Applied Biological Research 23(1): 13-20; (2021)
DOI: 10.5958/0974-4517.2021.00002.1

COMPARATIVE STUDY ON TOTAL SOLUBLE PROTEINS OF VARIOUS *Mucuna* SPECIES AND DISSECTING THEIR ROLE IN TRYPSIN INHIBITION

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(Received 14 August, 2020; accepted 28 October, 2020)

ABSTRACT

Plant seed proteins have pivotal health benefits. However, diminutive information is available on the presence of total soluble protein (TSP) levels in major crops. The present study was aimed to extract total soluble proteins from seven species/varieties of *Mucuna* and evaluate their activity for endogenous regulation of proteases. The total soluble proteins and protease inhibitory activity of various varieties/species of *Mucuna* were studied by both qualitative and quantitative methods. The total soluble proteins isolated were variable (18 to 29%) with maximum proteins in *Mucuna utilis* (29%) and minimum in *M. pruriens* V1 (18.7%). The total protease inhibitory activities were in the range of 9-18.25 TIU mg^{-1} with maximum in *M. pruriens* (18.25 TIU mg^{-1}) and minimum in *M. pruriens* V1 (9.78 TIU mg^{-1}). The TSP content and protease inhibitory activities in other species/varieties were: *M. pruriens* 27.4% and 18.25 TIU mg^{-1}, *M. utilis* 29% and 16.67 TIU mg^{-1}, *M. monosperma* 26.9% and 15.27 TIU mg^{-1}, *M. pruriens* V2 21.7% and 13 TIU mg^{-1}, *M. hirsute* 23.8% and 15.93 TIU mg^{-1} and *M. gigantea* 24.3% and 11.98 TIU mg^{-1}, respectively. Electrophoretic analysis revealed the presence of various trypsin isoinhibitors and multiple protein bands. The present analysis revealed that *Mucuna* seeds can be used as an alternative to protein source and their biological activity indicated their usefulness in medical field.

Keywords: *Mucuna* species, native PAGE, protease inhibitors, reverse zymography PAGE, seed storage proteins

INTRODUCTION

The proteins or peptides capable of inhibiting the catalytic activity of protease are classified as protease inhibitors. They are widely distributed in plants, animals and microorganisms (Mosolov and Valueva, 2005; Joanitti *et al*., 2006). Plants possess a variety of proteinase inhibitors (PI) for regulating the endogenous activity of proteinases as well as for their defence against herbivores. They are primarily stored in seeds and tubers, hence called as 'storage proteins', whose content varies from 6 to 10% (Ryan, 1990). Serine protease inhibitors (trypsin and chymotrypsin inhibitors) due to their abundance in plants, particularly in seeds of legumes, are the most studied one (Rustgi *et al*., 2008; Volpicella *et al*., 2011). Serine proteases play a vital role in physiological functions like blood coagulation, immune related activities, digestion and related domains as well as in biological processes; however, in case of over-expression or abundance of these enzymes they may cause severe complications (Sharony *et al*., 2010; Ohler *et al*., 2012; Esumi *et al*., 2015). Regulation of these enzymes either by systemic or external factors is much desired to understand control mechanisms (Di

Cera, 2009). Various beneficial protease inhibitions have biomedical applications like receptor signalling, carcinogenesis, growth factor-related activities, etc. (Habib and Fazili, 2007).

Plants including cereals and legumes are the rich source of bioactive compounds which contribute a wide range of significant physiological properties. The cereals and legumes in kingdom *Plantae* are classified under Angiosperms-Eudicots-Rosids, family Fabaceae (Group, 2009). The family Fabaceae has economically valued plants like legume, pea or bean with variable sized flowers. Among legumes, genus *Mucuna* has many species of annual and perennial climbing plants (vines and shrubs). *Mucuna* species are distributed world-wide, especially in Central America, South Africa, Nigeria and Asia (Duke, 2012). The Asian diets fulfil the protein requirements on daily basis by consuming *Mucuna* species. *Mucuna* plants are widely spread across the tropical forests of India especially in the Western Ghats. Many varieties of *Mucuna* are found in Karnataka, Kerala and Tamil Nadu which specialize as core tropical area suitable for the growth of this legume. *Mucuna* has about 100 species/varieties of which the prominent ones are *Mucuna pruriens* var. *hirsute*, *M. pruriens* var. *pruriens*, *M. pruriens* var. *sericophylla*, *M. pruriens* var. *utilis* and *M. pruriens* var. *thekkadiensis*, *M. gigantea*, and *M. monosperma*. These species/varieties differentiate themselves through characteristic pod pubescence, seed colour and pod harvest time. *Mucuna* is extensively used to control weeds and pests in agriculture and is considered a richest source of dietary proteins so is in great demand in food and pharmaceutical industries (Adebowale and Lawal, 2003). Some enzymes like carboxylesterase have been reported from the *M. pruriens* (Chandrashekharaiah *et al*., 2011). In addition, *Mucuna* is effective in the treatment of various neurological disorders, constipation, ulcers, elephantiasis, etc. (Lampariello *et al*., 2012). The seed extract of *M. pruriens* has potential antidepressant activity (Rana *et al*., 2014). *Mucuna* species are known to contain some active compounds like L-DOPA which is successfully used in the treatment of Parkinson's disease (Pulikkalpura *et al*., 2015). Recently, α-amylase inhibitor has been isolated and characterized from the seeds of *M. pruriens* (Bharadwaj *et al*., 2018). However, the plant is considered to be an under-utilized wild legume because of its non-availability i.e. it is only grown in the tropical and sub-tropical wild regions of the world.

The legumes express bioactive proteins which get accumulated in seed in the form of total soluble protein (TSP). The variations in total protein contents have been studied in many cereal crops (Drzewiecki *et al*., 2003). However, there is meagre information available on the variation of TSP levels of major legumes especially *Mucuna*. Therefore, it is imperative to understand the variation in TSP contents in *Mucuna* species/varieties. In present study we attempted to quantify TSP in *Mucuna* by using qualitative and quantitative methods such as native PAGE, Lowry assay, reverse zymography PAGE and protease inhibitory activity. The study was aimed to generate basic information about the diversity and quantity of total seed storage proteins and their protease inhibitory activity in different *Mucuna* species/varieties.

MATERIALS AND METHODS

Collection of seeds and chemicals

Seeds of seven varieties/species of *Mucuna* were collected from various parts of Karnataka and Kerala (*Mucuna pruriens* V1 from Somvarpete taluk, Karnataka; *M. hirsute* from Madikeri taluk, Karnataka; *M. pruriens*, *M. utilis*, and *M. pruriens* V2, from IIHR-Bangalore; *M. gigantae* and *M. monosperma* from TBGRI, Kerala). The procured seeds were stored at 4°C till use. All the chemicals used were of analytical grade. Acrylamide, N, N methylene bis-acrylamide, Tris, trypsin and BAPNA (N-benzoyl-DL-arginine p-nitroanilide hydrochloride) were purchased from Sigma, Missouri, USA.

Preparation of crude extract

The 10% protease inhibitor extracts of soaked dehulled seeds of *Mucuna* varieties/species were blended with 0.05 M sodium phosphate buffer, pH 7.0, followed by centrifugation at 10,000 rpm for 30 min (Chandrashekharaiah *et al.*, 2011). The supernatants were collected and used in the study.

Determination of total soluble protein

The total soluble proteins in all the extracts were determined by using bovine serum albumin (BSA) as standard (Lowry *et al*., 1951).

Determination of trypsin activity

The trypsin activity was determined by using casein as the substrate (Kakade *et al*., 1969). Trypsin (120 µg mL^{-1}) was dissolved in 0.001M HCl containing 20 mM $CaCl_2$. The enzyme assay solution consisting of trypsin (60 µg mL^{-1}) and 1% casein (1 mL) was incubated for 20 min at 37°C. The assay reaction was stopped after 20 min by adding 6% trichloroacetic acid (5 mL). The precipitate formed was filtered through Whatman No. 1 filter paper. The filtrate was analyzed for trypsin activity at optical density (OD) of 280 nm (UV-Visible spectrophotometer: SP 3000-Plus, Optima Tokyo Japan). One trypsin unit is defined as an increase in absorbance by 0.01 at 280 nm under normal assay conditions.

Determination of trypsin inhibitor activity

The trypsin inhibitory activity was determined by using casein as the substrate (Kakade *et al*., 1969). The inhibitory assay solution consisted of trypsin (60 µg) [pre-incubated with known aliquots of inhibitor extract] and incubated for 15 min, followed by the addition of 6% trichloroacetic acid (5 mL). Then, 1% casein (1 mL) was added to the mixture and incubated for 10 min. The precipitate formed was filtered through Whatman No. 1 filter paper. The filtrate was analyzed for trypsin activity at OD of 280 nm. Trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the normal assay conditions.

Electrophoretic analysis

A discontinuous cationic slab gel electrophoresis was carried out with 10% separating gel and 0.5% stacking gel for 3 h (Reisfeld *et al*., 1962). After electrophoretic run, the gel was removed and used for protein staining with 0.1% Coomassie brilliant blue R-250 and destained in 25% methanol and 10% acetic acid solution.

Reverse zymogram of protease inhibitors

Trypsin inhibitors were visualized on reverse zymogram PAGE co-polymerized with 1% gelatin (Felicioli *et al*., 1997). After 3 h of electrophoretic run, the gel was removed carefully and incubated with trypsin solution (100 µg mL^{-1}). The gel was washed with distilled water and stained with Commassie brilliant blue R-250. The presence of blue coloured bands against a transparent background after destaining with 10% acetic acid and 25% methanol indicated trypsin inhibitors.

Statistical analysis

All the experiments were conducted in triplicates. The data was analysed on graph pad prism 5.

RESULTS AND DISCUSSION

Total storage soluble proteins in Mucuna species/varieties

The cereals and legumes are important stable foods in different parts of the world and are mostly used because of their high content of proteins. The legumes are the best source of natural proteins (Bressani 1975; Eggum, and Beames, 1983). The natural proteins were isolated from the seeds of seven different species/varieties of *Mucuna*. The total storage soluble proteins (TSP) in *Mucuna* seeds ranged from 18 to 29% (Table 1). The TSP values varied significantly in different species/varieties. Among the different species/varieties analyzed, the protein content of *M. pruriens* (27.43 ± 2.14 mg mL^{-1}) and *M. utilis* (29.02 ± 3.76 mg mL^{-1}) were higher than other species/ varieties like *M. pruriens V1* (18.75 ± 0.65 mg mL^{-1}), *M. hirsute* (23.88 ± 2.66 mg mL^{-1}), *M. pruriens* V2 (21.70 ± 1.98 mg mL^{-1}), *M. gigantae* (24.34 ± 0.46 mg mL^{-1}), and *M. monosperma* (26.98 ± 1.32 mg mL^{-1}). The TSP content

in *Mucuna* species were higher than those of pigeon pea, common bean, lentil, green pea and chick pea, which was around 21.9% (Kumar *et al*., 1991; de Almeida Costa *et al*., 2006). The variation in TSP content in different *Mucuna* species may be attributed to their genetic makeup and environment. Since *Mucuna* species/varieties have a high content of proteins as compared to the other cereals and legumes; therefore, these legumes can replace *Mucuna* species/varieties. Due to the high protein content in *Mucuna*, there is the possibility of the presence of more sulfur-containing amino acid residues which help in the stabilization of proteins in different physicochemical conditions and also help the protein in slow digestion, which helps in treating various metabolic diseases. The quantity of proteins in *Mucuna* makes it favourable for use as protein supplement for bodybuilding.

Evaluation of trypsin inhibition form Mucuna species/varieties

Most of the biochemical and nutritional studies carried out with legumes have explored that some factors are involved in determining the quality of proteins (Bressani, and Elias, 1977). One such factor is the presence of anti-physiological substances of which trypsin inhibitor, amylase inhibitor and haemagglutinins are the most studied ones. In present study, the trypsin inhibitors were isolated from seven different species/varieties of *Mucuna* legume. The trypsin inhibitory potential of crude extracts of *Mucuna* species/varieties against standard trypsin were estimated (Table 1). The seeds of *Mucuna* species/varieties showed a considerable variation in trypsin inhibitor activity, ranging from 9.78 to 18.25 TIU mg^{-1} protein. The maximum inhibitory activity was in *M. pruriens* (18.25 TIU mg^{-1}) and minimum in *M. pruriens* V1 (9.78 TIU mg^{-1}). There was less change in the proportion of TIA (TIU mg protein) in different *Mucuna* species/varieties in relation to TIU mg^{-1} sample (Table 1). The content of trypsin inhibitory activity was much higher than that of Faba bean (Makkar *et al*., 1997) and *Caesalpinia mimosoides* (Lekha *et al*., 2019). Trypsin inhibitory proteins help in the endogenous proteolytic regulation of protease enzymes in seed as well as regulates the enzymatic action in insect gut, thereby hinders the metabolism of essential amino acids and makes the proteins nutritionally unavailable, ultimately resulting in inhibition in insect growth (Hilder *et al.*, 1993). The presence of trypsin inhibitor in legumes protects them against pathogenic attack during germination and hence acts as defense system by blocking the protease action in pathogens.

Trypsin inhibitors, in addition to their role as plant defense, have a greater role in clinical applications (QI *et al.*, 2005). Leguminous trypsin inhibitors have potent anti-carcinogenic and radio-protective properties that help in reducing the cancer of breast, colon, prostate and skin in those populations which consume them (Kennedy, 1998). This inhibition appears due to the regulation of proteolytic action caused by metastasis of cells, angiogenesis, tumour growth and proliferation (Hsieh *et al.*, 2010; Clemente *et al.*, 2010). Trypsin inhibitors also help in regulating the activity of some serine proteases such as neutrophil elastase. These proteases are generally involved in inflammations and their uncontrolled release cause severe tissue damage (Hiemstra *et al.*, 1998; Lendeckel *et al.*, 2003). Hence, on this basis, they can be a regulatory target for controlling gastric ulcers and pulmonary diseases (Ribeiro *et al.*, 2010; Oliveira *et al*., 2017). It has been reported that the trypsin inhibitor isolated from peanut showed a potent anti-glycemic effect in rats (Serquiz *et al*., 2016). Further, they reported that trypsin inhibitor supplemented to the healthy rats did not affect the glucose plasma, total cholesterol, triglycerides and liver transaminases in comparison to control (Komarnytsky *et al*., 2011; Nakajima *et al*., 2011; Chen *et al*., 2012). Another trypsin inhibitor reported in bitter gourd (*Momordica charantia*) showed anti-diabetic property. Some experiments on animals have shown that trypsin inhibitor help in controlling type I

Table 1: Total protein content and total trypsin inhibitor in different *Mucuna* species/varieties

Plant species	Total protein (mg mL^{-1})	TIA (TIU mL^{-1})
Mucuna pruriens	27.43 ± 2.14	18.25
Mucuna utilis	29.02 ± 3.76	16.67
Mucuna pruriens V2	21.70 ± 1.98	13.02
Mucuna pruriens V1	18.75 ± 0.65	9.78
Mucuna monosperma	26.98 ± 1.32	15.27
Mucuna gigantea	24.34 ± 0.46	11.98
Mucuna hirsute	23.88 ± 2.66	15.93

diabetes and reduces glucose level (Lo *et al*., 2014). Hence, it may be presumed that the trypsin inhibitor isolated in present study may help in regulating the satiety hormones and unwanted proteolytic action in other physiological process and thus may become an ideal candidate for the development of a natural therapeutic drug.

Native and reverse zymogram PAGE

Electrophoretic analysis of crude extracts of the seeds of *Mucuna* species/varieties on native PAGE revealed the presence of multiple protein bands (Fig. 1a). *M. pruriens* (6 major and 8 minor bands) and *M. utilis* (8 major and 8 minor bands) showed higher major bands than other species/varieties like *M. pruriens* V1 (4 major and 3 minor bands), *M. hirsute* (7 major and 9 minor bands), *M. pruriens* V2 (1 major and 1 minor bands), *M. gigantae* (1 major and 2 minor bands) and *M. monosperma* (9 major and 6 minor bands). Protein electrophoretic profiles of *Mucuna* species revealed almost identical patterns in both major and minor protein bands, which infers the stability of seed storage proteins within these species/ varieties. However, a considerable variation in protein patterns was observed in the seeds of *M. pruriens*, *M. pruriens* V1 and *M. pruriens* V2. This could be correlated to the different geographical regions of south India.

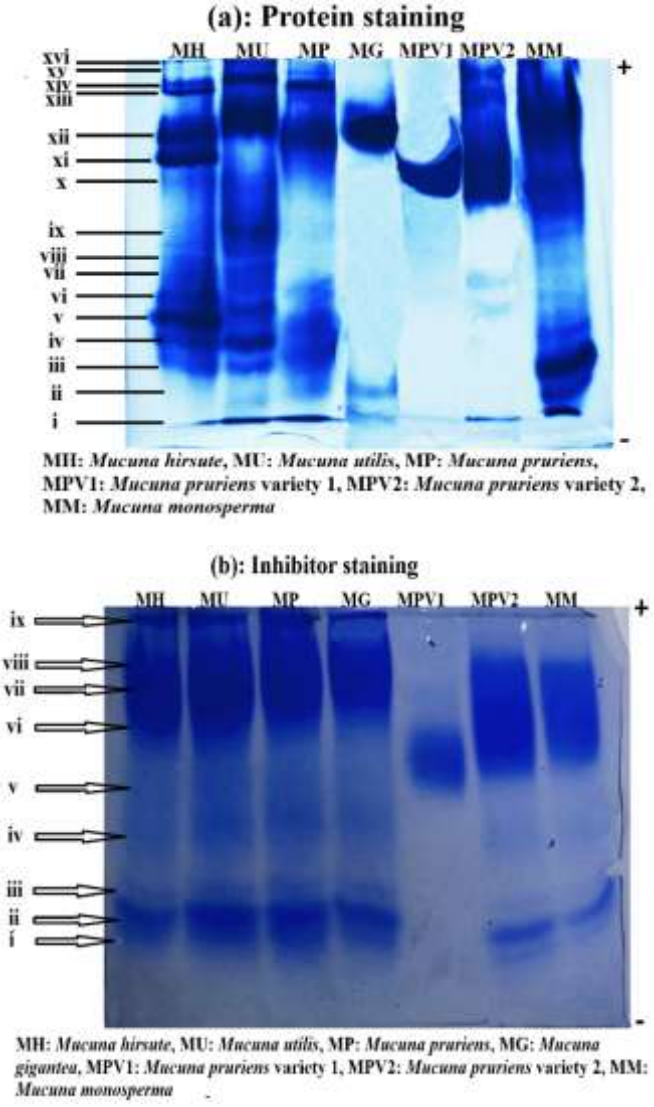

Fig. 1: Electrophoretic analysis of seeds extracts of *Mucuna* species a) native PAGE for proteins staining and b) reverse zymography PAGE for trypsin inhibitor staining

The electrophoretic analysis of trypsin inhibitors revealed the presence of 3-9 isoinhibitors in the seeds of *Mucuna* species (Fig. 1b). *M. hirsute, M. pruriens, M. gigantean, M. monosperma* and *M. utilis* had a maximum of 9 isoinhibitors of trypsin while *M. pruriens* V2 and *M. pruriens* V1 had 5 and 3 trypsin isoinhibitors, respectively.

Conclusion: The study revealed that all the evaluated *Mucuna* species/varieties were rich in soluble proteins and their protein profile depicted that these seeds can be used as a good food source. These plant seeds are widely used by the tribals of Kerala, Karnataka and Tamil Nadu in India. The presence of various trypsin isoinhibitors in *Mucuna* species/varieties were confirmed by reverse zymography PAGE. The *Mucuna* species/varieties with high TSP levels might be considered as potential source of therapeutic but inexpensive proteins in proteomics and biomedicine.

Acknowledgement: The authors are thankful to the Department of Biochemistry, Jnana Kaveri Campus, Mangalore University for research support.

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