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Research article

Studies on protease inhibitory peptides from the seeds of *Tamarindus indica*

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Key words: *Tamarindus indica* seeds, Proteins, Trypsin inhibitors, Germination, Fermentation, Cold storage, Variation.

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Abstract

Food processing techniques such as germination, fermentation and cold storage were employed to study the total soluble protein/peptide content and Protease inhibitory peptides from the seeds of *Tamarindus indica*. The seeds were germinated for seven days and seedlings were removed for every 24 hrs of interval. The soluble protein content of fourth day germinated seedlings was subjected to fermentation for different period of time using *Bacillus substilis* and fermented samples were removed for every 24 hrs of interval. The fermented soluble protein content was further subjected to cold storage (0 – 4^{o}C) for seven days. The germinated, fermented and cold stored samples were analyzed for both protein/peptide content and trypsin inhibitory activity. The total soluble proteins and trypsin inhibitory peptides were isolated from the germinated seeds and changes in both the contents were monitored. The protein content was gradually increased from first day of germination (7.8%) to fourth day and it was maximum (12%). It was followed by gradual decrease their onwards. It was observed that trypsin inhibitory activity was high in the initial period of germination (30.52 TIU/mg of protein) followed by gradual decrease throughout the germination period and inhibition was very less in the seventh day of germination (3.71 TIU/mg of protein). Fermented and cold stored fourth day sample showed high trypsin inhibitory activity, 23.09 TIU/mg and 20.71 TIU/mg respectively.Fermentation and protease treated cold storage process produced bioactive peptides showed significant proteolytic inhibition which can be used to treat several human pathologies.

Introduction

Protease inhibitors play significant role in many physiological processes such as blood coagulation system, compliment cascade, apoptosis, cell cycle and hormone processing pathways [1, 2]. They are also important in the treatment of much human pathology such as inflammation, hemorrhage and cancer [3, 4, 2]. Plant proteinacious protease inhibitors or plant derived protease inhibitors rich in seeds also play essential role in the regulation of endogenous proteinases and involved in defense mechanisms against insects, fungi and other pathogenic microorganisms [5-8].These are also involved in the regulation of proteolytic enzyme mediated biological process such as intracellular protein breakdown, transcription, cell cycle, cell invasion [9-12].Interest on protease inhibitors has increased because recent studies indicated that have also been employed as new drugs in highly active antiretroviral combination therapy (HAART), increasing life expectancy in HIV positive patients [13-15]. Peptides with biological activity can be produced from proteins by enzymatic hydrolysis with digestive enzymes, fermentation of proteins with proteolytic starter cultures of microorganisms and through the action of enzymes derived from proteolytic microorganisms.

Tamarindus indica is a leguminous tree belongs to family Fabaceae indigenous to tropical Africa, south India and other parts of the world. *T. Indica* is primarily used for its fruits, which are eaten fresh or processed, used as a seasoning or spice. It contains (40%) pod pulp which is rich in vitamin C and also reported some compounds such as tartaric, malic, and citric acids as well as sugars, has a sweet-sour flavour and is used in drinks, sweet meats, curries, and chutneys. It is an essential ingredient in Worcestershire sauce. The main acidulent used in food preparation in India is tartaric acid from Pulp with concentration (8 to 18%). Almost every part finds at least some use, either in textile, carpentry, nutritional or medical [16]. Since no work is carried out on the production of peptides with proteolytic inhibitory activity using food processing techniques, the present investigation is carried out and in this research work, production of peptides with proteolytic inhibitory activity employing germination, fermentation and cold storage is described.

Materials and Methods

Plant material

The seeds of *T. indica* were collected during the month of Jan-Feb, 2017 from Chikkaaluvara, Kodagu dist. Karnataka, India.

Chemicals
Trypsin, bovine serum albumin, casein, Acrylamide and bisacrylamide were obtained from Sigma chemical company, USA. All other chemicals used were of analytical grade.

Germination/ Crude protein extract
The seeds of *T. Indica* were soaked in distilled water for 24 hours and germinated for six days under standard conditions. The seedlings were removed for every 24 hrs of germination. The acetone powder (10%) of dry, soaked and germinated seeds of *T. Indica* were prepared according to the method of wetter (1977) [17]. Seeds/seedlings (10 g) were blended in a blender for 5 min using chilled acetone, then filtered using suction pump under vacuum and dried at 37°C. A 10% extracts of the dry, soaked and germinated seeds of *T. Indica* were prepared using sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 1.5 hrs at 4°C. The extract was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants were collected and used for qualitative and quantitative analysis of proteins and trypsin inhibitory activity. Total soluble Protein content of all the extracts were estimated according to the method of Lowry *et al* (1951) [18].

Fermentation
The crude protein extract of the fourth day geminated seedlings was fermented with *Bacillus subtilis* for seven days. The fermentation was carried out for every 24 hours with a constant stirring (200rpm) in an incubator shaker at 37°C. After fermentation, it was centrifuged at 8000rpm for 20 minutes. The supernatant was used for trypsin inhibitory assay.

Cold storage after fermentation
The crude protein extract of the fourth day geminated seedlings was subjected to cold storage treatment at 0^{o} to 4^{o}C with protease isolated from microbial source for different time of interval. Then these samples were removed for every 24 hours of incubation and centrifuged at 8000rpm for 20 minutes. The supernatant was used for trypsin inhibitory assay.

Trypsin and trypsin inhibitor assay
Trypsin and trypsin inhibitory activity was determined as described by Chandrashekharaiah (2013) [2]. The trypsin activity was determined using casein as the substrate [19]. Forty µg of trypsin was taken in 2.0 ml of sodium phosphate buffer, pH 7.6 containing 0.15 M NaCl. The reaction was initiated by the addition of 2.0 ml of 2% casein at 37°C. The reaction was stopped after 20 minutes by the addition of 6% trichloroacetic acid (6.0 ml) and after standing for 1 hr, the suspension was filtered through whatman no. 1 filter paper. Absorbance of the filtrate was measured at 280 nm using spectrophotometer. One trypsin unit is arbitrarily defined as an in-crease in absorbance by 0.01 at 280 nm under conditions of assay. The trypsin inhibitor activity was determined using casein as the substrate [19]. Enzyme solution (40µg of trypsin was pre-incubated with known aliquots of the inhibitor extract in a total volume of 2 ml at 37°C for 10 min in 0.01 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl. The residual enzyme activity was determined as described above. Trypsin inhibitory unit is defined as the number of trypsin units inhibited under the assay conditions.

SDS- PAGE Polyacrylamide Gel Electrophoresis
SDS-PAGE (15%) was done according to the method of Lammelli [20]. The electrophoresis was performed for 3-4 hr by applying 100mA current using tris – glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye. After the run, the gels were removed and stained for proteins using staining solution (0.02% Coomassie brilliant blue R-250) for 1 hour and de-stained in 10% acetic acid and methanol.

Results and Discussion

Germination is the early and important stage during the growth and development of the plants. Several complex biochemical and physiological changes occur during germination that brings metabolically inactive cells into active cells. Seeds contain storage proteins that provide energy and nitrogen required for germination and subsequent growth of plants. Biochemical and physiological changes occurred during the germination can be correlated with variation in the expression of several types of proteins and hydrolytic enzymes (Syed Ajmal Ali *et al.,* 2013) [21]. During germination of seeds, the high activities of hydrolytic enzymes such as proteolytic enzymes required for the hydrolysis of storage proteins to provide energy as well as amino acids required for the synthesis of new proteins. Germination and seedling stage of the plant is more prone to insect or pest attack results in crop loss in agriculture. Plants during germination produce several proteinacious protease and amylase inhibitors to protect themselves against insects and pests or these classes of inhibitors may be produced during proteolytic action on storage proteins find applications in the treatment of several human pathologies. During the germination of seeds of *T. Indica*, the protein content was gradually increased from first day, reached maximum on the fourth day of germination and decreased gradually thereafter. Similarly, proteolytic inhibition was gradually decreased throughout the germination. Similar results were obtained in the germination of seeds of Mucuna (Chandrashekharaiah, 2013) [2].

Fermentation is one of the promising food processing techniques used to produce bioactive peptides of physiological significance. Several microorganisms are used in the fermentation process and microbial fermentation is the cheapest technique compared to enzymatic hydrolysis for the production of bioactive peptides (Agyei and Danquah 2011) [22]. *T. Indica* seeds were fermented for seven days using B. Substlis and fermented samples were removed for every 24 hrs and analyzed for both protein/peptide content and proteolytic inhibition. The results indicated that proteolytic inhibition was gradually increased from first of day of fermentation and maximum on the fourth day fermentation. This increased proteolytic inhibition can be probably correlated with the activities of microbial proteases produced during the fermentation resulted in the production of bioactive peptides of physiological significance. Similar results were obtained in the fermentation of soy proteins (Meguma Kuba *et al.,* 2003) [23]. Similarly, soluble proteins isolated from the *T. Indica* seeds were cold stored with proteases for seven days and protease treated cold stored samples were analyzed for both protein/peptide content and proteolytic inhibition. The results indicated that maximum proteolytic inhibition was observed on the fifth day of protease treated cold stored protein sample.

Table 1. Protein and trypsin inhibitory profile of *Tamarindus indica* seeds during germination

Days	Protein (mg/gm of acetone powder)	Trypsin inhibitory activity (TIU/mg of protein)
1	78.625	30.52 ± 1.5
2	102	21.17 ± 3.0
3	116	15.80 ± 0.3
4	120	10.04 ± 0.6
5	93.5	8.90 ± 1.4
6	78.625	7.75 ± 1.2
7	104.125	3.71 ± 1.5

Table 2. Protein and trypsin inhibitory profile of *Tamarindus indica* seeds during fermentation

Days	Protein (mg)/mL	Trypsin inhibitory activity
1	14	210 ± 2.0
2	18	320 ± 1.2
3	22	480 ± 1.8
4	22	508 ± 1.2
5	18.5	360 ± 1.3
6	16.25	356 ± 1.3
7	23	326 ± 1.3

Table 3. Protein and trypsin inhibitory profile of fermented *Tamarindus indica* seeds during cold storage

Days	Protein (mg)/mL	Trypsin inhibitory activity
1	18.75	219 ± 0.4
2	23.5	346 ± 0.4
3	28.185	502 ± 0.4
4	27.125	562 ± 0.23
5	33.625	580 ± 0.4
6	18.625	356 ± 0.4
7	21.75	326 ± 0.4

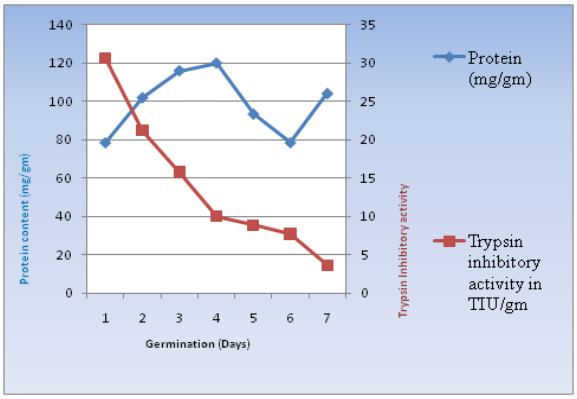

Figure 1. Variation of proteins and protease inhibitor activity during germination

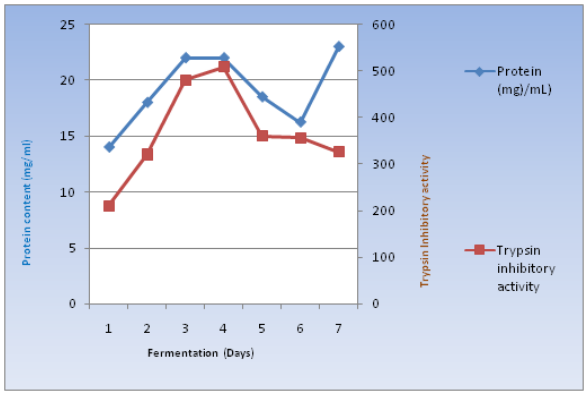

Figure 2. Variation of proteins and protease inhibitor activity during Fermentation

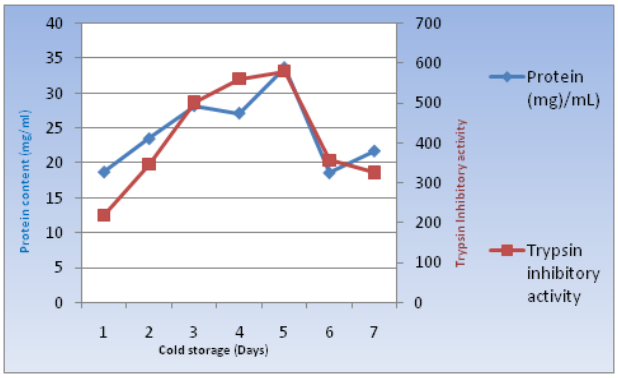

Figure 3. Variation of proteins and protease inhibitor activity during cold storage

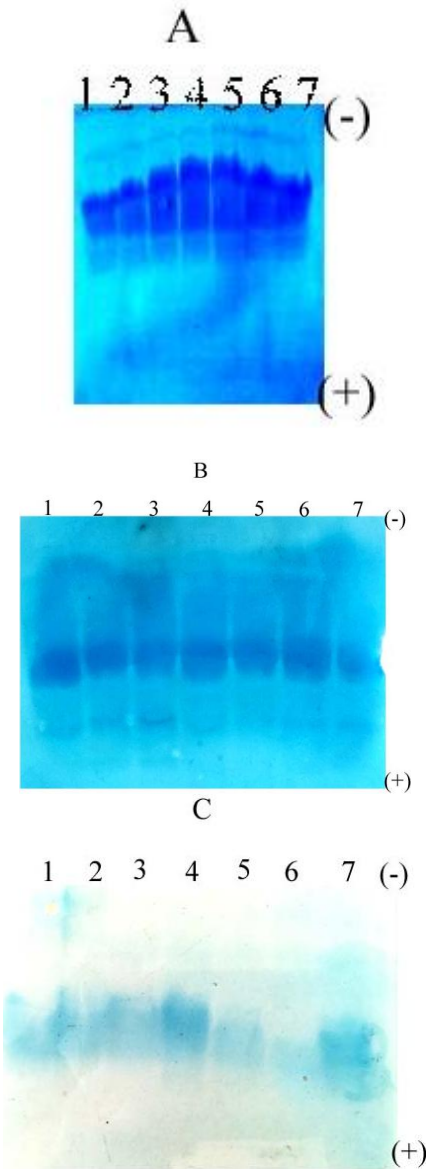

Figure 4. A, B &C: Peptide profile of germination, fermentation and cold storage of *T. Indica* seeds

Conclusion

The seeds of *T. Indica* seeds were subjected to food processing techniques such as germination, fermentation and protease treated cold storage. Fermentation and protease treated cold storage process produced bioactive peptides showed significant proteolytic inhibition which can be used to treat several human pathologies.

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