ISOENZYME ANALYSIS OF VIGNA RADIATA LEAVES BY USING DIFFERENT MANURES

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ABSTRACT
Molecular study of plants give the better idea for the cultivation of plants which having importance commercially. Legumes play a critical role in natural ecosystems, agriculture, and agro forestry, where their ability to fix Nitrogen in symbiosis makes them excellent colonizers of low-Nitrogen environments, and economic and environmentally friendly crop, pasture, and tree species. The protein content of green gram is very high and commercially it having more in demand. By the help of better manure if yielding will be high then the economical condition will be increased. Manures are the substances which provide proper nutrients for proper growth of plants. Manure is anything that has been added to the soil to increase its fertility for plant growth. Now a days green manuring is gaining popularity as a method that successfully improved soil productivity. Green manuring with legumes, adds organic residues conserves, recycles plant nutrients and protects the soil from erosion. Isoenzyme is a multiple forms of enzymes arising from genetically determined differences only in the primary structure. In this study the researcher have done the comparison of protein profiling and isoenzyme analysis in green gram leaves by three different organic manures. The study was made with the objectives of observing the growth of plants by using different manures such as seaweed manure, coirwaste and peatmass. Further to observe any molecular differences for betterment of crop production

Key Words: Isoenzyme analysis, Ammonium sulphate Precipitation, Peroxidase analysis, Native Page

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INTRODUCTION

Legumes form a symbiotic association with certain soil bacteria such as rhizobia. This bacteria colonize the root hairs of legumes and multiply swellings which become nodules. The bacteria benefit from the legume which convert nitrogen into ammonium salts in the soil. The routine use of chemical fertilizer spoils the fertile nature of land. Therefore now a days use different organic manures such as seaweed manure, cow dung, vermicompost, coir waste and peatmass have been used to increase the fertility of the soil. The organic manure helps to grow the plants faster when compared with chemical fertilizer.

Isoenzyme analysis is based on the existence of enzymes with similar or identical specificity, but different molecular structure e.g., Peroxidase. Peroxidases are those enzymes are involved in the transfer of oxygen from peroxides to substance to be oxidized. This enzyme catalyses a host of reactions in which hydrogen peroxide is a specific oxidizing agent and a wide range of substrates act as electron donors.

MATERIALS AND METHODS

A) PROTEIN EXTRACTION FROM LEAVES OF GREEN GRAM (Vigna radiata):

Leaves were collected from different pots which were grown by using three different manures. Then the leaves were grinded with phosphate buffer (pH 7.5) and it was centrifuged for 15 minutes. After centrifugation, the supernatants were collected and protein concentration was estimated by Lowry's method.

B) ESTIMATION OF PROTEIN BY LOWRY'S METHOD:

First 0.5ml of three different samples was taken in six different test tubes. Then volume of the test tubes were adjusted up to 1ml by adding of 0.5ml of distilled water. Then 5 ml of alkaline copper reagent added to each test tube and incubated at room temperature for 10 minutes. To each test tube 0.5 ml folin phenol reagent was added and contents were mixed well. Blue color developed was read in calorimetrically at 640 nm.

C) DESALTING

Desalting columns are pre-packed, ready to use columns for group separation between high and low molecular weight substances. The Desalting columns are prepared by packing size exclusion matrix. The matrix is beds of cross linked dextran with epichlorohydrin. The matrix allows excellent resolution with good flow rates. The fractionation range for globular proteins is between 1000-5000Da. The desalting columns are made of biocompatible polypropylene which is no interactive with biomolecules. The top and bottom frits are made of porous polyethylene. The column is fitted with top and bottom caps. The use of a eluant containing a buffer salt for substances carrying a charged groups.

C. (i) COLUMN EQUILIBRATION

The column had a void volume of 3.5 ml. This column was equilibrated by first washing with 10 ml of distilled water followed by washing with 20 ml of Phosphate buffer (pH 7)(approximately 6 times the volume of the void volume). This was done in refrigerator condition.
C. (ii) SAMPLE PREPARATION:

500 mg of leaves from three different plants were weighed. The weighed samples were grinded properly followed by the addition of 1.5 ml of Phosphate buffer (pH7). This was then churned properly. The following was then centrifuged for 15 minutes and the supernatant was collected. 150 µl of the supernatant was used in finding the enzyme activity, protein content and the specific enzyme activity of the crude enzyme and the remaining volume was subjected to gel filtration and subsequent measurement of the enzyme activity of the filtered enzyme.

C. (iii) AMMONIUM SULPHATE PRECIPITATION AND SAMPLE LOADING:

According to the volume of the supernatant that was available after 150µl was used for determining the specific activity of the crude enzyme, 1 M Ammonium Sulphate was added to each sample. After adding the reagent it was kept in ice for a time period of 20 minutes, with constant shaking to initiate pellet formation. After this time period it was centrifuged and supernatant was discarded. The pellet formed was collected and was dissolved in 500 µl of 0.1M Phosphate buffer (pH 7). This sample was loaded gently and was allowed to drain completely. After this the recommended amount of buffer was added and the eluting amount was collected in small fractions and was monitored for protein content by UV absorption.

(D) ISOLATION OF PEROXIDASE BY NATIVE PAGE:

Technically, the sample extract is electrophoresed in starch or polyacrylamide buffered (non denaturating) stack gel at a low temperature (4-8)°C. Each lanes should be loaded with equal amount of proteins after normalizing the proteins content in exact in as small volume as possible (25-50µl) later electrophoresis the gel is incubated in a solution containing all the necessary components for enzyme reaction. The colored reaction product stains the gel where the enzymes were located.

The sample leaves were collected from the plants which were grown by three different manures such as seaweed, coirwaste and peatmass. 1 gm of sample was homogenized with phosphate buffer, centrifuged and the supernatant were collected. 30µl of supernatant was mixed with 5µl of native PAGE sample buffer. The sample was loaded carefully and gel was running using a power pack. The entire setup was maintained in a refrigerator at 4°C because the protein does not get denatured. Then the gel was removed from the glass plates and incubated in staining solution (7% acetic acid). It was kept on a rocker for 10 min and the zymogram obtained was photographed.

D. (i) ESTIMATION OF PEROXIDASE ACTIVITY:

\[
\text{Guaicol} + \text{H}_2\text{O}_2 \rightarrow \text{oxidised guaicol} + 2\text{H}_2\text{O}
\]

The resulting oxidase (dehydrogenated) guaicol was probably more than one compound and depends on the reaction condition. The rate of formation of guaicol dehydrogenation product is a measure of the POD activity and was assayed spectrophotometrically.
3 ml of buffer solution, 0.05 ml of guaicol solution, 0.1 ml of enzyme extract and 0.03 ml of H₂O₂ solution was pipetted out in a cuvette. The buffer solution was bought to 25°C before the assay. The absorbance was allowed to increase by 0.05. After the rise, a stop watch was used and the time required in minutes (Δt) to increase the absorbance by 0.1 was noted. Since the extinction coefficient of guaicol dehydrogenation product at 436 nm under the condition specified is 6.39 per molecule, the enzyme activity per litre of the extract was calculated as

\[
\text{Enzyme activity units/litre} = \frac{3.18 \times 0.1 \times 1000}{6.39 \times 1 \times \Delta t} = \frac{500}{\Delta t}
\]

Where Δt was the time required in minutes to increase the absorbance by 0.1.

RESULTS AND DISCUSSION

Extraction of protein and isolation of isoenzyme was done successfully with the purification from the leaves of green gram (\textit{Vigna radiata}). The concentration of protein in each group was analyzed. In table – I various types of manures used in the growth of \textit{Vigna Radiata} plant were placed. The Table- II reveals that growth of plants was faster in case of plants having manures in comparison to the control. The germination of seeds appeared on second day of plantation. The flowering and fruitening were occurred on the age of 51st day of plants. The extraction and isolation of peroxidase have been carried out on 48th day. Table III shows the manures enhance the growth of the plants. Among the four different samples the protein concentration was found to be higher in plant with coirwaste than other plants. Plants with seaweed manure showed somewhat better in protein concentration than peatmass and Table- IV reveals that the activity of peroxidase and its specific activity which was found to be higher in sample 4 when compared with other samples

DISCUSSION:

Several authors have studied on peroxidase. Schoenbein (1855) was the first who searched vigorously on peroxidase. Fig peroxidase was the first one isolated and investigated in 1936 by summer and Howell. Peroxidases have been purified from such diverse sources as horseradish, yeast, sweet potato, turnip, and wheat during 1942-1956. One of the first successful methods in purification and crystallization of purified peroxidase was performed by Shannon and his colleagues in 1966. He separated about 7 isoenzymes, from horseradish by ammonium sulfate precipitation and using ion-exchange chromatography. Delincee reported about 20 peroxidase isoenzymes by thin layer isoelectric focusing. In 1968, Mazza and his colleagues worked on purification of turnip peroxidases and they separated about seven isoperoxidases. Ion exchange and affinity chromatography have already been used for purification of peroxidase from horseradish.

Comparing with crude extract, the recovery of purified enzyme was not very high, but purity, as was shown by SDS-PAGE analysis and RZ, was very high. Therefore this method may be useful for purification of peroxidase from sources with high protein content.
It has been reported that peroxidase isoenzymes from various sources have different molecular weights ranging from 30,000-60,000 Dalton, e.g. four isoenzymes of turnip peroxidase have molecular weights between 37,000 and 57,000 Dalton and 30,000-54,000 for Japanese radish isoperoxidases. The molecular weight (MW) of BOC-POD was about 45,000 Dalton, which are in the range of MW of most the known peroxidases.

<table>
<thead>
<tr>
<th>SL.NO.</th>
<th>MANURES USED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control without manure</td>
</tr>
<tr>
<td>2</td>
<td>Seaweed+aminoacid</td>
</tr>
<tr>
<td>3</td>
<td>Peatmass</td>
</tr>
<tr>
<td>4</td>
<td>Coirwaste</td>
</tr>
</tbody>
</table>

Table I: TYPES OF MANURES USED

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>AMOUNT OF SAMPLE TAKEN (mg)</th>
<th>PROTEIN CONCENTRATION (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>2880</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>3441</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>3360</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>3680</td>
</tr>
</tbody>
</table>

Table II: CRUDE PROTEIN CONCENTRATIONS OF LEAVES

The Table- II reveals that growth of plants was faster in case of plants having manures in comparison to the control. The germination of seeds appeared on second day of plantation. The flowering and fruitening were occurred on the age of 51st day of plants. The extraction and isolation of peroxidase have been carried out on 48th day.

Table III: PARTIAL PURIFICATION OF PROTEIN USING DESALTING AND ITS CONCENTRATION

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>AMOUNT OF SAMPLE TAKEN (mg)</th>
<th>PROTEIN CONCENTRATION (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>548</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>520</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>660</td>
</tr>
</tbody>
</table>

Table III shows the manures enhance the growth of the plants. Among the four different samples the protein concentration was found to be higher in plant with coirwaste than other plants. Plants with seaweed manure showed somewhat better in protein concentration than peatmass.

Table IV: PARTIAL PURIFICATION OF PEROXIDASE

<table>
<thead>
<tr>
<th>SL NO.</th>
<th>SAMPLE</th>
<th>VOLUME (ml)</th>
<th>PROTEIN CONTENT</th>
<th>TOTAL ACTIVITY</th>
<th>SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>3</td>
<td>2880</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>PP</td>
<td>3</td>
<td>390</td>
<td>0.091</td>
<td>0.233</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>3</td>
<td>3441</td>
<td>0.093</td>
<td>0.027</td>
</tr>
<tr>
<td>4</td>
<td>PP</td>
<td>3</td>
<td>527</td>
<td>0.124</td>
<td>0.235</td>
</tr>
</tbody>
</table>

Table- IV: Reveals that the activity of peroxidase and its specific activity which was found to be higher in sample 4 when compared with other samples

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Figure I: THE NATIVE PAGE BANDS FOR PEROXIDASE AFTER ITS PARTIAL PURIFICATION

Figure observed the native PAGE band for enzyme specifically the peroxidase. The appearance of band explained the presence of peroxidase isoenzyme.

CONCLUSION

Protein profiling and isoenzyme analysis has done successfully. The protein content of green gram (*Vigna radiata*) in sample no.4 is higher than the other plants. The coirwaste manure shows the better yield than the other manures.

The activity of peroxidase enzyme is occurring in case of green gram (*Vigna radiata*) plants. Mostly from this work we concluded that the enzymatic activity of sample no5 leaves is higher than the other pots.

The coirwaste manure contain high amount of C: N ratio, cellulose and lignin. So it act as a good fertilizer containing NPK having greater effect than other manures. Seaweed manure shows the better yield than the peatmass.

The activity of peroxidase enzyme in crude extract is lesser than the partially purified sample. Hence it concluded that the partial purification of enzymes successfully done by the followed process. The peroxidase isoenzyme having a significant effect on the green gram plant (*Vigna radiata*).

REFERENCES


