Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Studies on proteolytic inhibitory peptides from the seeds *Ricinus communis*

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Key words: *Ricinus communis* seeds, food processing methods, bioactive peptides, protease inhibitor peptides.

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Abstract

The search for dietary compounds that prevent many life style diseases is deemed crucial to tackle this major health problem worldwide, and recent developments in the field of health have suggested that increased protein consumption, particularly from plant sources, might reduce many life style diseases. Nowadays, interest among researchers has been emerging to identify, isolate and characterize bioactive peptides from plant and animal sources. Bioactive peptides are considered as specific protein fragments that are inactive within the sequence of the parent protein and they exhibit various physiological functions after released from their parent protein. Bioactive peptides were produced by germination, fermentation and cold storage from the seeds of *Ricinus communis*. The seeds were germinated for various days and germinated seedlings were subjected to fermentation for different period of time using *Bacillus substilis*. The total protein and protease inhibitor activity were monitored during germination. The fermented soluble protein content was further subjected to cold storage (0 – 4^{o}C) for seven days. The protein content was gradually increased from first day of germination (9.18%) and it was maximum on the third day (11.73%) followed by gradual decrease their onwards. It was observed that proteolytic inhibitory activity was less in the initial period of germination (23.32 TIU/mg of protein) followed by gradual increase up to fourth day (32.04 TIU/mg of protein) and decrease their onwards during the germination. Similarly, fourth day Fermented and fifth day cold stored sample showed high proteolytic inhibitory activity, 408 TIU/mg and 286 TIU/mg respectively.

Introduction

Plants and animals become resistant to pathogenic conditions and developed defense mechanisms against microorganisms such as viruses, bacteria and fungi. This happens only by the production of biochemical compounds, before or after the pathogen attack to the host at outer-side or inner-side, which complexes with proteases and inhibit their hydrolytic activity [1]. These biochemical compounds may be proteins or other metabolites which inhibit pathogen secreted proteins by different mechanisms. Some of these inhibit the proteolytic enzymes and hence called a pathogenesis-related (PR) protein which acts as inhibitors. Therefore also termed as protease inhibitors. This inhibition may be because of proteinatious or other metabolite compounds which inhibit pathogen secreted proteins by various mechanisms. Protease inhibitors play significant role in many physiological processes such as blood coagulation system, compliment cascade, apoptosis, cell cycle and hormone processing pathways [2,3]. They are also important in the treatment of many human pathologies such as inflammation, hemorrhage and cancer [3-5]. Most PIs forms protease-inhibitor complex by targeting proteases and binds to the active site of the protease resulting and is incapable of enzymatic activity [6].

The biochemical compounds derived from the seeds are rich in proteinacious protease inhibitors or plant derived protease inhibitors which plays an essential role in the regulation of endogenous proteinases and involved in defense mechanisms against insects, fungi and other pathogenic microorganisms [7-10,3]. These are also directly involved in the regulation biological processes such as intracellular protein breakdown, transcription, cell cycle, cell invasion mediated by proteolytic enzymes [11-14]. 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 [15-17]. Bioactive-peptides can be produced from proteins with biological activity by enzymatic hydrolysis with digestive enzymes, fermentation of proteins with proteolytic starter cultures of microorganisms, through the action of enzymes derived from proteolytic microorganisms and by use of cold storage methods.

Ricinus communis, the castor-bean or castor plant belongs to the spurge family, Euphorbiaceae, is a species of perennial flowering plant. Castor's origin is south-

eastern Mediterranean Basin, Eastern Africa and India, but also found in tropical regions and grown as an ornamental plant widely [18-20]. The *R. communis* plant parts have different medicinal properties. The stem and leave extracts shows antioxidant activity due to the presence of flavonoids and also some chemical constituents like Methyl ricinoleate, Ricinoleic acid, 12-Octadecadienoic acid and methyl ester produces antioxidant activity [21,22]. Themethanolic extract of leaves shows antinociceptive activity due to the presence preliminary Phytoconstituents like saponins, steroids and alkaloids [23]. The hexane and methanol extracts of roots showed maximum antimicrobial activity where the aqueous extracts has no significant antimicrobial properties [24]. The ethanolic extract of roots of *Ricinus communis* (RCRE) is potent phytomedicine for diabetes [25]. The methanolic extract of leaves and roots in *R. communis* shows anti-inflammatory activity was due to the presence of flavonoids because the flavonoids have the protective effect against carragennan-induced paw edema in rats [26-28]. The *R. communis* leaf extract have molluscicidal activity and the seed extracts showed better insecticidal and insectistatic activity against Lymnaea acuminate as compared to leaf extracts against S. Jefrugiperda due to the active ingredients like castor oil and ricinine [29-31]. The sex hormone being steroidal compound's (phytosterols) and the presence of steroids in methanolic extract of *Ricinus communis* seed produces anti-fertility effects [32,33]. 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.

Experimental

Materials and Methods

Plant material

The seeds of *R. communis* were collected during the month of Jan-Feb, 2017 from Hebale, Kodagu dist. Karnataka, India under the supervision of Botany Department, Mangalore University.

Chemicals

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

Gemination/ Crude protein extract

The seeds of *R. communis* were soaked in distilled water for 24 hours and germinated for seven 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 *R. communis* were prepared according to the method of wetter (1977) [34]. 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 *R. communis* 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) [35].

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 (200 rpm) 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 Chandrshekharaiah (2013) [2]. The trypsin activity was determined using casein as the substrate [36]. 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 [36]. 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, con-taining 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. The assay for each sample was done in triplicates.

SDS- PAGE Polyacrylamide Gel Electrophoresis

SDS-PAGE (15%) was done according to the method of Lammelli [37]. The electrophoresis was performed for 3-4 hr by applying 100 mA 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

In the life cycle of the plant, the most important stage for growth and development is the germination process. During this period, various changes occurs in various complex biochemical and physiological processes that results in the conversion of metabolically inactive cells into active form. The only source for the food in the seeds during germination period. Seeds contain storage proteins that provide energy and other macronutrients like nitrogen etc required for the germination and subsequent growth of the plants. During germination, variation in the expression of several types of proteins and hydrolytic enzymes can be correlated with the biochemical and physiological changes (Syed Ajmal Ali *et al.,* 2013) [38]. The germination period requires enzymes with high activities like hydrolytic enzymes to break storage proteins into simple forms for providing energy as well as amino acids required for transamination process as well as biosynthesis of new proteins. This stage is more prone to insects and pathogens attack results in the crop damage in agriculture. For this reason, the plants produces more proteinacious protease as well as amylase inhibitors to protect themselves and these classes of inhibitors can be produced during proteolytic action on storage proteins which finds application in the treatment of several human pathologies. During the germination of seeds of *R.communis*, the protein content was gradually increased from first day, reached maximum on the third day of germination and decreased gradually thereafter. Similarly, proteolytic inhibition was gradually increases from first day to the fourth day (maximum) and then continuously decreases 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 (Agyeiand Danquah 2011) [39]. *R.communis* 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 and then decreases continuously till end. 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 and after fourth day the bacteria produced little peptides may be due to the reason of less nutrition available and more resistant shown by seeds to this period. Similar results were obtained in the fermentation of soy proteins (MegumaKuba *et al.,* 2003) [40]. 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 and may be due to the cold environment for the bacteria and seeds releases peptides for its survival. Similar results were obtained in the cold storage of *R. communis* seed proteins (KS Chandrashekharaiah *et al.,* 2017) [41].

Table 1. Protein and trypsin inhibitory profile of *Ricinus communis* seeds during germination

Days	Protein (mg/gm of acetone powder)	Trypsin inhibitory activity (TIU/mg of protein)
1	153	23.32 ± 0.2
2	163.625	16.10 ± 0.61
3	195.5	28.00 ± 0.2
4	146.625	32.04 ± 0.21
5	170	31.90 ± 0.16
6	174.25	17.61 ± 0.42
7	163.625	11.30 ±0.42

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

Days	Protein (mg/mL)	Trypsin inhibitory activity
1	29.75	260 ±02
2	30.75	292 ±02
3	30.5	320 ±0.8
4	29.62	408 ±04
5	34.75	360 ±04
6	32.5	346 ±04
7	38	223 ±02

Table 3. Protein and trypsin inhibitory profile of fermented *Ricinus communis* seeds during cold storage

Days	Protein (mg/mL)	Trypsin inhibitory activity
1	19.5	136 ±02
2	19.9	164 ±02
3	20.5	168 ±01
4	18.75	226 ±0.6
5	22.25	286 ±02
6	18.37	236 ±03
7	23.45	226±02

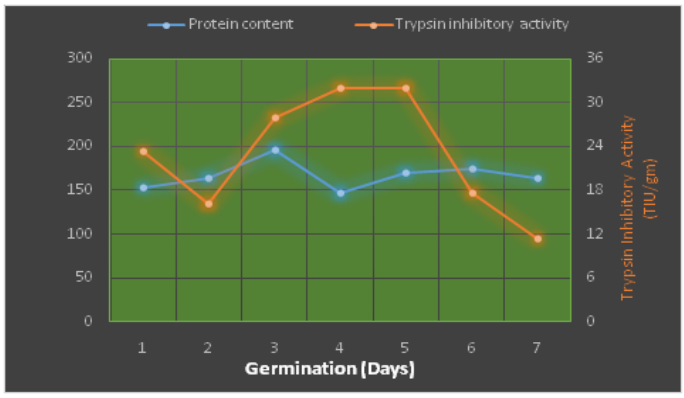

Figure 1. Variation of proteins and protease inhibitor activity during germination

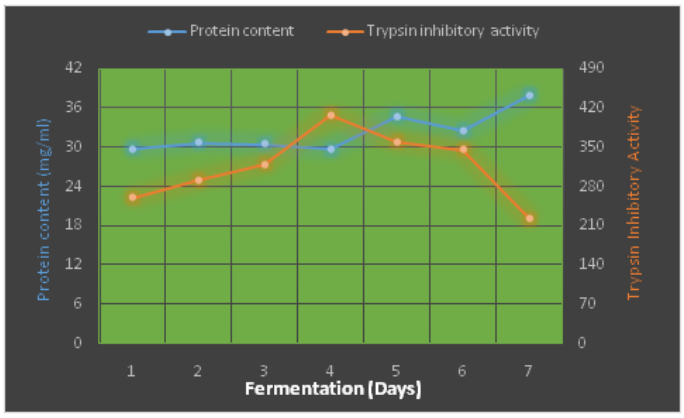

Figure 2. Variation of proteins and protease inhibitor activity during Fermentation

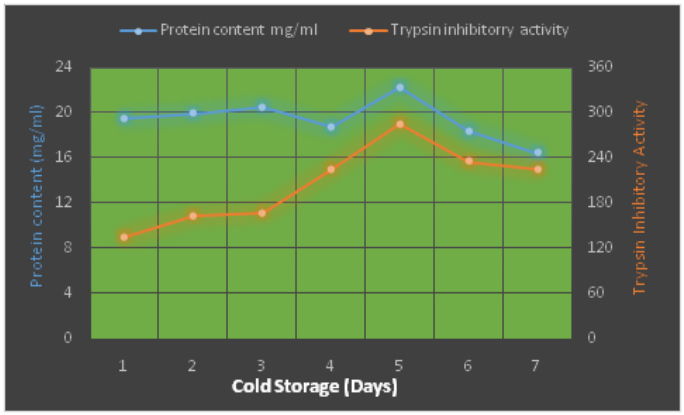

Figure 3. Variation of proteins and protease inhibitor activity during cold storage

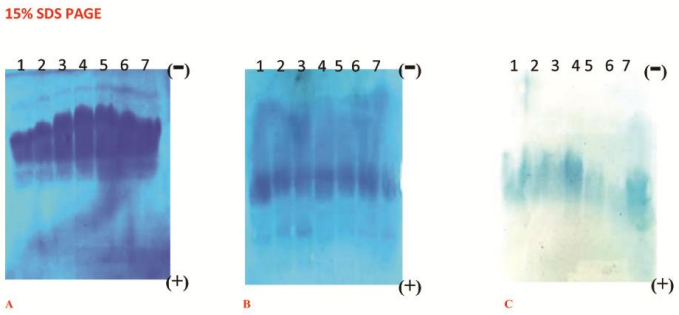

Figure 4. A, B, C: peptide profile of germination, fermentation and cold storage of *Ricinus communis*

Conclusion

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

Acknowledgement

We would like to thank Mangalore University, Mangalore for research support

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