, 0.5ml of (0.1% Trypsin) enzyme solution was mixed with 0.5 ml sample (extracted from seeds) then added 1.0ml of Na-phosphate buffer (pH=7.5). The reaction mixture was thoroughly mixed and incubated at 35°C for 10 minutes. After 10 minutes the residual protease activity was assayed by adding 0.5ml of substrate (1% soluble casein) and the reaction mixture was incubated for another one hour. After one hour incubation, 2.5ml of 15% TCA (Tri-

chloro acetic acid) was added to stop the reaction and then kept for 5 min at room temperature for complete precipitation. The precipitates were removed by centrifugation at 4000 rpm for 5 minutes. In 1.0ml of supernatant 4.0ml, of 0.5N NaOH (sodium hydroxide) and 1ml of Folin phenol reagent (1:1 v/v) were mixed. The total volume was made up to 10.0ml by adding 4.0ml de-ionized water, the absorbance of blue color was read after 5min at 625nm by spectrophotometer.

One protease inhibitory unit is defined as the amount of inhibitor required inhibiting one unit of protease activity.

Protease activity: Protease activity in defatted seeds sample was determined by the method of Penner and Aston (1967). Half milliliter of sample (plant extract), 1.5ml of phosphate buffer (pH 7.6) and 0.5ml of casein solution (1% PH 7.6) were added and incubated at 35°C for one hour. 2.5ml of 15% trichloroacetic acid was added in the above reaction mixture after one hour. The precipitates were removed by centrifugation at 6000 rpm for 5 minutes.

In 1.0 ml supernatant, 4.0ml of 0.5 N sodium hydroxide and 1.0 ml of diluted Folin phenol reagent (1:1v/v) were added and the total volume was then made up to 10.0 ml by the addition of 4.0 ml de-ionized water. A blue color developed was determined after 5 minutes at 625nm.

One unit of protease activity was defined as the amount of enzyme that liberated 1.0mg of tyrosine under the standard assay condition.

Determination of protein: protein content of seeds extracted samples was determined by the method of Lowery et. al., (1951) with bovine serum albumin as a standard.

RESULT AND DISCUSSION

In this study, seeds of different plants were used to check protease inhibitory activity such as *Ricinus communis, Glycin max, Triticum astevium, Zey mays, Helianthus annus* which contained 95%, 90%, 50%, 45% and 35% inhibition activity respectively. It is clearly seen (Table-1) that Ricinus contained higher inhibition activity in comparison to other tested seeds. However, *Pennisetium typheides, Avena sativa, Brassica compestris* and *Moringa olifera* showed activation of protease activity. Therefore communis was selected for subsequent experiments for protease inhibition activity.

Table-1: Protease Inhibitor Activity of different plants seeds

Plant names	Botanical name	Prot. conc.	% of activity	% of inhibition
Enzyme control Without sample		Mg/ ml	100	---
Castor	*Ricinus communis*	0.023	5	95
Soybean	*Glycin max*	0.164	10	90
Wheat	*Triticum aestivum*	0.072	50	50
Maize	*Zey mays*	1.01	55	45
Sunflower	*Helianthus annus*	1.0	65	35
Sohanjno	*Moringa oleifera*	0.605	376.5	No inhibition
Millet	*Pennisetum typheides*	0.390	165	-ve
Barley	*Avena sativa*	0.105	125	**-ve**
Mustered	*Brassica compestris*	0.312	230	-ve

Effect of Incubation Period: The time period plays an important role for

inhibition, because some enzymatic reactions complete in short time while the other take longer time for completion. Results shown in Table-2 indicate that the highest inhibition was achieved in 60 minutes and then it decreases in *Ricinus communis* samples. The reaction time 10 minutes reported by Thomsen and Bayne (1990), and Erlanger, (1961) in case of protease inhibitor from legume and buckwheat seeds respectively.

Table-2: Effect of Incubation period on (*Ricinus communis*) protease inhibition activity

Time period (minutes)	% of activity	% of inhibition
Enzyme control	100	---
15	46	54
30	41	59
45	36	64
60	4.89	95
75	40	60

Effect of substrate concentration: Effect of substrate concentration on the protease inhibition activity was checked by using soluble casein as a substrate of different concentration ranging from 0.5-2.5%. It was concluded from the results given in Table-3. The optimum inhibition occurred at 1% substrate concentration and then protease inhibition activity was decreased. Inhibitor (I) binds reversibly with enzyme (E) at active site to form E-I-Complex. This dead end complex can not go on to form product, however, when substrate concentration is increased, the inhibition is overcome by pulling the free enzyme (E) via breakdown of the E-I-complex, which equilibrate with free enzyme and free inhibitor. Murachi, (1970) and Sumathi and Pattabiraman (1975) have used soluble casein as a substrate in their work, while Erlanger (1961), Thomsen and Bayne (1990) have used N-α-benzoyl-DL-arginine -p-nitroanilide as a substrate for maximum protease inhibition activity.

Table-3: Effect of substrate concentration on (casein) castor bean protease inhibitor activity

% of Substrate conc:	% of activity	% of inhibition
Enzyme control	100	---
0.5	86	13.4
1	5.07	95
1.5	66.5	37.5
2	75.6	24.4
2.5	89.95	10.08

Effect of enzyme concentration: The effect of enzyme concentration on the protease inhibition was determined in the range of 0.05 to 0.25%. This was observed from the Table-4 that the optimum inhibition occurs at 0.1% enzyme using trypsin enzyme of different concentration and then inhibition was decreased may be due to change of enzyme substrate ratio. These results were also found in accordance with reported method of Erlanger et. al., (1961) and Shibata, at. al., (1986), who used trypsin type II from procine pencrease, trypsin and substilisin like enzymes from fungi respectively for identification of protease inhibition activity.

Table-4: Effect of enzyme concentration (trypsin) on (Ricinus communis) protease inhibitor activity

Enzyme conc:	% of activity	% of inhibition
Enzyme control	100	---
0.05	48	52
0.1	5.20	95
0.15	69.7	30.2
0.2	83.85	16.15
0.25	93.75	6.25

Effect of sample concentration: The protease inhibition activity was checked by using different concentration of sample (extracted from *Ricinus communis*) ranging from 5-25%. It was observed from Table-5 that 10% sample concentration was optimum for the inhibition of protease activity and then inhibition was decreased due to substrate and sample concentration ratio. Tsybina (2001) and Veerappa, (2002) were used legume and buck wheat seeds respectively for maximum results.

Table-5: Effect of sample concentration (Ricinus communis) on protease inhibition activity

% Sample conc:	% of activity	% of inhibition
Enzyme control	100	---
5	75	25
10	4.32	95.68
15	60.86	39.17
20	75.37	24.63
25	94.17	5.83

Effect of Temperature: Effect of temperature on protease inhibition was checked by using different temperatures ranging from 15-45°C, as shown in Table-6. It was observed from the results that initial inhibition was increased by increasing the temperature but after optimum temperature 35°C, the inhibition was decreased sharply. The decrease in inhibition activity after optimum temperature may be suggested due to the denaturation of protein at high temperature. There is little difference in case of Thomsen and Bayne, (1990) and Erlanger, 1961), who observed maximum results at 37 ºC.

Table-6: Effect of temperature on (Ricinus communis) protease inhibitor activity

Temperature conc:	% of activity	% of inhibition
Enzyme control	100	---
15°C	73.5	26.5
20°C	65.71	34.29
25°C	51.4	48.58
30°C	43.8	56.20
35°C	9.05	91
40°C	85.7	14.3
45°C	98.57	1.4

Effect of pH: The inhibition activity of enzyme is determined on their pH values. The pH of the system in which reaction process operate, each inhibitor has its own optimum pH i.e. H^+ concentration at which inhibitor react at maximum level (stop/slow the enzyme reaction). In this study different pH values ranging from 7-11 of sodium phosphate buffer was used. It was observed from result (Table-7) that maximum protease inhibition obtained at pH 7.5 while in acidic and alkaline pH the protease inhibition activity is low. Similar results were also reported by different group of scientists, [Pattabiraman (1975), Thomsen and Bayne, (1990) and Erlanger, (1961)] using Tris-HCl and sodium phosphate buffer respectively.

Table-7: Effect of pH on (castor bean) protease inhibition activity

pH	% of activity	% of inhibition
Enzyme control without sample	100	---
7.0	97	33
7.5	7.5	92.5
8.0	1015	76.2
8.5	970	55.6
9.0	98	2
9.5	72.5	27.5
10.0	74	26
10.5	98	2
11.0	99	1

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