Storage Proteins and Trypsin Inhibitors of an Underutilized Legume, *Mucuna*: Variability and Their Stability during Germination

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ABSTRACT

The proteins and trypsins inhibitors were isolated from the seeds of different varieties/accessions of an underutilized legume, *Mucuna*. The crude protein content of all the germplasms of *Mucuna* is varied from 15% - 26%, showed little variation and contain higher crude protein when compared with other *Mucuna* species reported earlier and the pulse crops commonly consumed in India. The seeds of all the varieties of *Mucuna* exhibited trypsins inhibitor activity. The trypsins inhibitor activity varied from 11 - 14 TIA/mg of protein. Not much variation was observed in trypsin inhibitory activities in soaked seeds compared to dry seeds. Germination of *Mucuna pruriens* has been carried out and the change in the protein content and trypsins inhibitors were monitored. The protein content of the endosperm increased up to 72 hrs of germination and then decreased. The trypsins inhibitor activity decreased with increase in germination time. The trypsin inhibitor activity was decreased from 14.81 TIA/mg to 2.62 TIA/mg (82% reduction in the trypsins inhibitor activity) after 144 hrs germination.

Keywords: *Mucuna* Seeds; Proteins; Trypsin Inhibitors; Germination; Variation

1. Introduction

The seeds of the plants belonging to the family Leguminosae generally rich in proteins required for human consumption in developing countries. Although legumes are rich in proteins, they have limited utilization because of the presence of various anti-nutritional factors including enzyme inhibitors such as trypsin and chymotrypsin inhibitor. Trypsin inhibitors when ingested in large quantities may disrupts the digestive process and leads to undesirable physiological reactions. The suggested functions of protease inhibitors are including acting as storage proteins, regulation of endogenous proteinases, or acting as protective agents against insects or microbial predators. Germination is an inexpensive and effective technology for improving the quality of legumes by reducing the content of anti-nutritional factors such as protease inhibitors [1] and decrease in storage proteins and enzyme inhibitors of seed is commonly observed during germination. In part, this decrease may be explained by the physical loss of active inhibitor from the seed into the medium [2]. It is generally assumed that the bulk of this decrease is due to proteolysis of the inhibitor by seed proteinases. A slight decrease in trypsin inhibitor activity during germination was reported from navy beans and soybeans [3,4]. Similarly, a decrease in trypsin inhibitor activity of about 16% in case of horse gram and 40% in case of moth bean after 72 hrs of germination was observed [5].

*Mucuna* is one of the lesser known under utilized legume, grown as a minor food crop by tribal and ethnic groups of Asia and Africa. The immature pods and leaves serve as vegetables, while seeds as condiment and main dish by ethnic groups in Nigeria [6]. The genus *Mucuna* belongs to the family fabaceae (leguminosae) includes up to 150 species and is annual or perennial legumes of pan tropical distribution. More than 15 varieties/accessions are available in southern India. *Mucuna* seeds are relatively good source of protein and have a relatively favorable amino acid composition. They contain high amounts of certain minerals, including Ca (calcium), Mg (magnesium) and Fe (iron) [7,8].
seeds are a promising source of protein and to meet the protein demands in developing countries like India, there is a need to source proteins from under utilized legume, *Mucuna*. However, Protein digestibility is affected by the presence of protease inhibitors which inhibits the proteolytic enzymes. A high level of trypsin inhibitors in the diet stimulates pancreatic juice secretion and causes pancreatic hypertrophy and poor growth performance in animals [9]. Soaking and germination of the seeds slightly reduces the levels of trypsin inhibitory activity (TIA). Germination has been suggested as an inexpensive and effective technology for improving the quality of legumes by enhancing their digestibility [10], increasing the level of amino acids [11] and reducing the content of antinutritional factors [12].

In the present study, the variability of proteins and trypsin inhibitors of eight varieties of *Mucuna* seeds and their stability during germination have been studied.

2. Materials and Methods

The seeds of different varieties/species of *Mucuna* (*Mucuna hirsute*, *Mucuna cochinensis*, *Mucuna cochinensis* MP9, *Mucuna pruriens* MP7, *Mucuna species NRC*, *Mucuna* species IIHR MP5, *Mucuna* species IC2199 and *Mucuna utilis* IC25333) were collected from different parts of Karnataka, Tamilnadu, Kerala and IIHR, Karnataka, India. Bovine pancreatic trypsin, a-chymotrypsin, Casein, N-acetyl-DL-phenylalanine-β-naphthyl ester (APNE), acrylamide, N,N methylene bis acrylamide were obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

3. Methods

3.1. Preparation of Acetone Powder

The seeds of eight varieties of *Mucuna* were soaked in distilled water for 12 hours and germinated for six days under standard conditions. The acetone powder (10%) of dry, soaked and germinated seeds of eight varieties of *Mucuna* were prepared according to the method of [13]. Seeds (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 8 varieties of *Mucuna* were prepared using 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.

Protein was estimated according to the method of Lowry *et al.* (1951) [14]. 0.2 to 1.0 ml aliquots of standard bovine serum albumin (200 µg/mL) was pipette into a series of test tubes and volume made up to 1.0 mL in each case. 5 mL of alkaline copper reagent was added to all the test tube. The test tubes were allowed to stand at room temperature for 10 min followed by the addition of 0.6 mL of FC reagent. The absorbance was read at 660 nm after 30 min against reagent blank.

3.2. Determination Trypsin and Trypsin Inhibitory Activity

The trypsin activity was determined using casein as the substrate [15]. 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 increase in absorbance by 0.01 at 280 nm under conditions of assay. The trypsin inhibitor activity was determined using casein as the substrate [16]. Enzyme solution (40 µg of trypsin was preincubated 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.

3.3. Polyacrylamide Gel Electrophoresis

An anionic disc gel electrophoresis was carried out essentially according to the method of Davis and Ornstein [17]. A discontinuous gel system consisting of 8% separating gel and 4% spacer gel was used. The electrophoresis was carried out in cold applying a current of 20 - 25 mA for 4 hours using tris-glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye. After the electrophoresis, the proteins were stained with coomassie brilliant blue R-250 for 1 hour and destained using 7% acetic acid. Visualization of trypsin inhibitor in polyacrylamide gel was performed according to Filho and Moriera [18]. The gel was stained for trypsin inhibitory activity separately by incubating the gel in 100 µg trypsin/ml in 0.1 M phosphate buffer, pH 7.6 for 20 min at 37°C and then stained using 0.1 M phosphate buffer pH 7.6 containing 0.8 mM N-acetyl-DL-phenylalanine-β-naphthyl ester (APNE) and 0.5 mg Diazo Blue B/ml for 1 hr. The gels were stored in 7% acetic acid.

4. Result and Discussion

The protein content of eight varieties of *Mucuna* samples
ranged between 15% - 26% (Table 1). Among the different varieties tested, the protein content of *Mucuna pruriens* and *Mucuna utilis IC25333* was higher than that of commonly consumed legumes, such as chick pea (*Cicer arietinum*), green pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*) and lentil (*Lens culinaris*) which ranged from 18.5% to 21.9% [19,20]. The protein content of the *Mucuna* seeds (*Mucuna pruriens* and *Mucuna utilis IC25333*) compared well with that of cowpea (*Vigna unguiculata*) at 29.3% and mung bean (*Phaseolus aureus*) at 26.5% [21,22]. Pugalenthi *et al.* [23] reported the protein content of *Mucuna* bean grown in different locations of the tropics and subtropics between 21.0% - 30.3%. The variation may be attributed to interaction between genetic make up and the environment.

Seeds of different varieties of *Mucuna* contain trypsin inhibitor activity (Table 1) and range from 11.24 - 14.81 TIU/mg of protein, which is higher than that of 9.32 TIU/mg reported by Mugendi *et al.* [24]. Trypsin inhibitory activity observed in *Mucuna* seeds is lower than that of soybean (29.1 - 30.2 mg/g) [25] and far higher than levels of 1.7 - 3.6 mg/g reported for faba bean [26].

Electrophoretic analysis of trypsin inhibitors revealed the presence of five to seven iso inhibitors in the seeds of different varieties of *Mucuna* (Figures 1(a) and (b)). The *Mucuna hirsute* and *Mucuna pruriens* contained a maximum of seven trypsin iso inhibitors. Analysis of proteins during germination of eight varieties of *Mucuna* seeds are shown in Table 2. Analysis of protein profile of raw and germinated seeds of different varieties of *Mucuna* revealed that there is no significant change in the protein content up to 48 hrs of germination. After 72 hrs of germination, among eight varieties, *M. utilis*, *M. hirsute* and *M. pruriens* showed increase in the protein content by 8.7%, 9.4% and 12.2% respectively, whereas *M. cochinensis*, *M. sps. NRC* and *M. cochinensis MP9* showed increase in the protein content by 19.8%, 23% and 24.5% respectively. A remarkable increase in the protein content was observed in *M. pruriens MP7* (34%) and *M. sps. IIHR MP5* (39%). During 92 hrs of germination, *M. hirsute*, *M. utilis IC25333*, *M. pruriens MP7*, *M. pruriens MP9*, *M. sps. NRC* and *M. pruriens* showed decrease in the protein content, however, increase in the protein content was observed in *M. cochinensis* (21.4%) and *M. sps. IIHR MP5* (45%). The seeds of all the varieties/species of *Mucuna* showed decrease and increase in

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**Table 1. Protein and protease inhibitor profile of seeds of different varieties of Mucuna.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Samples</th>
<th>Total protein (mg/gm)</th>
<th>TIA (TIU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mucuna hirsute</td>
<td>206</td>
<td>12.46</td>
</tr>
<tr>
<td>2</td>
<td>Mucuna cochinensis</td>
<td>188</td>
<td>13.24</td>
</tr>
<tr>
<td>3</td>
<td>Mucuna utilis IC25333</td>
<td>261</td>
<td>12.91</td>
</tr>
<tr>
<td>4</td>
<td>Mucuna sps. IIHR MP5</td>
<td>162</td>
<td>11.46</td>
</tr>
<tr>
<td>5</td>
<td>Mucuna pruriens MP7</td>
<td>211</td>
<td>14.31</td>
</tr>
<tr>
<td>6</td>
<td>Mucuna cochinensis MP9</td>
<td>161</td>
<td>12.01</td>
</tr>
<tr>
<td>7</td>
<td>Mucuna sps. NRC</td>
<td>154</td>
<td>12.32</td>
</tr>
<tr>
<td>8</td>
<td>Mucuna pruriens</td>
<td>251</td>
<td>14.81</td>
</tr>
</tbody>
</table>

TIA: trypsin inhibitor activity; TIU: trypsin inhibitor unit.
Table 2. Protein profile of raw and germinated seeds of different varieties/species of Mucuna.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Dry seeds</th>
<th>Soaked seeds</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
<th>96 hrs</th>
<th>120 hrs</th>
<th>144 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucuna hirsute</td>
<td>203</td>
<td>206</td>
<td>206</td>
<td>213</td>
<td>222</td>
<td>219</td>
<td>158</td>
<td>179</td>
</tr>
<tr>
<td>Mucuna cochinensis</td>
<td>182</td>
<td>188</td>
<td>191</td>
<td>208</td>
<td>218</td>
<td>221</td>
<td>114</td>
<td>142</td>
</tr>
<tr>
<td>Mucuna utilis IC25333</td>
<td>254</td>
<td>261</td>
<td>253</td>
<td>272</td>
<td>276</td>
<td>181</td>
<td>108</td>
<td>121</td>
</tr>
<tr>
<td>Mucuna sps. IIHR MP5</td>
<td>151</td>
<td>162</td>
<td>162</td>
<td>178</td>
<td>210</td>
<td>219</td>
<td>144</td>
<td>179</td>
</tr>
<tr>
<td>Mucuna pruriens MP7</td>
<td>206</td>
<td>211</td>
<td>223</td>
<td>240</td>
<td>276</td>
<td>219</td>
<td>132</td>
<td>156</td>
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<tr>
<td>Mucuna cochinensis MP9</td>
<td>159</td>
<td>161</td>
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<td>179</td>
<td>198</td>
<td>119</td>
<td>111</td>
<td>123</td>
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<tr>
<td>Mucuna sps. NRC</td>
<td>143</td>
<td>154</td>
<td>153</td>
<td>170</td>
<td>176</td>
<td>149</td>
<td>114</td>
<td>164</td>
</tr>
<tr>
<td>Mucuna pruriens</td>
<td>246</td>
<td>251</td>
<td>253</td>
<td>260</td>
<td>276</td>
<td>219</td>
<td>124</td>
<td>161</td>
</tr>
</tbody>
</table>

The protein content after 120 and 144 hrs of germination. The observed changes in soluble protein content indicated that these were intimately involved in the process of germination.

The electrophoretic protein band pattern obtained for different varieties of Mucuna are shown in Figure 2(a). The proteins were numbered from anodic end. The protein banding pattern revealed the presence of total 11 bands on anionic polyacrylamide gel electrophoresis. Mucuna cochinensis and Mucuna cochinensis MP9 were identical in both number of bands (total of 09 bands) and banding pattern. Mucuna species NRC and Mucuna species IC2199 were identical in both number of bands (total of 10 bands) and banding pattern. The intensity of bands 4, 5, 7, 8 and 9 of Mucuna species IC2199 was more than Mucuna species NRC. Mucuna species IIHR MP5, Mucuna utilis IC25333 and Mucuna pruriens were identical in both number of bands (total of 11 bands) and banding pattern. However, a change in the intensity of band 7, 8 and 9 were observed among these samples. Mucuna hirsute showed similarity with Mucuna species IIHR MP5, Mucuna utilis IC25333 and Mucuna pruriens in the total number of bands present but differed in intensity. The major proteins corresponded to intense band numbers 2, 7, 8 and 9 while the minor proteins were medium intensity bands 1, 3, 6 and 11 followed by very light intensity bands corresponding to band number 4, 5 and 10.

The electrophoretic protein band pattern during germination of Mucuna pruriens is shown in Figure 2(b). Electrophoretic analysis of proteins revealed the presence of total 11 bands in the soaked seeds of Mucuna. After 24 hrs of germination, there are total 12 protein bands and intensity of the band 9 was increasing and was maximum at 48 hrs of germination. The intensity gradually decreased there onwards till 120 hrs of germination. The intensity of the band 9 increased again on 144 hrs of germination. Similar results were observed for band 7. The protein band 8 disappeared during 72, 96 and 120 hrs of germination.

Figure 2. (a) Electrophoretic pattern of proteins isolated from 1) Mucuna hirsute; 2) Mucuna cochinensis; 3) Mucuna utilis IC25333; 4) Mucuna sps. IIHR MP5; 5) Mucuna pruriens MP7; 6) Mucuna cochinensis MP9; 7) Mucuna sps. NRC; 8) Mucuna pruriens; (b) Electrophoretic pattern of Proteins during germination of seeds of Mucuna pruriens: D: dry, S: soaked, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs and 144 hrs of germination.
hrs of germination and reappeared on 144 hrs of germination. The intensity of the minor bands increased on germination. In addition to band 6 new bands appeared after 72 hrs of germination and disappeared thereafter. The intensity of band 10 and 11 gradually decreased up to 48 hrs of germination and disappeared up to 120 hrs of germination. These bands were again expressed at 144 hrs of germination. The intensity of the bands 4 and 5 increased gradually throughout the germination. Electrophoretic protein profiles of different accessions of the same subspecies showed identical or similar patterns, confirming the stability of seed storage proteins within these subspecies. However, considerable variation of protein patterns was observed among the seeds of different varieties of Mucuna pruriens, Mucuna cochinensis and Mucuna hirsute. This could be correlated to different geographical origins.

The seeds of M. pruriens and M. utilis IC2533 showed higher amount of proteins, trypsin inhibitory activity. M. pruriens is one of the major legume seed commonly available and it was chosen for further studies to analyze the effect of germination on proteins and trypsin inhibitory activity. The proteins and trypsin inhibitor profile of dry, soaked and germinated seeds of M. pruriens is shown in Table 3. There is no significant decrease in TIA level was noticed up to 48 hrs of germination and gradual reduction followed by significant reduction with the increase of germination time. After 144 hrs of germination, TIA was significantly reduced (TIA was decreased by 82%) compared to the levels in the dry seeds of Mucuna pruriens. The results obtained in this study agree with those reported for kidney bean [27], lima bean [28], Mung bean [29], lentil [30-32], pigeon bean [33], faba bean and kidney bean [34]. Electrophoretic analysis of trypsin inhibitors during germination of Mucuna pruriens are shown in Figures 1(c) and (d). It is observed the gradual decrease in the intensity of the trypsin isoinhibitor bands up to 4th day of germination and steep decrease in the intensity of the bands thereafter.

Soluble proteins are the physiologically active fractions which constitute the major bulk of enzymes involved in plant metabolism. During germination and plant development, specific metabolic changes have been observed by many investigators. The analysis of zymogram of protein banding pattern revealed the variation in the protein pattern. This result suggests the requirement of these for the development of the plant. The protein content also increased at 72 hrs of germination and decreased thereafter. Analyses of protein banding pattern revealed the wide variation in banding pattern. The intensity of some of the major protein bands gradually decreased and few bands were disappeared followed by the appearance of small molecular weight new protein bands during the germination. The electrophoretic studies showed that high molecular weight polypeptide bands disappeared with the appearance of new low molecular weight polypeptide bands in the endosperm proteins of the germinating seeds.

5. Conclusion

The protein profile of Mucuna germplasms suggests that mature Mucuna bean seeds can be used as food source and that the bean merits wider use by tribals of Karnataka and other parts of India. The high protein content in the seeds of Mucuna indicated that seeds are good source of proteins, if the seeds are properly processed. The presence of trypsin inhibitors as anti-nutritional factors identified in the current study should pose a problem in human consumption if the beans are not properly processed. Germination is one such seed processing method and this method can be used to process Mucuna beans before consumption. However, the presence pharmacologically active compound L-DOPA (L-3,4-dihydroxy phenylalanine) is potentially toxic if large amounts are ingested.

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