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A Study of Estimation of the activity of cellololytic and pectolytic enzymes secreted by the fungal pathogens in jute

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Abstract:
In the present Article Enzyme secreted by the fungal pathogens in jute, cellulolytic and pectolytic enzymatic activity of Colletotrichum capsici f. capsularis (C / o of Anthracnose), Macrophomina phaseoli (C / o of Stem rot) and Sclerotium rolfsii (C / o of Soft rot) was worked out. The following are the proposed enzymes.Cellulolytic enzymes, Pectolytic enzymes, Amylolytic enzyme, Proteolytic enzyme secreted by the pathogens. these enzymes are secreted by the pythogens for the entry into the host tissue.The spores falling on the surface of leaf and stem cause infection.
Keyword: Enzyme, Infection, Pathogens, tissue, glucose, sucrose.

Introduction:

The pathogenic fungi are capable of secreting enzymes for the entry into the host tissue. The spores falling on the surface of the leaf and stem cause infection. This is the case of air borne spores. The soil borne fungi also get entry into the host (eg, Fusarium spp causing wilt disease). In the aerial part of the plant the first barrier is the cuticle found as acelluar layer of cutin. This polymeric high carbon chain fatty acid compound is degraded into smaller carbon compounds (Graniti, 1962, Ende and Linskens, 1974). Next to the cuticle layer is the cellulosic cell wall which forms the effective fence surrounding the protoplast. During infection, the cuticle is dissolved followed by the dissolution of the cellulosic material for approach to the protoplast. The dissolution of this material is achieved by cellulolytic enzyme complex acting sequentially on cellulose to make this polymer into simple sugar, the glucose (Hussain and Kelman, 1959; Wood, 1967). This produce is utilized by the pathogens as nutrient. Hemicellulose is also degraded to its simple compounds (Cochrane, 1958; Lilly and Barnett, 1951; Reese, 1963; Gould, 1969; Conn and Stumpf, 1976).

The degradation of the cellulosic wall facilitates the pathogen to form haustoria in the host cells for absorbing nutrient from the cytoplasmic content. The middle lamella that binds cells into tissue is made of pectic substance which is also degraded during infection for facilitating the pathogens to lodge in the intercellular space and penetrating deep into the tissue and organ. The pulpiness or softness of tissue in rotting is due to secretion of pectolytic enzymes by the pathogens. The pectolytic enzyme is also a complex of many enzymes hydrolyzing pectic substances in different ways (Sadasivan and Subramanian, 1963; Bateman and Miller, 1966; Wood, 1967). Wheeler (1975) has observed that all the purified pectic lyase and hydrolyses, endo- PGTE, PMTE and endo- PG of the pectolytic complex, capable of tissue maceration, cause death of the cells also. Good account of the role of pectolytic enzyme complex has been given by Mahadevan and Kathirvelu (1967), Sadik et al. (1983) and Khare et al. (1994). Thus the foregoing paragraphs affecting the role of cellulolytic and pectolytic enzymes in the successful infection. The part of the mycelium that enter into the cell i.e haustorium tends to enzymatically degrade the synthesized starch, protein and lipid to their respective simple compounds and absorb them for their nutrition. The enzymes to degrade these are amylase, protease and lipase respectively. In this way the enzymes secreted by the pathogens prove the competant tool for entry and establishment into the host and deriving nutrition for their survival. Sucrose is degraded to glucose and fructose by invertase and maltose by maltase. Amino acid degrading enzymes such as amino acid

decarboxylase and non - oxidative deaminase of particular amino acids have been found to be secreted in galled tissue of coriander by Protomyces macrosporus (Prasad et. Al., 1989) besides oxidase of amino acids. Cytoplasmic DNA and RNA have been reported to be hydrolysed by DNAse and RNAse (Griffin, 1981; Singh, 1984)

Materials And Methods :

A. ESTIMATION OF CELLULOLYTIC ENZYME ACTIVITY OF C.CAPSICI F. CAPSULARIS

In vitro cellulolytic enzyme activity of the pathogen was estimated after growing it in medium A.

100 ml of the medium omitting agar was warmed at 50 ° C and ingredients were dissolved. 2 g of carboxy methyl cellulose was added to make its 2% concentration. The medium was taken in plugged conical flasks and autoclaved. The pH was adjusted to 6.5 on cooling.

The spore suspension of the pathogen was prepared in 5% solution of Tween 20 (Sarbitol monolaureate). This solution was prepared in autoclaved distilled water. 5 ml of the solution so prepared was taken on the culture of the pathogen growing on the slant of the medium B. The culture tube was shaken and the number of spores per ml of the suspension was adjusted to 1x103 after measuring with the help of haemacytometer . The conical flasks containing the autoclaved medium was inoculated using 1ml of the spore suspension aseptically. The inoculated medium in the conical flasks was incubated at 25 + 1 ° C for 10 days.

Extraction of enzyme

After expiry of the incubation the metabolite was filtered on Buchner funnel using Whatman No.1 filter paper. The filtrate was centrifuged at 10,000 rpm for 15 min. The supernatant was saved. It was dialyzed in cellophane tubing against distilled water at 4 ° C for 24 hr. The water was changed at 8 hr interval. This preparation is enzyme extract.

1.Measurement of endoglucanase (= Cx) activity

Cx activity was measured viscometrically using viscometer size 150 with minimum efflux time of 20 sec for distilled water.

Substrate: -

Carboxy methyle cellulose (CMC) 0.5 g of CMC was dissolved in 100 ml of the buffer by warming at 50 ° C. It was blended at low speed for next 5 min and was filtered on Buchner funnel using Whatrnan No.1 filter paper.

4 ml of CMC solution, 1 ml of the buffer and 2 ml of the enzyme were pipetted in the viscometer. The content were mixed by drawing air gently through the large arm of the viscometer. Suction was applied to the small arm and efflux time was measured. Per cent loss in viscosity of CMC was calculated by the formula

$$V = \frac{To - T}{To - Tw} x100$$

Where,

V = Per cent loss in viscosity , T_0 = Flow time in second at Zero time

T = Flow time of reaction mixture T_w = Flow time of distilled water

For the control the liquid medium was taken in place of the enzyme. The result was recorded in Table A

2.Measurement of the activity of endo - ß 1, 4- glucanase (or Carboxy methyl cellulase)

This enzyme attacks the 1, 4. B glucosidic linkage of cellulose molecule randomly and releases reducing sugars which can be estimated with Dinitro-salicylic acid Reagent (DNS reagent).

(a) DNS reagent

1 g of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulphate (Na2SO4) were placed in a beaker with 100 ml of 1 % sodium hydroxide (NaOH) by stirring,

(b) Rochelle salt (Potassium Sodium Tartarate): 40% solution in water

Substrate: 0.5 / CMC in the buffer.

In a test tube 4ml of CMC solution, Iml of buffer and 2 ml of the enzyme prepared in case of determining the activity of Cx, were taken and the mixture was incubated at 30 ° C for 5 min. 1 ml of the aliquot was withdrawn from each tube and 3 ml of DNS reagent was added. The tubes were heated in boiling water bath for 5 min. It was slightly cooled and Iml of Rochelle salt solution was added. The tubes were cooled under running tap water and the OD was measured at 575 nm. For the control only the filtered medium was used in place of the culture filtrate, the enzyme preparation.

0.1% glucose solution after diluting serially was used for drawing calibration curve for the estimation of reducing sugar. The result was noted in Table A.

3. Estimation of the activity of cellobiase

The amount of reducing sugar released from cellubiase by cellobiase was used to assay the enzyme activity.

Reagents:

Alkaline copper tartarate

(a) 2.5 g of unhydrous sodium carbonatse (Na2CO3), 2 g of sodium bicarbonate (NaHCO3), 2.5 g of potassium sodium tartarate and 20 g of unhydrous sodium sulphate (Na2SO4) were dissolved in 80 ml of distilled water and the volume was made to 100 ml.

(b) 15 g of crystalline copper sulphate (CuSO4.5H20) was dissolved in small volume of water. Two drops of H2SO4 was added and the volume was made to 100 ml.

96 ml of a and 4ml of b were mixed together before their use.

Arsenomolybdate reagent

2.5 g of ammonium molybedate was dissolved in 45 ml of distilled water. 2.5ml of H2SO4 was added and mixed. Then 0.3 g of dissodium hydrogen arsenate was dissolved in 25 ml of water. These were mixed well and incubated at 37 ° C for 24hr.

1 ml of the aliquot was taken in a test tube and 1ml of distilled water was added.distilled water was added. 2 ml of distilled water was taken in a separate tube to use it as blank (at zero aborbance). In the tube containing the aliquot, 1 ml of copper tartarate reagent was added. The tube was placed in boiling water bath for 10 min. The tube was cooled and 1 ml of arsenomolybdate reagent was mixed. The volume of reaction mixture was made to 10 ml with water. After 10 min the absorbance of blue color was read at 620 nm. The amount of reducing sugar released was calculated with the help of calibration curve prepared from 0.1% of glucose after serial dilution. The result was noted in Table A.

B. ESTIMATION OF THE ACTIVITY OF PECTOLYTIC ENZYMES

Pectic substances are found in primary cell wall and in the middle lamella. The simplest monomer of pectin is galacturonic asid and it is linked in an 1, 4 - fashion. The pectin substances are categorized according to the degree of polymerization of galacturonic acids. The simplest classification of pectic substances is (a) pectic acid, (b) pectin and (c) Protopectin. Pectin acid is composed of about 100 galacturonic acid units. Their large number of carboxyl groups (-COOH) react with polyvalent cations such as Ca ++.

Pectin (= pectinic acid) is made up of Ca 200 polygalacturonic acid units and the -COOH groups are esterified with methoxy groups.

Protopectin is the major component of pectin. It is composed of Ca 1000-2000 units of esterified -COOH groups and has bound Ca ++ and PO4.

Pectin as such is not utilized by the pathogens but it is degraded to galacturonic acid which is readily utilized. Therefore, breakdown of pectic substance into smaller units of galacturonic acid is the first act of the pathogen. The cleavage of pectin is mediated by both hydrolytic and nonhydrolytic enzymes.

Pectic substance $\frac{Hydrolytic cleavage}{at 1,4-gly\cos idic linkage}$ Galacturonic acid of varying sizes

Following is the category depending on substrate specificity.

(a) Enzyme that cleaves pectinic acid is Polymethyl galacturonase (PMG)
(b) Enzyme that cleaves pectic acid is Polygacturonase (PG)
(c) Enzymes that removes methoxy groups is Pectinmethyl esterase (PME).

The nonhydrolytic breakdown of pectic substance involves the participation of two enzymes noted below.

(a) PTE (Pectin transeliminase) - It acts on pectin
(b) PATE (Pectic acid transeliminase) - It acts on pectic substance.

In the present part of this chapter PG, PMG, PME and PTE will be estimated in C.capsici f. capsularis.

Estimation of the activity of PG

100 ml of the medium A containing 2.0 g pectin, pH 6.5, was taken in conical flask of 500 ml capacity, autoclaved and adjusted the pH to 6.5, and then inoculated aseptically with 1 ml of the spore suspension (1x103 spore / ml). The culture was incubated at 25 + 1 ° C for 10 days. The culture filtrate was filtered with one layer of sterilized cheese cloth and then centrifuged at 1,5000 rpn for 10 min. The supernatant was dialyzed in cellophane tubing against distilled water in a refrigerator at 4 ° C for 24 hr. The water was changed at every 3 hr. This is the enzyme preparation (EP).

Procedure

Sodium polypectate

750 mg of sodium polypectate was dissolved in 100 ml of the buffer by warming at 60 ° C. It was blended at low speed for 3 min and at high speed for next 3 min. The solution was filtered through two layers of cheese cloth. The pH was adjusted to 5.2 with the help of 1 NHCl or 1 N NaOH and by reading in pH meter.

The activity of PG was measured as loss of viscosity of the substrate using Ostwald viscometer. 4 ml of the substrate was pipetted into the viscometer. 1 ml of the buffer was added followed by 2 ml of the enzyme preparation. The content was gently mixed. The solution was sucked through the small arm. The efflux time of the substrate, distilled water and the mixture was noted. The mean of three reading was recorded.

Percent reduction in viscosity of the substrate was calculated by the formula given below.

$$V = \frac{Tc - T}{To - Tw} x100$$

Where,

V = Per cent loss in viscosity, To= flow time in second at zero time
T = Flow time of the reaction mixture, Tw = Flow time of the distilled water

The result was noted in Table A.

Estimation of the activity of PMG (Polymethyl galacturonase)

for this purpose the medium A was supplement with pectin as substrate so as to make its concentration 2.0%. 100 ml of the medium was taken in conical flask of 500 ml capacity and the pH was adjusted to 6.5 and incubated at 25 +1 ° C for 10 days after inoculation of the medium with the pathogen.

Extraction of the enzyme

After the expiry of the incubation, the culture filtrate was filtered with two layers of autoclaved cheese cloth. The filtrate was Centrifuged at 15,000 rpm for 15 min. The supernatant was dialyzed in cellophane tubing in refrigerator at 4 ° C for 24 hr changing the distilled water at every 3 hr. This is the enzyme extract.

Estimation of the activity

The polymethyl galacturonase acivity was measured in term of per cent loss of the viscosity of the substrate by the enzyme.

1 g of pectin was dissolved in 100 ml of acetate buffer, pH 5.2 noted earlier by warming at 60 ° C kept in a blender. For 3 min the blending was done at low speed and then at high speed for next 3 min. It was filtered with two layers of cheese cloth. The pH was adjusted to 5.2 using 1 N HCl or 1 N NaOH.

The per cent loss in viscosity was measured as noted for PG earlier. The result was noted in Table A.

Estimation of the activity of PME (Pectin methyl esterase)

PME cleaves pectin resulting in pectinic acid and methynol

Pectin $\xrightarrow{\text{PME}}$ Pectinic acid + Methanol

Pectinic acid is neutralized by continuous titration method using 0.02N NaOH.

The pathogen was cultured in medium A supplimented with 2.0% pectin as noted for the estimation of PG earlier

The enzyme was extracted as noted for the estimation of PG.

Procedure:

Substrate: 1.0% pectin dissolved in 0.15 M sodium chloride (NaCl).

Sodium hydroxide

0.02 N NaOH was prepared from N NaOH on the day of use.

20 ml of pectin solution was taken in 50 ml of beaker. The pH was adjusted to 7 and measured with the help of pH meter. 10 ml of the enzyme extract was added. The pH was immediately adjusted to 7 using 0.02 N NaOH. This is zero time. The beaker was placed in a water bath at 30 ° C. The control was maintained with boiled enzyme.

At every 15 min, the pH was cheked. If it fell below 7, 0.02 N NaOH was added with pipette drop by drop and the mixture was stirred. At each interval the volume of the alkali used to eutralize the reaction mixture, was noted. 1 hr period was spared for reaction. The result was noted in Table A.

Estimation of the activity of PTE (Pectin transeliminase or pectin lyase)

PTE splits peetin releasing unsaturated galacturonic acid residue. The rate of release of unsaturated galacturonic acid is estimated by the loss in viscosity of pectin.

The pathogen was cultured on medium A supplimented with 2.0% of pectin, the procedure of which has been described earlier.

Extraction of the enzyme: The enzyme was extracted as noted earlier for PG.

Procedure:

Substrate: 1 g pectin was dissolved in 100 ml of the said buffer. Warmed at 60 ° C and was blended at low speed for 3 min in warm condition. Pectin was filtered through two layer of cheese cloth. The pH was adjusted at 8.7.

4 ml of the pectin solution, 1 ml of the buffer and 2 ml of the enzyme were taken in the viscometer. A gentle suction was applied through the large arm of the viscometer to mix the contents. Suction was applied to the small arm. The per cent loss in viscosity was determined adopting the procedure and formula as noted for PG. The result was noted in Table A.

Table A: In vitro activity of cellulolytic, pectolytic, amylolytic and proteolytic enzymes of C.capsict f. capsularis

Enzymes	Value
Cellulolytic enzymes	
Endoglucanase (% loss in viscosity)	10.12
Carboxy methyl cellulose (mg glucose released / ml 0.98 enzyme / 30 min)	0.98
Cellobiase (mg glucose released / ml enzyme / 30min)	0.81
Pectolytic enzymes	
Polygalacturonase (% loss in viscosity)	15.23
Polymethyl galacturonase (% loss in viscosity)	12.15
Pectin methyl esterase (ml of 0.02N NaOH used / ml 3.17 enzyme / 30 min	3.17
Pectin transeliminase (% loss in viscosity)	8.62
Amylotytic enzyme (mg of reducing sugar released / ml enzyme / 30 min)	6.42
Proteolytic enzyme (mg amino acid released / ml enzyme/30 min)	7.63

It appears that activity degree of endoglucanase, carboxy methyl cellulase and cellobiase of cellulolytic enzyme complex, polygalacturonase, polymethyl galacturonase, pectin methyl esterase and pectin transeliminase of pectolytic enzyme complex, amylolytic and proteolytic enzyme was observed in C. capsici f. capsularis indicating that the said pathogen is capable of degrading cellulose, pectic of substance, starch and protein

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