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Protease inhibitory activity from five common dried fruits by dot blot method using X-Ray film

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Abstract
Dried fruits are fruits from which majority of the original water content has been removed either naturally, through sun drying or through the use of specialized dryers or dehydrators. A dried fruit has a long tradition of use dating back to the fourth millennium BC and is prized because of its sweet taste, nutritive value and long shelf life. Also these Dried fruits were recognized for their physiological and medicinal properties but along with this benefits, dried fruits are also known to posses the inhibitory activities against digestive enzyme, trypsin. These may lead to many digestion related troubles such as indigestion, acid reflux and other digestive troubles.

Presence of protease inhibitors in five dried fruits was described for the first time. These proteins were detected in considerable amounts 43.47% and 39.77% in two dried fruits, namely pista (Pistacia vera) and walnut (Juglans regia) respectively, where as 26.32%, 23.36% and 17.39% in chironji (Buchanania lanzan), cashew (Anacardium occidentale) and almond (Prunus dulcis) respectively, the protein was detected at low concentrations.

Keywords: protease, inhibitor, dry fruits, almond, cashew, walnut. pista, charoli

Introduction

Protease inhibitors (PI) are one of the abundant and most studied proteins, and are present in almost all life forms. These inhibitors, either specifically or nonspecifically, inhibit a wide range of enzymes. They are found to inhibit nearly all of the four types (serine, sulphydryl, metallo and acidic) of proteolytic enzymes. Trypsin is an important serine protease of the digestive tract of animals and insects (Richardson M, 1977) [1]. Dried fruits are healthy addition to your diet, high in fiber which improves regularity and promote bowel health. A small portion size of dried fruit makes it easy to gain weight. It takes an excess of 3,500 calories in your diet to gain 1 pound (Erlanger B, 1984). Along with this benefits, dried fruits are also known to posses the inhibitory activities against digestive enzyme, trypsin. There have been no reports on identification of protease inhibitors in dried fruits.

The objective of the present study was to identify the inhibitor proteins against trypsin enzymes from five dried fruits, namely almond (Prunus dulcis) and cashew (Anacardium occidentale), walnut (Juglans regia), pista (Pistacia vera) and charoli (Buchanania lanzan).

Material and Methods

All the dried fruits used were procured from local market of Akola, Maharashtra and ground to fine powder and extensively defatted with hexane and acetone. The solvents were filtered off and powders were air dried.

Extraction of trypsin inhibitor

Inhibitor were extracted in six volumes of distilled water containing 1% polyvinylpyrollidone (PVP) and kept frozen until needed. The suspension was centrifuged at 10,000g for 15minutes at 4^0 C; the supernatant was used as an inhibitor source. Protein in the extracts was determined by Lowry's method (Lowry *et al.* 1951).

Dot–Blot Assay

X-ray films were purchased from AGFA, Selvas Photographics Limited, Silvassa, India. X-ray film contact print method developed by Pichare and Kachole (1994) was used to detect inhibitory activity against trypsin. Three varying concentration of enzyme and inhibitor viz., 3:1, 1:1, 1:3 v/v were prepared. The volume of reaction mixture was adjusted with Glycine-NaOH buffer for trypsin. The film with spots was incubated for 20 min depending upon extent of gelatin hydrolysis; the film was washed with cold or tap water. Hydrolysis of gelatin was visually monitored.

Trypsin inhibitory assay

Trypsin inhibitor activity against trypsin was assayed according to the method described by Kunitz with some modifications (Kunitz, 1947). The TCA soluble fraction formed in this method by action of trypsin on the protein substrate Hammerstein casein was measured by the change in absorbance at 280nm. In the presence of inhibitor, the residual caseinolytic activity of the trypsin, at 37°C, was used as a measure of inhibitory activity; appropriate blanks for enzyme, inhibitor, and substrate were also included in the assay along with the test.

Reagents:-

1. Trypsin (SRL, India) 0.5mg/ml (1000 units/mg) (Prepared in 0.l M phosphate buffer pH 7.0)
2. Harnmerstein Casein 1% in 0.l M phosphate buffer
3. Tri chloro acetic Acid 0.44 M in DW
4. Phosphate Buffer (pH-7.0) 0.1M

The assay method included the following steps

a) One ml aliquot of trypsin was pre incubated with 1 ml of suitable dilution of inhibitor at 37°C for 15 minutes.
b) To the above mixture 2ml of 1% Hammerstein casein was added and incubated at 37°C for 30 minutes.
c) The reaction was terminated by the addition of 2.5 ml of 0.44 M tri chloro acetic acid (TCA) solution.
d) The reaction mixture was transferred to centrifuge tube and the precipitated protein was removed by centrifugation, at 10,000 rpm for 15 minutes.
e) The absorbance of the clear supernatant was measured at 280nm in UV-Visible spectrophotometer (Shirnadzu, Japan) against appropriate blanks. The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of inhibitor were quantified.
f) Trypsin activity of one unit was defined as the amount of enzyme that liberated 1 ug of tyrosine per milliliter of the reaction mixture per minute under the assay conditions.
g) Inhibitor activity of one unit was defined as to decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by trypsin action at 280nm per minute at 37°C in the given assay volume.

In terms of percent inhibition the trypsin inhibitory activity was expressed and it was calculated as

Inhibitory activity (%) = Amount of tyrosine released without inhibitor

$$\frac{\text{- Amount of tyrosine released with inhibitor}}{\text{Amount of tyrosine released without inhibitor}} \text{ X } 100$$

Estimation of Protein

Estimation of protein content was determined according to the method of Lowry *et al.*, (1951) using Bovine Serum Albumin (BSA) or egg albumin as the standard and the concentration was expressed in milligram per milliliter (mg/ml).

Estimation

The sample was made up to 500 µl with distilled water and added with 2 ml freshly prepared working reagent (2% (w/v) sodium carbonate, 0.1N sodium hydroxide, 0.5% (w/v) cupric sulphate, 1% (w/v) sodium potassium tartarate/distilled water.), mixed thoroughly, and incubated for 10 minutes. Later, 250 µl of Folin Ciocalteau's phenol reagent was added, incubated for 30 minutes and the absorbance was measured at 750 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

Specific activity

The sample Specific activity was calculated by dividing the percent inhibitory activity with the protein content and was expressed as percent specific activity/mg protein.

$$\text{Specific activity} = \frac{\text{Inhibitory activity (\%)}}{\text{Protein (mg/ml)}}$$

Results

Spot test was carried out to determine the potency of inhibitors, to block the trypsin. Out of 5 samples screened, 2 samples showed promising results. These samples were capable of inhibiting the trypsin at the three concentrations used viz., 3:1, 1:1 and 1:3 v/v. Following samples were used for inhibitory studies listed in table 1 and the inhibitory profile of dried fruits using dot blot assay is represented in the table 2.

Table 1: Amount of protein, trypsin inhibitor activity and Specific activity of dried fruits.

S. No	Name	Amount of protein mg/ml	Amount of inhibitor %	%Specific activity/mg protein
1.	Almond (Prunus dulcis)	350	17.39	0.049
2.	Cashew (Anacardium occidentale)	300	23.36	0.077
3.	Walnut (Juglans regia)	167	39.77	0.238
4.	Pista (Pistacia vera)	283	43.47	0.153
5.	Chironji (Buchanania lanzan)	317	26.32	0.083

Table 2: Spot test for trypsin Inhibitor in dried fruits

S. No	Name	Conc. of inhibitor 1:3	Conc. of inhibitor 1:1	Conc. of inhibitor 3:1
1.	Almond (Prunus dulcis)	N	Y	Y
2.	Cashew (Anacardium occidentale)	Y	Y	Y
3.	Walnut (Juglans regia)	Y	Y	Y
4.	Pista (Pistacia vera)	Y	Y	Y
5.	Charoli (Buchanania lanzan)	Y	Y	Y

Where Y = Inhibition
N = No inhibition

Conclusion

Our results suggest that dried fruits are acting against trypsin which is important enzymes of digestive system. Though not all dried fruits show the inhibitory activities, against the enzyme trypsin. Dried fruits are healthy addition of diet, but if you don't control your portion, they may have adverse effects. It can be concluded that dried fruits should be used in small concentrations to avoid abdominal cramping, bloating, constipation or possible diarrhea. Avoid these negative effects by keeping your dried fruit intake to a small amount each day or split your portion into two separate servings.

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