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Studies of Zymography for Detection of Proteolytic Enzymes in Testa of Some Cucurbitaceae Members

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ABSTRACT

Zymography is an important electrophoretic technique that is used to detect hydrolytic enzymes. The understandings of presence of the hydrolytic enzymes in testa of some Cucurbitaceae members are very much important as the seeds are edible. The intake of edible seed testa contains hydrolytic enzymes that catalyze chemical bond by hydrolysis of biomolecules. The standard marker was observed with bands on the gel at 66 kDa, 43 kDa, 29 kDa and 14 kDa. Cucumis sativus was shown one white band at 33 kDa corresponding to standard marker. Cucurbita maxima have shown two major white bands based on varying gelatinolytic activity at 33 kDa and 39 kDa corresponding to standard marker. Citrullus lanatus was shown a white band at 48 kDa corresponding to standard marker. The work using Zymography was shown that the testa of some Cucurbitaceae members are essential in controlling molecular mechanism within the living system.

KEYWORDS : Zymography, Cucurbitaceae members, hydrolytic enzymes

INTRODUCTION

Zymography is an electrophoretic technique used for detection of hydrolytic enzymes on the basis of substrate degradation (Vandooren, 2013; Manchenko, 2002). In biochemistry, a hydrolytic enzyme is an enzyme that has the capability to catalyze a chemical bond by hydrolysis of proteins, fats, nucleic acids, starch and other complex biomolecules.

One of the most popular techniques is by separating the protein mixture with polyacrylamide gel electrophoresis (PAGE) wherein a substrate is included by the polyacrylamide gels (Righetti and Drysdale, 1973). The protein substrates in the gel are degraded by the various proteases in the sample which are activating during the incubation period (Kleiner and Stetlerstevenson, 1994). Staining with coomassie blue shows the proteolytic cleaved sites as white apparent bands on a dark blue background on the gels (Rouet et al., 1999). The essential safety measures are taken to avoid the enzyme from denaturation during the procedure (Cohen, 1973). The tested samples were isolated, standard and non-reducing loading buffers used for SDS-PAGE. There is no reducing agent or boiling necessary since substrates would interfere with refolding of the enzyme.

Vacuoles isolated from pea (Pisum sativum), and wheat (Triticum aestivum) leaf protoplasts contained considerable activities of electrophoretically highly mobile exoamylases (Ziegler and Beck 1986). The complete barley seeds of α-amylase (Hordeum vulgare L. cv. Himalaya) attain maximum concentration during the germination within 3 to 4 days (Jones and Armstrong, 1971).

MATERIALS AND METHODS

Preparation of Crude extract

The crude extract was prepared based on the protocol proposed by Pichare and Kachole,

Collection of Seed Coat Powder from Cucurbitaceae Members

In the present experimentation, Citrullus lanatus, Cucurbita maxima and Cucumis sativus fruits was collected from the fields of Visakhapatnam district, AP, India during March to June 2011. The plants are authenticated by Dr. P.V. Arjun Rao, Ethanobotanist, Dept. of Botany, Phytopharma Technology Laboratory, Visakhapatnam (No. Res/2 dated 21-09-2010). Citrullus lanatus., Cucurbita maxima and Cucumis sativus respectively.

Preparation of Crude Extract

The seeds present in the fruits are collected and dried for 2 days. The testa is separated and crushed to fine powder using motar and pestle. The fine seed coat powder is selected for the present experimentation.

The testa powder was homogenized in 100 ml of 0.1M phosphate buffer pH 7.0 and the extract was prepared in 500 ml conical flask. The homogenate was mixed by incubating the extract in a rotary shaker at 120 rpm for 30 minutes at room temperature. Then the cell debris was removed by the slurry filtered through cheese cloth. Filtrate was collected and centrifuged at 10,000 rpm for 15 minutes at 4°C (Pichare and Kachole, 1994). The crude extract was used for detection of proteolytic enzymes in some Cucurbitaceae members by gelatin zymography method.

Gelatin Zymography

In the Gelatin Zymography has been used substrate gelatin or casein is embedded in the resolving gel throughout preparation of the acryl amide gel. After Electrophoresis, SDS is detached from the zymogram (or gel) by incubation using Triton X-100, gel was incubated in an appropriate developing buffer at 37°C. The zymogram is stained (Coomassie Brilliant Blue). The substrate has been ruined by using the enzyme and appears as clear bands against a darkly stained back ground. Gelatin Zymography was conducted for find the proteolytic enzymes present in the seed coat of some Cucurbitaceae members.

Reagents

The common reagents used in the present experimentation are proposed below.

Stock solutions

A stock solution is one that is used in the preparation of formation and developing of gel. The stocks solutions require in zymograpy method were discussed below.

Acrylamide 30%

About 29.2 g acrylamide and/0.8 g Bisacrylamide was makeup to 100 ml of double distilled water. The solution was filtered through 0.2 µm filter.

Tris ((hydroxymethyl) aminomethane) 1.5 M

The solution was prepared as 36.3 g Tris ((hydroxymethyl) aminomethane) dissolved in 100 ml of water. pH Adjusted to 8.8 (with 1M HCL) and makeup to 200ml with distilled water and adjusted pH 8.8.

Trishydrolytic Enzymes 0.5 M
Solution was prepared as 6 g of Tris in 40 ml water. Adjust pH to 6.8 (with 1M HCL) and then make upto 100 ml solution with double distilled water and adjust pH to 6.8.

Ammonium Persulfate 10%
Add 0.1 g of ammonium persulfate in 1 ml of double distilled water.

SDS 10%
Add 0.1 g of SDS in 1 ml of double distilled water.

Sample Buffer (2X)
To 2.5 ml of 0.5 M Tris-HCl and add other various reagents like 4ml of 10% (w/v) SDS, 2ml of Glycerol, 0.5 ml of 0.1% Bromophenol Blue in a conical flask and make up to 10 ml solution with double distilled water (pH 6.8).

Reservoir Buffer for SDS-PAGE
About 3 g of Tris buffer is to be taken and add 14.4 g of Glycine and 1.0 g of SDS. The solution is to be dissolved and makeup to 1L with double distilled water. The solution is prepared (equal to 10X concentration), adjust pH to 8.3 and stored at 4°C.

Coomassie R-250 Staining solution
Prepare 0.4% comassive brilliant blue (R-250) in a solution of methanol, acetic acid and water in the ratio of 40:10:50.

Destaining solution
A solution of 40ml Methanol and 10ml acetic acid is prepared and add 50 ml of distilled water for getting a 100ml of destaining solution..

Renaturing Buffer (10X)
Prepare 2.5% (v/v) of Triton X-100 in distilled water.

Developing Buffer (10X)
Add 12.1 g of 50mMTris base, 63 g of Tris-HCl, 117g of 0.2M NaCl, 7.4 g of 5mM $CaCl_2$ and adjust to 1L with distilled water.

Gel percentage
The gel percentage was prepared based on the David Bowtell, 2006 protocol. Table 1 shows the gel percentages that can be used to prepare gels.

Table 1: Gel percentages used in preparation of gels for zymograms

Component	18% Gel
Water	1.24 ml
1.5M Tris,pH 8.8	3.0 ml
10%SDS	0.12 ml
Acrylamyde	7.2 ml
TEMED	5.0 µl
10% Persulfate	35 µl

Stacking Gel 4%
Add 6 ml water, 2.5 ml of 0.5 M Tris pH 6.8, 0.1 ml of 10% SDS,1.17 ml Acrylamide, 15 µl TEMED and 200 µl of 10% Ammonium persulfate for the preparation of 4% stacking gel.

R_f Value Calculation from Gelatin Zymography
Calculation of relative mobility's of the protein (Marker and samples) is done by using the formulae:

R_f = distance migrated by protein/distance migrated by tracking dye

Experimental Method for Gelatin Zymography
Prepare gels (18%) according to the standard procedure (David Bowtell, 2006). The running gel is prepared by adding gelatin stock solution 0.2% (20 mg of gelatin/ml of H_2O) (Liota and settler, 1990).

Sample buffer (2X) was added to the sample and stand for 10 minutes at room temperature. The sample should not heat the sample solution. The gel run with the 1x Tris-Glycine SDS running buffer to the standard running conditions (constant voltage, ~125V). Run time (3 to 4 hrs) will depend on the gel percentage, running buffer concentration and pH. Stop the power, when the tracking dye (bromophenol blue) reaches to the bottom of the gel. After running, the zymogram 1X (10X renaturing buffer in 1:9 ratio dilute with distilled water) develop gel in the buffer (for 100 ml to one or two mini-gels) through gentle agitation for nearly 30 minutes by room temperature.

The zymogram decants and renaturing buffer and zymogram incubates in the 1X developing buffer (100 ml). The gel Equilibrate for 30 minutes at room temperature by gentle agitation, after that restore through fresh 1X zymogram developing Buffer and develop to over night at 37°C for maximum sensitivity. Decant the developing buffer and Stain through Coomassie Blue R-250 above 30 minutes. For obtaining maximum contrast should use 0.5% (w/v) stain instead of the usual 0.1% concentration. Gels should be destained with suitable Coomassie R-250 destaining solution. The clear bands represents protease activity protease has digested the substrate against a dark blue background.

RESULTS AND DISCUSSION
Zymography is a Protein breakdown has been recognized as essential for the adaptation of plants to environmental conditions (Vierstra, 1996). The main players in carrying out and regulating protein breakdown are proteases, together with specific endogenous inhibitors that regulate their activities (Vaseva et al 2012).Protein inhibitors of proteinases present in multiple forms in plants, animals and microbes which are ubiquitous in nature (Laskowski and Kato, 1980). Protein inhibitory activity from plant and animal components provide a significant role in medicinal usage in control of various diseases. The present work has been conducted to find the protease inhibitor effect of Citrullus lanatus, Cucurbita maxima and Cucumis sativus testa extracts.

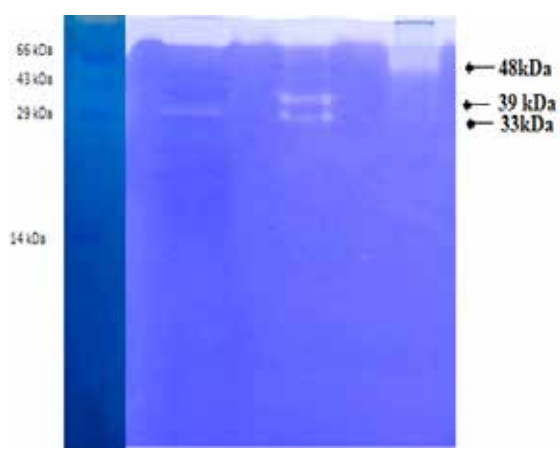

Figure 1: Proteolytic enzymes identification in seed coat extracts of Cucurbitaceae members by zymography

Cucumis sativus was shown one white band at 33 kDa molecular weight. Cucurbita maxima have shown two major white bands based on varying gelatinolytic activity at 33 kDa and 39 kDa. Citrullus lanatus has showed a white band at 48 kDa corresponding to serum marker. The marker was observed with bands on the gel at 66 kDa, 43 kDa, 29 kDa and 14 kDa (Figure 1).

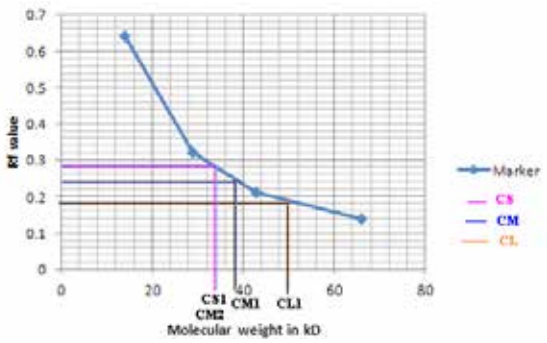

Figure 2: Standard curve for estimation of molecular weight of protein

Figure 2 has shown R_f values for the marker and the Cucurbitaceae members. Marker has shown R_f values as 0.64, 0.32, 0.21, and 0.14. Cucumis sativus has shown protein/Peptide at R_f value as 0.28. Cucurbita maxima have shown two major white bands of Protein/peptide with R_f values as 0.24 and 0.28.Citrullus lanatus has showed Protein/Peptide at R_f value 0.18.

The Electrophoretic technique used to identify the proteolytic enzymes activity are separated in polyacrylamide gels beneath various non reducing conditions .The nonreducing SDS-PAGE sample buffer is loaded on gelatin- containing gels. In SDS-PAGE zymography, SDS polyacrylamide gels containing 20mg/ml gelatin or casein is to be added and kept at 37°C for the identification of proteins. The presence of the gelatinolytic bands is the result of enzymatic activity.

In zymography, Gels stained with Coomassie blue R 250 will reveal zones of lysis within the gelatin as molecular-weight-activated forms were detected. The gelatin zymograms showed different pattern of hydrolysis with band formation. As there is high protein content in 90% of isolate samples, the protein sample has been used for further studies like Zymogaphy, reverse zymography and antimicrobial activities.

The enzymes have different molecular weights and can be visualized in zymograms (Kleiner and Stetlerstevenson, 1994). In the latest two decades, the protease activity of developing and dry seeds was shown great interests that are received by plant proteases. Serine proteases are present particular in cucurbits, cereals and trees (Antão and Malcata, 2005). Three papain class cysteine protease has been identified and has shown different patterns of activities during flower development on zymograms (Wagstaff et al., 2002).

Two major endopeptidases were previously identified in cotyledons of germinating Vigna mungo seeds by the zymogram after PAGE (Mitsuhashi et al., 1986). A number of diverse disciplines have been applied on a crop plant, soybean (Glycine max L. Merr.) whose seed is cherished as a source of protein and oil by using electrophoresis. PAGE is relatively simple technique that quickly screen for a huge number of genetic markers. The Electrophoretic systems identify seed storage proteins and in excess of 25 enzyme systems encoded with more than 60 genes from the past decades.

Protein electrophoresis has been continued to be a commanding tool in Cucurbitaceae research. Globulin is a common protein present in cotyledons of dormant seeds of Cucurbitaceae family. The two peptides with molecular weights of 18500 and 20000 have been produced by O'Kennedy et al., 1979 in Cucurbitaceae members using SDS-PAGE (O'Kennedy et al., 1979). A chitinase was purified from the seeds of Benincasahispida, a Cucurbitaceae member by Shih et al., 2001.

CONCLUSION

Studies on protease analysis using Zymography is an important method carried out for detection of hydrolytic enzymes in the present experimentation. Citrullus lanatus was shown 48 kDa proteinase, Cucurbita maxima was shown two major bands at 33 kDa and 39 kDa as proteinases, and Cucumis sativus was shown 33 kDa band proteinase based on zymography. As there are proteolytic enzymes in the testa of C. lanatus, there may be also presence of protease inhibitors to inhibit the proteolytic activity during seed dominance. As preliminary analysis on Cucurbitaceae members was shown good serine protease inhibition activities in testa of C. lanatus, further studies on isolation, prurification and charecterization has to be conducted.

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