STUDIES ON POLYGALACTURONASE FROM *ASPERGLLUS FLAVUS*

Mohan B. Gewali*, **Jyoti Maharjan***, **Sambhu Thapa*** and **Jaya Krishna Shrestha***
*Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Abstract: Crude polygalacturonase was obtained from *Aspergillus flavus* cultured in Glucose Free Medium (GFM) by precipitating cell free broth with 66% cold acetone. Effect of different parameters such as temperature, pH incubation time and enzyme concentration on this polygalacturonase preparation was studied. Furthermore, some inhibition studies on this enzyme preparation were conducted.

Key words: Aspergillus flavus; Polygalacturonase.

INTRODUCTION

Our interest in polygalacturonase stemmed from the fact that polygalacturonase (pectinolytic enzymes) are involved in causing soft rot disease in fruits and vegetable. Finding natural inhibitors of these phytopathogenic enzymes constitute a novel way of controlling such disease. In pursuit of finding natural inhibitors of phytopathogenic polygalacturonase , we have studied polygalacturonase from three soft rot causing plant pathogens, *Geotrichum candidum*[1], *Botrytis cinerea*[2] and *Aspergillus niger*.[3]

Phytopathogenic fungi are known to cause tissue maceration and soft rot diseases in the post harvest fruits and vegetables. The extra cellular polygalacturonase produced by these pathogens is responsible for causing tissue maceration. The substrates for these pectolytic enzymes are pectic substances. In the plant cell, the pectic constituents are the primary constituents of the middle lamella and are composed of high molecular weight polymer consisting of a backbone of α-4 linked D- galactopyranose interspersed with 1,2-α-linked rhamnopyranose. There are two main groups of pectolytic enzymes that attack pectin substances-esterases and depolymerases. The esterase split the methyl ester group of esterified poly-D-galacturonic acids and is referred to as pectin esterase or pectin methylesterase. The depolymerizing enzymes are either hydrolyses or lyases. Pectolytic enzymes act upon the pectin substances and bring about tissue maceration resulting in the soft rot diseases. Thus certain fungal and bacterial diseases are caused by secretion of tissue hydrolyzing enzymes, which play a key role in the pathogenic process of penetration, infection, and tissue maceration. Our aim is to find and develop products, which will intervene in the disease process by specifically inhibiting these pathogenic enzymes without poisoning the pathogen.

In course of our study on phytopathogenic enzymes, we got hold of a strain of *Aspergillus flavus* and from this strain of *Aspergillus flavus*, crude polygalacturonase was obtained. In this article, we report the production of polygalacturonase from *Aspergillus flavus*, its characterization and attempted inhibition studies.

MATERIALS AND METHOD

Plant Materials

Sample or remedies being tested were purchased or obtained locally. Each of the samples was made powder, weighed 5-10 gm and extracted three times with boiling methanol. The solvent was removed and dried extract was used for sample preparation.

Microbial Strain

Pure strain of *Aspergillus flavus* was obtained from Mrs. Geeta Rajbhandari, Amrit Science Campus, Tribhuvan University, Kathmandu, Nepal.

Growth Condition

YGP medium: A starter YGP (0.1% yeast extract, 0.1% glucose and 0.1% pectin) medium was prepared in 50 ml of doubled distilled water. A small agar plate disc (-5mm) was cut from a PDA plate containing sporulating fungus and transferred to the starting medium. The flask was kept in a water bath shaker at 30°C for 3-4 day. This inoculum from the starter medium was transferred to the same YGP medium (10% volume). Samples were taken at various times for biomass and enzyme activity.

Glucose free medium (GFM): A small agar disc (-5mm) was cut from PDA plate containing sporulating fungus and was transferred to a glucose free starter medium containing 0.5 g pectin, 0.5 g $NaNO_3$, 0.25 g KH_2PO_4, 0.12 g $MgSO_4$, $7H_2O$, 0.05 g $CaCl_2$ and traces of $FeCl_3$ in 50 ml of doubled distilled water. The flask was kept in water bath shaker at 30°C for 2-3 days. This inoculum from the starter medium was transferred

Author for Correspondence: Mohan B. Gewali, Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

to the same glucose free general culture (10% Volume). Samples were taken at various times for biomass and enzyme activity.

Growth study: Growth of fungus in culture medium was studied in terms of turbidity of solution. For turbidity measurement, culture samples were taken at various interval of time and turbidity was measured at 600 nm using distilled water as blank.

Preparation of Crude Enzyme

Mycelia of the broth were removed by centrifugation followed by filtration on Whatman 1 filter paper. Protein was precipitated either by adding NH_4SO_4 or by cold acetone. Precipitation with ammonium sulphate was not satisfactory. Cold acetone was added to the clarified supernatant (cell free broth) to 20% (by volume) concentration and precipitate was centrifuged off. The supernatant was adjusted with acetone to 66%(v/v) and centrifuged for ten minutes. The precipitate was dissolved in 0.1 M sodium acetate buffer (pH 4.2). DNS assay and protein measurement on crude enzyme preparation revealed increased specific activity.

Polygalacturonase Assay [4]

Reducing groups determination (DNS assay) polygacturonase activity was determined by measuring the amount of released reducing groups by pectin hydrolysis with 3,5-dinitrosalycilic acid (DNS) reagent. Mono-D-galacuronic acid was taken as standard. The assay system contain 3-4 tube containing 0.1 ml, 0.2 ml, 0.3 ml, 0.4ml of crude enzyme solution and 0.1 M sodium acetate buffer pH 4.2 was added in each tube and incubated at 30° C. After 5 minutes 4 ml of DNS reagent (NaOH 1 %, DNS 1 %, phenol 0.2 %, sodium sulfite 0.05%) was added and boiled for 15 minutes. After cooling, absorbance was measured at 575 nm. Reaction mixture also contained boiled enzyme, which was used as control. One unit of enzyme was that amount of enzyme, which catalyzed the release of 1 mg of mono galacuronic acid per hr.

Tissue Maceration Assay[5]

For potato, carrot and turnip discs: They were locally purchased and gently wiped with a mist sponge to remove dirt. They were soaked for 15 minutes in hypochlorite solution in order to destroy contaminating microorganism and washed several times with water. Disc of about 6 mm were excised at random with cork borer to a depth of 5 mm. The discs were rinsed twice with 0.1 M sodium acetate buffer pH 4.2 and blotted with filter paper before use. In an assay vial containing 5 ml of acetate buffer were incubated at water bath shaker at 30°C. Polygalacturonase was added at different from 0.5 U/ml to 4U/ml. Enzyme mediated maceration was expressed at the net turbidity measured at 540 nm.

For cucumber discs: Green cucumber fruits were washed thoroughly and sliced perpendicular to their long axis into several pieces. Cylinder of white pericarp tissue was removed by a 6 mm diameter cork borer. These cylinders were sliced into 6 mm long section using a blade. Section (5 slices) was stored in distilled water for 1 hour and were gently blotted and weighed. Net weight loss determined by correcting for weight changes for slices incubated in identically prepared boiled solution of enzyme or solution containing no enzyme.

Inhibitor Activity Assay[4]

Two methods were used for inhibition studies

The inhibitory activity was assayed by measuring the residual Polygalacturonase activity after the enzyme was preincubated with inhibitor solution for fifteen minutes at 30Â° C. The assay system contained 1-2 units of enzyme with different concentration of inhibitor solution and 0.1 M sodium acetate buffer pH 4.2 up to 0.5 ml which was incubated with 0.5 ml of 0.5% pectin dissolved in 0.1 M acetate buffer pH 4.2 for 5 minutes at 30° C. A control sample without inhibitor solution was incubated in parallel with test sample. Percent inhibition of polygalacturonase activity was calculated from the equation A-B/A x 100% when A and B are values of

Table 1: Polygalacturonase production *by Aspergillus flavus* in GFM.

Time (hour)	Vol. of Culture broth	Vol. for DNS Assay	Unit./ml	Total unit
0	275	0	0	0
24	260	200	0.8	208
72	230	200	2.54	584
120	200	200	13.25	26.50
168	170	200	4.0	680

Table 2: Partial Purification of Polygalacturonase from *Aspergillus flavus*.

Purification step	Enzyme (U/ml)	Protein (Mg/ml)	Specific activity
Cell free broth	6	0.002	3000
66% acetone precipitate	24	0.004	6000

Table 3: Effect of Temperature on polygalacturonase activity.

Temperature	0	10	20	30	40	50	60	80	100
Activity %	100	100	100	100	100	70	55	25	0

Table 4: Effect of pH on polygalacturonase activity.

pH	2.2	3.2	4.2	5.2	6.2	7.2
Activity %	18	70	100	85	22	18

Table 5: Effect of Enzyme concentration of polygalacturonase activity.

Enzyme concentration (Unit/ml)	1	2	4	6	8	10
Absorbance ×' 575 nm	0.08	0.14	0.28	0.41	0.54	0.54

Table 6: Effect of Incubation Time on polygalacturonase activity.

Incarnation time (Min)	0	5	10	20	30	60	120	240
Mole of reducing μg/ml	1.47	1.58	1.70	1.94	2.0	2.05	2.17	2.17

galacturonic acid (expressed in absorbency at 575mm) in the absence and the presence of inhibitor respectively.

Cup-plate assay also used for inhibitory studies. In the cup, a mixture of enzyme and the potential inhibitor (1.2 v/v) were added and incubated for 18 hour at 30° C. The plates were flooded with a mixture of concentrated HCl and distilled water (1:1 v/v). Inhibition was determined by comparing with control (enzyme only) in a semi transparent holozone that was formed.

Mycelial Growth Inhibition Study

Five different micro-organisms, Penicillin species, Bacillus species, Micrococcus species and two unidentified micro-organisms isolated from a home made compost were tested on this study. On 5 mm thick MY plates, 100 μg of 48-72 hours old liquid culture of micro-organism were sprayed. This was covered with approximately 10 mL of PDA at 40^0C. A 3-5 mm diameter mucelial disc of sporulating *Aspergillus favus* was placed in the mddle of the plate and incubated at 30^0

C for several days. A PDA plate without test micro-organism served as a control.

RESULT AND DSCUSSION

For the production of polygalacturonase from *Aspergillus flavus*, the fungi was grown in two liquid culture media namely yeast glucose pectin (YGP) and glucose free medium (GFM). On repeated experiments, polygalacturonase was not found to be produced in appreciable amount in YGP medium. However, polygalacturonase was found to be produced in GFM medium and its production was optimum after 120 hours of incubation. (Table 1)

Polygalacturonase was harvested from the culture broth when its production was maximum. Extraction of enzyme was done by precipitating cell free broth from GFM first with 20% cold acetone followed by 66% cold acetone. No precipitation was obtained from cell free broth of YGP medium. The specific activity of the crude enzyme obtained after precipitation with cold acetone was found to be increased by two folds. (Table 2).

The gradual viscosity loss of pectin solution brought about by this crude enzyme preparation was observed. This assay was carried out in the test mixture containing 2ml of crude enzyme and 8ml of 0.5% pectin solution in Sodium acetate buffer (0.1M, pH 4.2). The loss of viscosity of pectin solution was measured at different interval of time. The gradual loss in viscosity of pectin with time was observed due to hydrolysis of pectin by enzyme. About 82% viscosity loss was observed at 120 minutes. Further more, Dingle's cup plate assay of this polygalacturonase preparation revealed the linear relationship between halo ring and concentration of enzyme.[6]

We also subjected this enzyme preparation to the Tissue Maceration Assay. As opposed to polygalacturonase from *Botrytis cinerea*, this enzyme preparation did not bring about substantial tissue maceration in pumpkin, radish, turnip, potato discs, however only slight tissue maceration in orange peels were observed.

This enzyme preparation was found to be stable in the temperature range 0°C-40°C and at 100°C, the enzyme was completely deactivated. (Table 3)

The optimum pH for polygalacturonase activity was 4.2 (Table 4)

Velocity of reducing group liberation by polygalacturonase was linear up to 6 unit/ml of enzyme concentration (Table 5) and up to 20 minute of incubation time. (Table 6)

With this polygalacturonase preparation with known characteristics on hand, next we undertook inhibition studies. Eighteen Kathmandu home herbal remedies i.e. *Acorus calamus, Allium Sativum, Areca Catechu, Azardirachta Indica, Cumin cyaminum, Cinnamomum tamala, Cinnamomum zeylanicum, Curcuma longa, Euphorbia thymifolia, Ferula narthex, glycyrrhia glabra, Myristica fragrance, Nardostachys Jatamasi, Swertia chirata, Sizygium Aromatica, Terminalia Belarica, Terminalia chebula* and *Zanthoxylum Armatum* were selected to be candidates for inhitbtion studies. For inhibition studies, both reducing group determination (DNS method) and cup plate methods were employed. None of them were found to inhibit the activity of polygalacturonase produced by *Aspergillus flavus*. Even extracts of A. *catechu*, which inhibited the activity of polygalacturonase, produced by the fungi B. *cinerea*[2] and A. *niger*[3] failed to inhibit the enzyme activity. It has been known that the nature and action of polygalacturonase differ depending upon their source. As such the polygalacturonase produced by *Aspergillus flavus* may have different

composition active site and structure from the enzyme produced by B.*cineara* and A. *niger*

Furthermore, twelve different metal-ions were tested for their inhibitory activity toward the enzyme. For this study also both DNS assay and cup plate method were applied. Only five metal-ions, out of twelve, were found to possess inhibitory properties towards enzyme activity. They were, lead acetate [$Pb(OAc)_2$], Mercuric Chloride ($HgCl_2$), Cobalt chloride ($CoCl_2$) Ethylene diamine tetra amine (EDTA) and Ammonium Sulphate $(NH_4)_2SO_4$. It was found that lead acetate and mercuric chloride had 100% inhibition activity in 5 µg /ml and 20µg /ml concentration respectively.

Next, we directed our attention to mycelial growth inhibition of *Aspergillus flavus* by other microbial populations. Out of five different micro-organisms tested against the growth of *Aspergillus flavus*, only *Bacillus* species was found to inhibit the mycelial growth of *Aspergillus flavus* Mechanism for the inhibition is not clear. It has been reported that *Bacillus subtilis* produces antibiotic which inhibits the growth of *F*. oxisporum and *F. udum.*[7] Possibly due to the similar reason the growth of *Aspergillus flavus* was inhibited by *Bacillus* species.

In summary, crude polygalacturonase was obtained from *Aspergillus flavus* cultured in Glucose Free medium. Maximum production of PG was found at 120 hours after inoculation from starter medium at 30°C. Specific activity of this polygalacturonase preparation was increased by two fold after precipitating with 66% cold acetone. Enzyme activity was stable upto 40°C. About 74% loss in activity was observed at 80°C. Enzyme exhibited maximum activity at pH 4.2. Degradation of pectin by crude enzyme was linear up to 6 unit/ml and up to 20 minute of incubation time. Enzyme mediated maceration was observed only on orange peels. Five metal-ion solutions i.e.$Pb(OAc)_2$, $HgCl_2$, $CoCl_2$, $(NH4)_2SO_4$, and EDTA inhibited the polygalacturonase activity. Furthermore, A liquid culture broth of Bacillus species was found to inhibit the mycelial growth of *Aspergillus flavus*.

ACKNOWLEDGEMENT

We thank Professor A.P. Sharma, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu Nepal for valuable suggestion and comment. This work was supported by a CDR grant.

REFERENCES

1. Gewali, M.B., Thapa, S., Baral, A., Bhattarai, S. and Shrestha, J.K. 1994. *Proceeding of Second National Conference on Science and Technology*. 947-958. Royal Nepal Academy of Science and Technology, Kathmandu, Nepal , June 8-11.
2. Gewali, M.B. 1995. *Proceedings of the 4th Symposium on Traditional Medicines in Toyama*. Toyama, Japan. 217.
3. Gewali, M.B., Manandhar, S., Thapa, S., Banskota, A. and Shrestha, J.K. 1997. *Journal of Nepal Chemical Society*. **16**: 23-26.
4. Miller, G.L. 1959. *Anal.Biochem.* **31**: 426.
5. Bateman, D.F. 1968. *Neth.J.Plant Path.* ***25***: 161.
6. Dingle, J., Reid, W.W. and Soloman, G. 1950. *J.Sc. Ed. Agric.* **1**:234.
7. Vasudeva, R.S., Subbain, T.V., Sastry, M.L.N., Rangaswami, G. and Iyengar, M.R.S. 1958. *Ann. Appl. Biol*. **46**: 336.