Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio Tech. 49B (2012) 10178-10181

ISSN: 2229-712X

Detection and purification of Mucuna Pruriens seed protease inhibitors.

Vinod Borde[1], Vandana Hivrale[2] and Manvendra Kachole[2]

[1]Department of Biotechnology, Vinayakrao Patil College, Vaijapur, Dist. Aurangabad, M.S. 431003. India.

[2]Department of Biochemistry, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, 431004, India.

ARTICLE INFO

Article history:

Received: 30 May 2012;

Received in revised form:

16 August 2012;

Accepted: 31 August 2012;

Keywords

Protease inhibitor,
Try sin Inhibitor,
Mucuna Pruriens,
Ammoniumsulphate Precipitation,
Gel filtration,
Dot-blot assay,
X-ray film contact print method.

ABSTRACT

Trypsin protease inhibitor have been identified and purified from extracts of *Mucuna Pruriens*. *Mucuna Pruriens* seed sample were purified using ammonium sulphate precipitation, Saphadex G-75 gel filtration chromatography technique including analysis of trypsin inhibitors by dot-blot method, and electrophoretic analysis and visualization of protease inhibitor bands by gel X-ray film contact print method. Saphadex G-75 gel filtration chromatography identified a small molecular weight trypsin inhibitory fraction. In the present paper we described, a simple and inexpensive procedure to detect protease inhibitors of seed by the gel-x-ray film contact print technique.

Introduction

Protease inhibitors are ubiquitous molecules. They are present in all organisms as a means of regulating physiological proteolysis and predation. Proteinase inhibitor interactions are involved in protein digestion, blood coagulation, and pathological processes such as cancers and hypertension. These proteinase inhibitors are found in animals, plants, and microbes. Protease inhibitors from a wide variety of sources have been purified and characterized. Seeds contain a high amount of protease inhibitors [Koiwa R.A., 1997]. A number of techniques and methods have been developed and are used to detect protease inhibitors extracted from seeds and microbes [Raser K.J., 1995, Pichare M.M., 1994]. Many techniques such as electrophoretic analysis, chromatographic techniques, enzyme-linked immunosorbent assay plates, etc. are used to detect protease inhibitors. Gel electrophoretically separated protease inhibitors have been detected by incubating the gel in protease solution and then in chromogenic substrates [Michuad D, 1993], by incorporating gelatine, casein [Laemmli U. K., 1970], or fibrinogen [Jongsma M.A., 1996] in acrylamide or agarose gels, and incubating the gel in protease solution after electrophoresis [Felicioli R, Davis B. J., 1964]. Several other methods are also available for the detection of electrophoretically separated proteinase isoforms, which can also be used for quantitation of activity by scanning the gel [Michuad D, 1993. & Maerki W.1984]. A major disadvantage of these methods is that the proteinases can degrade the substrate during an electrophoretic run in native and in denaturing gels (SDS-PAGE) [Jongsma M. A., 1996]. Moreover, these methods are tedious and time consuming and require staining and distaining of the gel.

Gelatine, a denatured form of collagen, is a substrate commonly used to detect proteolytic activity [Kliener D. E., 1994]. Cheung *et al.* [Cheung A. L., 1991] have demonstrated the use of gelatin coating present on x-ray film as a substrate for detecting aggregate proteolytic activity in a dot-blot assay. With the help of x-ray film assay variety of proteolytic enzymes including serine proteinases, metalloproteinase, thiol proteinases, and acid proteinases has been demonstrated [Cheung A. L., 1991]. The use of x-ray film for the detection of electrophoretically separated proteinases on native gel and SDS-PAGE has also been reported [M. M. Pichare M. M., Kachole M.S., 1994].

Present studies we purify protease inhibitors from *Mucuna Pruriens* seed using different fractionation and chromatographic techniques including analysis of trypsin inhibitors by dot-blot method, Electrophoratic analysis and visualization of protease inhibitor bands by gel-x-ray film contact print method.

Material and Methods: -

Equipment and Reagents: -

Vertical slab gel electrophoresis (Broviga Basic model, Balaji Scientific Services Madras-17) was used. PC-based UV/VIS spectrophotometer (JASCO V- 500 series). Glass column, trays for incubation, test tubes, dialysis tubing, Eppendorf tubes, and micropipettes, bovine serum albumin, and dialysis tubing were purchased from (HiMedia Laboratories Pvt.Limited Mumbai, India). Ammonium sulphate, acrylamide, bisacrylamide, TEMED, ammonium persulfate, polyvinylpyrrolidine, acetone, and hexane were purchased from Sisco Laboratory, Mumbai, India. X-ray films were purchased from AGFA, selvas photo graphics Ltd. Silvassa. All other reagents used were of analytical grade.

Collection of seed material: -

Seed samples which are used for studies are from Davasaaj, medicinal plant seller, shahaganj, Local market Aurangabad 431005. Seed samples (50-100 g) of plants have been obtained.

Defatting and Extraction of Inhibitor: -

Dry seeds of *Mucuna Pruriens* were ground to fine powder.100 g of fine flour of the seed sample was washed with 300 ml of chilled acetone (four to five times) and later with 200 ml of chilled hexane (two times) to defat the flour. The solvents were

Tele:
E-mail addresses: avigoplani1@rediffmail.com

filtered off and the seed powders were kept at room temperature for air drying.

Inhibitor proteins in the dry defatted seed powder were kept for extraction in 1:6 ratio of seed flower:1% polyvinylpyrrolidine (w/v) (1% polyvinylpyrrolidine was added to the mixture to remove the phenols present in the flour).The resulting extract was then centrifuged at 10,000 rpm at 4 °C for 15 min. The clear supernatant was dialyzed extensively against distilled water. The resulting supernatant was used as crude seed extract of inhibitor.

Estimation of protein Concentration: -

Proteins in the crude seed extract preparation were estimated by Lowry's method. (Lowry et al., 1951). Bovine serum albumin was used as standard.

Ammonium Sulphate Precipitation of Trypsin Inhibitor from Seed Extract:-

The clear supernatant obtained from defatting and extraction of the inhibitor was saturated in three stages to precipitate the protein. Ammonium sulphate saturation was carried out from 0 to 40%, 40 to 60%, and 60 to 90% as follows: solid ammonium sulphate was slowly added to the supernatant in the cold. The solution containing the precipitated protein, which had been kept overnight from each step, was centrifuged at 10,000 rpm at 4 °C for 15 min. The protein pellet obtained was dissolved in a minimum quantity of distilled water and dialyzed extensively, using a membrane with a cut off range of *M*r 12,400 (Himedia), against 10 mM Tris-HCl buffer (pH 7.6) overnight. The buffer was changed every 4 h. The protein obtained was stored in small (5-ml) vials and was used for further studies. The amount of protein present in each step was estimated by Lowry's method. (Lowry, O. H., 1951) as described in the estimation of protein concentration.

Preparation of Saphadex G-75 Column (Gel Filtration Column):-

Glass column (50 cm in length and 1 cm in diameter) was used for gel filtration. The column was packed with Saphadex G-75 matrix (spherical composite of cross linked agarose and dextran) that has the capacity to separate 3,000 to 70,000 molecular weight compounds. The matrix was initially washed with 20% alcohol and degassed for 1 h under suction. It was then prewashed with distilled water to remove the traces of alcohol and equilibrated with 10 mM Tris-HCl buffer (pH 7.8) and loaded onto the column so as to fill the entire column (50 cm in length). It was then washed with 10 mM Tris-HCl buffer (pH 7.8) (five times the column length), and the column was ready to use. The concentrated ammonium sulphate fraction which show all majority Ti bands on x-ray film loaded (500 µl) and eluted out by 10 mM Tris-HCl buffer (pH 7.8). 2-ml fractions were collected and the protein was monitored at 280 nm. To confirm the results, the fractions collected were analysed again using a PC-based UV/VIS spectrophotometer (JASCO V- 500 series), and the graph was plotted. The fractions collected were tested for trypsin inhibitor by the dot-blot method.

Analysis of Trypsin Inhibitor by the Dot-blot Method: -

The fractions collected were analysed for their inhibitory activity as follows: 10 µl of inhibitor was mixed with 10 µl of trypsin and spotted onto a strip of x-ray film. The trypsin does not degrade the gelatine on the x-ray films, where the inhibitor is present. And a clear zone is formed at the site of sample application on the x-ray film, if the inhibitor is absent.

Electrophoretic Analysis of Protease Inhibitors: -

Crude seed extracts, ammonium sulphate precipitation extracts, and the protease inhibitors obtained from gel filtration chromatography were all subjected to electrophoretic analysis by PAGE in a vertical slab electrophoresis (Broviga Basic model, Balaji Scientific Services Madras-17.). Electrophoresis was carried out according to Davis [Davis B. J.,1964] and Laemmli [Laemmli U. K.,1970] methods. Basic gel electrophoresis was carried out in 10% acrylamide solution using the Davis buffer system. Denaturing gel electrophoresis *i.e.* SDS-PAGE (12.5%) was carried out according to the Laemmli method except that in some cases the sample buffer did not contain 2-mercaptoethanol, and/or the samples were not heated in boiling water. The non denaturing gels, after electrophoresis, were stained for their activity using trypsin solution. The denaturing gels were washed with 1% Triton X-100 (three washes) before staining.

Visualization of Protease Inhibitory Bands by the Gel-x-ray Film Contact Print Method: -

After electrophoresis the gel was processed for protease inhibitory activity by the gel X-ray film contact print method. The gel was washed in 0.1 M Tris-HCl buffer (pH 7.6) for 15 min, followed by incubation in 0.1% trypsin solution (SRL) for 15 min at room temperature. The gel was then briefly rinsed in Tris buffer to remove the excess trypsin. The gel and the x-ray film were placed in a tray and incubated at 37 °C. The gel was removed after 10 min, and the extent of hydrolysis of the gelatine was monitored visually. Depending on the extent of gelatin hydrolysis the x-ray film was washed with either tap water or warm water. The same gel was overlaid three to four times with different pieces of x-ray films. Occasionally the x-ray film was also rubbed gently to remove the hydrolysed gelatine. Protease inhibitory bands appeared as unhydrolyzed gelatine against the background of hydrolysed gelatine.

Result and Discussion: -

Ammonium sulphate precipitation:-

In ammonium sulphate precipitation showed that the crude extract contained all the inhibitors and that the ammonium sulphate fractions showed different isoforms of inhibitors. In particular, the 40–60% ammonium sulphate precipitated protein showed all majority of inhibitors. Therefore, from the above observations, we can designate the 40–60%-saturated protein contain all majority inhibitors. (*in vitro* analysis confirms these results). Fig.1 shows the gel-x-ray film contact print photograph and activity staining of fractions. *Lane 1* shows the crude extract, *lane 2* shows the 0–40% ammonium sulphate precipitation, *lane 3* shows the 40– 60% ammonium sulphate precipitation, and *lane 4* shows the 60–90% ammonium sulphate precipitation.

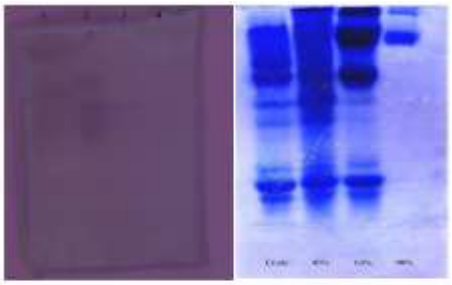

Fig 1 Gel X-ray film contact print technique and activity staining of fractions. Lane 1, Crude extract of the seed; lane 2, 0-40% ammonium sulphate precipitated protein, Lane 3, 40-60% ammonium sulphate precipitated protein, Lane 4, 60-90% ammonium precipitated protein. Electrophoresis was carried out at 150 V (20 mA).

Saphadex G-75 Column (Gel Filtration Column chromatography):-

The concentrated ammonium sulphate fraction which show all majority Ti bands on x-ray film was loaded (500 µl) and eluted out by 10 mM Tris-HCl buffer (pH 7.8). 2-ml fractions were collected and the protein was monitored at 280 nm. The fractions collected were analysed again using a PC-based UV/VIS spectrophotometer, and the graph was plotted. The fractions collected were tested for trypsin inhibitor by the dot-blot method. Fig.2 show the elution profile of 40–60% ammonium sulphate precipitation protein that was loaded onto a Saphadex G-75 column (concentrated protein was applied to the column) and eluted out with 10 mM Tris-HCl buffer (pH 7.8).

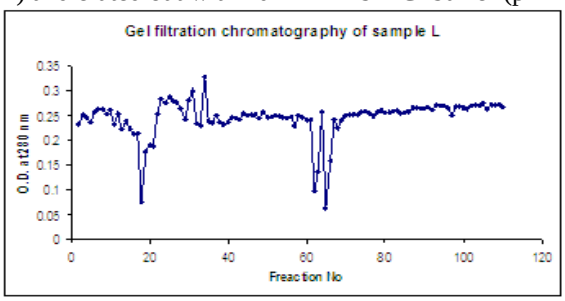

Fig.2 Elution profile of 40-60% ammonium sulphate precipitated protein loaded onto a gel filtration column (Saphadex G- 75). The protein was eluted out with 10 mM Tris- Hcl buffer (pH 7.8). 2ml fractions were collected and analyzed for their inhibitory activity using the dot-blot method.

Dot-blot assay method:-

The fractions collected were analysed for their inhibitory activity. The trypsin does not degrade the gelatine on the x-ray films, where the inhibitor is present. And a clear zone is formed at the site of sample application on the x-ray film, if the inhibitor is absent. Fig.3 Dot-blot detection of fractions collected after gel filtration chromatography using (Saphadex G-75). The fractions obtained were tested for their inhibitory activity by the dot-blot method. The tubes (numbers 12-35) showed the inhibitory activity. These tubes were used for electrophoretic analysis.

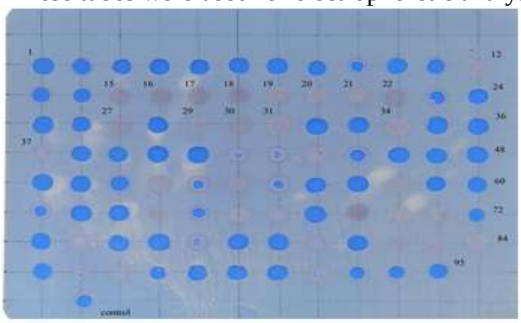

Fig.3 Dot-blot detection of fractions collected after gel filtration chromatography using (Saphadex G-75)

Electrophoretic Analysis of Protease Inhibitors:-

Crude seed extracts, ammonium sulphate precipitation extracts, and the protease inhibitors obtained from gel filtration chromatography were all subjected to electrophoretic analysis by PAGE in a vertical slab electrophoresis. Fig.4 shows the electrophoretically separated gel filtration fractions (numbers 12-35). Fractions 20, 21, 22 were loaded onto a gel (non-denaturing) and were stained for activity using 0.1% trypsin as a substrate. Only one inhibitor was observed depending upon the time of development. No extra bands were detected even when the gel was incubated with the x-ray films for more than 30 min. Fig.4a show the gel X-ray film contact print technique and activity staining of fractions collected Lane1-4, test tube number20,21,22, fractions showing inhibitory activity. Electrophoresis was carried out on native PAGE for 6 hrs at 150 V (20mA).

Fig.4 Gel X-ray film contact print technique and activity staining of fractions collected Lane1-4, test tube number20, 21, 22, fractions showing inhibitory activity. Electrophoresis was carried out on native PAGE for 6 hrs at 150 V (20mA).

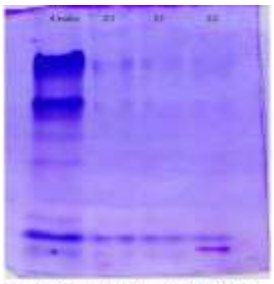

Fig.4a Gel X-ray film contact print technique and activity staining of fractions collected Lane1-4, test tube number20, 21, 22, fractions showing inhibitory activity. Electrophoresis was carried out on native PAGE for 6 hrs at 150 V (20mA)

Conclusion: -

In conclusion, a single trypsin inhibitor was isolated from the seed extracts of *Mucuna Pruriens*. Detection of the Mucuna Pruriens seed inhibitor was done with X-ray contact print method. and purification of seed inhibitor was done with (0-40%, 40-60% and 60-90%) ammonium sulphate fractionation. 40-60% fraction show majority of inhibitors isoforms in the seed sample that was further loaded for purification on Saphadex G-75 gel filtration Colum and fraction collected were analysed for trypsin inhibitor detection by dot-blot assay. And the visualization of the inhibitor in the fraction was done with X-ray film contact print method. Fractions 20, 21, 22 were loaded onto a gel (non-denaturing) and were stained for activity using 0.1% trypsin as a substrate. Only one inhibitor was observed in this fraction. No extra bands were detected even when the gel was incubated with the x-ray films for more than 30 min.

We report a simple method that demonstrates protein purification techniques. The method do not require complex or expensive instruments. The x-ray contact film technique is sensitive, simple, rapid, and cheap. This method will be useful for screening samples for the presence of protease inhibitors.

References:-

1 A. L. Cheung, P. Ying, V. A. Fischetti (1991) A method to detect proteinase activity using unprocessed X-ray films, *Anal. Biochem.* 193, 20–23.

2 B. J. Davis (1964) Disc electrophoresis II. Methods and application to human serum, *Ann. N. Y. Acad. Sci.* 121, 404–427.

3 D. E. Kliener, W. G. Stetler-Stevenson (1994) Quantitative zymography: detection of picogram quantities of gelatinases, *Anal. Biochem.* 218, 325–329.

4 D. Michuad, L. Faye, S. Yelle (1993) Electrophoretic analysis of plant cysteine and serine proteinases using gelatin-containing polyacryalamide gels and class-specific proteinase inhibitors, *Electrophoresis* 14, 94–98.

5 H. Koiwa, R. A. Bressan, P. M. Hasegawa (1997) Regulation of proteinase inhibitors and plant defense, *Trends Plant Sci.* 2, 379–384.

6 J. Hejgaard (1981) Isoelectric focusing of subtilisin inhibitors: detection and partial characterization of cereal inhibitors of chymotrypsin and microbial proteases, *Anal. Biochem.* 116, 444–449.

7 J. Lewosz, D. Rys, S. Reda (1981) Electrophoretic method for the determination of molecular forms of trypsin inhibitors of potato tubers, *Anal. Biochem.* 115, 27–29.

8 J. S. Hanspal, G. R. Bushell, P. Ghosh (1983) Detection of protease inhibitors using substrate-containing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, *Anal. Biochem.* 132, 288–293.

9 J. Uriel, J. Berges (1968) Characterization of natural inhibitors of trypsin and chymotrypsin by electrophoresis on acrylamide-agarose gels, *Nature* 218, 578–560.
10 J. X. Filho, R. de Azevedo Moreira (1978) Visualization of proteinase inhibitors in SDS-polyacrylamide gels, *Anal. Biochem.* 84, 296–303.
11 K. J. Raser, A. Posner, K. K. W. Wang (1995) Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents, *Arch. Biochem. Biophys.* 319, 211–216.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J.Biol.Chem 193: 265.
13 M. A. Jongsma, J. Peters, W. J. Stiekema, D. Bosch (1996) Characterization and partial purification of gut proteinases of *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae), *Insect Biochem. Mol. Biol.* 26, 185–193.
14 M. A. Jongsma, P. L. Bakker, W. J. Stiekema (1993) Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay, *Anal. Biochem.* 212, 79–84.
15 M. M. Bradford (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72, 248–254.
16 M. M. Pichare, M. S. Kachole (1994) Detection of electrophoretically separated protease inhibitors using X-ray film, *J. Biochem. Biophys. Methods* 28, 215–224.
17 R. Felicioli, B. Garzelli, L. Vaccari, D. Melfi, E. Balesteri (1997) Activity staining of protein inhibitors of proteases on gelatin-containing polyacryalamide gel electrophoresis, *Anal. Biochem.* 244, 176–179.
18 T. M. Leber, F. R. Balkwill (1997) Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels, *Anal. Biochem.* 249, 24–28.
19 U. K. Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227, 680–685.
20 W. Maerki, W. Zimmermann, M. Faupel, E. Von Arx (1984) Bioautography of proteinase inhibitors of microbial origin. A simple enzymatic detection procedure on casein agar plates, *J. Chromatogr.* 283, 406–411.